Antimicrobial Resistance in Indonesia

Prevalence, determinants and genetic basis

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Antimicrobiële resistentie in Indonesië

Prevalentie, determinanten en genetische basis

Thesis

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by

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Untuk yang tercinta dan tersayang
Suamiku Achmad Fuadi dan anak-anakku mas Qorri dan dik Vaza

Aan mijn ouders
Voor Marcelo en Sibolt

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Contents

Chapter 1

Introduction and outline of the thesis

INTRODUCTION

In the past 65 years, antibiotics have been critical in the fight against infectious diseases caused by bacteria. Penicillin was discovered in 1928, but it was only by the end of the 1940s that it became generally available. This was soon followed by the discovery and development of new antibiotics, including chloramphenicol and tetracycline. However, shortly after the introduction of each of these new antibiotics, resistance was detected. Nowadays, disease-causing microbes that have become resistant to antibiotic drug therapy are a major health problem worldwide, both in hospitals and the community (1-3, 5, 9). Infections with resistant bacteria increase the health care costs, length of hospital stay, and mortality, compared to infections caused by bacteria that are susceptible to standard antibiotics (4, 6). The problem is particularly pressing in developing countries, where the infectious disease burden is high and cost constraints restrict the application of newer, often more expensive and more toxic agents (7, 8).

Emergence of resistant bacteria is driven by the selective pressure that antibiotics exert on bacterial populations. Through person-to-person transmission these bacteria may be further spread. Surveillance of resistant microorganisms and knowledge of their molecular epidemiology and resistance mechanisms are essential for understanding how best to treat patients suffering from an infection by these bacteria, and how to develop effective resistance management strategies. Such resistance-control programs should include both the promotion of the prudent use of antibiotics and the improvement of infection control practices. Knowledge on the risk factors for carriage of and infection with resistant bacteria in a specific geographic region will determine specific targets for these actions in that location.

Although Indonesia is the world's fourth most populous country, with a population of over 230 million inhabitants, very few data on antimicrobial resistance are available from that country. The general aim of the studies presented in this thesis was to explore the prevalence, risk factors, molecular epidemiology, and mechanisms of antimicrobial resistance among commensal bacteria from almost 4,000 patients and healthy persons in two urban regions on the island of Java, Indonesia. All studies were performed as part of the Antimicrobial Resistance in Indonesia: Prevalence and Prevention (AMRIN) study.

OUTLINE OF THE THESIS

The chapters of the thesis are presented in five sections.

Part I: Background

In **chapter 2**, an overview of the presence and emergence of antimicrobial resistance in Southeast Asia among bacterial species that commonly cause infection is presented. The design and rationale of the AMRIN study is described in **chapter 3**.

Part II: Prevalence of antimicrobial resistance in Indonesia

In **chapter 4**, the possibility of using the agar diffusion method with antibiotic impregnated disks for antimicrobial susceptibility testing in a surveillance study in Indonesia is explored by comparing the accuracy of disk diffusion zone diameters obtained by manual zone measurements to that by automated zone measurements. Discrepancies were analyzed by the microdilution broth method. In **chapter 5**, data on the prevalence of antimicrobial resistance among commensal isolates of *Escherichia coli* and *Staphylococcus aureus* carried in the Indonesian population inside and outside hospitals are presented.

Part III: Focus on *Staphylococcus* spp.

Studies in this part of the thesis are focused on the mannitol-fermenting *Staphylococcus* spp. cultured during the population-based survey on nasal *S. aureus* carriage in Semarang and Surabaya, Indonesia.

In **chapter 6**, an analysis of possible associations of recent antibiotic use as well as demographic, socioeconomic, disease-related and healthcare-related determinants with nasal carriage of resistant *S. aureus* in the Indonesian population inside and outside hospitals is presented. **Chapter 7** describes the molecular epidemiology and population structure of *S. aureus* carriage isolates from Semarang and Surabaya. During the survey, we unexpectedly cultured *Staphylococcus sciuri*, a mannitol-fermenting staphylococcus that has commonly been described as a commensal of animals, from a number of individuals. In **chapter 8**, the risk factors for nasal carriage of methicillin-resistant and -susceptible *S. sciuri* are determined. The molecular characterization of these strains is described as well.

Part IV: Focus on Enterobacteriaceae

Studies in this part of the thesis deal with the Enterobacteriaceae cultured during the population-based survey on rectal carriage of resistant Gram-negative microorganisms in Semarang and Surabaya, and during a prospective survey of clinical isolates in an academic hospital in Surabaya.

Chapter 9 describes the analysis of possible determinants for carriage of resistant *E. coli* in the Indonesian population inside and outside hospitals. The determinants include recent antibiotic use, and demographic, socioeconomic, healthcare-related and disease-related variables.

During the population-based survey, resistance to fluoroquinolones and extended-spectrum β-lactams among commensal *E. coli* from patients that were discharged after a hospitalization of five or more days was of special concern. In chapters 10 to 13, these resistance types are studied in more detail. **Chapter 10** is devoted to the molecular epidemiology, phylogenetic background and virulence profile of the fluoroquinolone-resistant commensal *E. coli*. The resistance mechanisms involved in quinolone resistance in these isolates are described in **chapter 11**. In **chapter 12**, the molecular characterization of extended-spectrum β-lactamases (ESBLs) in commensal Enterobacteriaceae are presented. In **chapter 13**, the results are presented of a prospective survey to investigate the molecular epidemiology and genetic characteristics of clinical ESBL-producing *E. coli* and *Klebsiella pneumoniae* isolates from an academic hospital in Surabaya, Indonesia.

Part V: Discussion

In **chapter 14**, the main findings of the studies in this thesis are discussed and suggestions for further research regarding antimicrobial resistance in Indonesia are given.

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Part I

Background

Chapter 2

Antimicrobial resistance among pathogenic bacteria in Southeast Asia: A review

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INTRODUCTION

Bacterial antimicrobial drug resistance is a worldwide problem in both developing and developed countries (5, 24, 57, 83, 87), in hospitals as well as in the community (15, 56). Infections with resistant bacteria adversely affect treatment outcome, treatment costs, disease spread, and duration of illness (57). In 2001, the World Health Organization (WHO) launched the first global strategy to counter this phenomenon, one of the recommendations of which is to monitor trends in antimicrobial resistance using standardized microbiological methods. Many data exist about the emergence of antimicrobial resistance in Southeast (SE) Asian countries, but this information is fragmented since it has been published in separate articles from each country spanning several decades.

SE Asia is geographically divided into two regions, namely Mainland SE Asia and Maritime SE Asia. Mainland SE Asia includes Cambodia, Lao People's Democratic Republic or Laos, Myanmar or Burma, Thailand, and Vietnam. Maritime SE Asia includes Brunei, East Timor, Indonesia, Malaysia, the Philippines, and Singapore. The region is a mix of developed and developing countries, with East Timor and Myanmar belonging to the Least Developed Countries according to the United Nations Conference on Trade and Development (www.unctad.org).

For the present review we have gathered and evaluated all available information in Pubmed on the presence or emergence of antimicrobial resistance among bacterial species that commonly cause infection in SE Asian countries to present an integrated overview of the situation in this region.

METHODS

A review of the literature was conducted using the PubMed database. The search strategy was a combination of the following keywords, subjects and title words or combination between "antimicrobial resistance", "antibiotic resistance", "MRSA", and "ESBL" and SE Asian countries including "Brunei Darussalam" or "Brunei", "Cambodia", "Indonesia", "Lao People's Democratic Republic" or "Laos" or "Lao PDR", "Malaysia", "Myanmar" or "Burma", "Philippines", "Singapore", "Thailand", "Timor Leste" or "East Timor", and "Vietnam". Articles published between January 1, 1995 to January 1, 2007 were included. Articles and abstracts were limited to studies written in English only. Studies were classified / extracted by species, country, year of publication, year of sample collection, sources, method of antimicrobial susceptibility testing, quality control, number of strains and percentage of resistance for each species against tested antimicrobial agents. A scoring system was developed for inclusion and

exclusion of papers: use of quality control strains (0=no/not described and 1=yes/described), antimicrobial susceptibility testing according to an internationally approved method such as that published by the Clinical Laboratory Standards Institute (CLSI) e.g. disk diffusion, E-test, micro/macro/agar dilution (0=no and 2= yes), use of a well evaluated method for identification e.g. Vitek (bioMérieux), Phoenix (Becton Dickinson), API (bioMérieux), molecular (0=no; 1=yes). Only a paper with a total score of 2 or more was included for analysis. Per bacterial species a minimum of 10 strains had to be tested (16). Only major pathogenic bacterial species for humans were included, except for *Mycobacterium* spp. Data representing a mix of strains from several countries were not included. Clinical material and non-clinical material were analyzed separately (Supplementary Table S1 and Supplementary Table S2). Intermediate resistance was not classified as resistance. When resistance data for a bacterial species were available from only one country, these were excluded.

Figures were made to present trends in resistance among the most important pathogens over the years by country. For each country data collected in the same period but published in different articles or journals were merged. When isolates were collected during a period of more than one year, the median year was chosen to reflect the whole period. When the year of sample collection could not be extracted from the text, the year of publication was used instead.

For mapping purposes, data from multiple sites in geographically small countries were merged into one figure. Data from multiple sites in large countries were presented separately.

RESULTS AND DISCUSSION

Resistance among clinical isolates: Gram-positive bacteria

Streptococcus pneumoniae

S. pneumoniae is a major pathogen causing various infections in children and adults including pneumonia, meningitis, otitis media, and septicemia (72). Antibacterial resistance in pneumococci is increasing worldwide, primarily against β-lactams and macrolides (71). Prevalences of penicillin-non-susceptible *S. pneumoniae* (PNSP) in SE Asian countries are presented in Figure 1 and Figure 2a. Data are mainly extracted from studies by the Asian Network for Surveillance of Resistant Pathogens (ANSORP), and a number of other studies. In Malaysia, the PNSP rate increased from 9% in 1996 to 39% in 2000. In Singapore, PNSP levels increased from 23% and 24% in 1996 and 1997, respectively, to more than 40% in the year 2000 and beyond. In Thailand, the rate of PNSP was stably high, ranging from 47% in 1997 to 69% in 2000. In Vietnam,

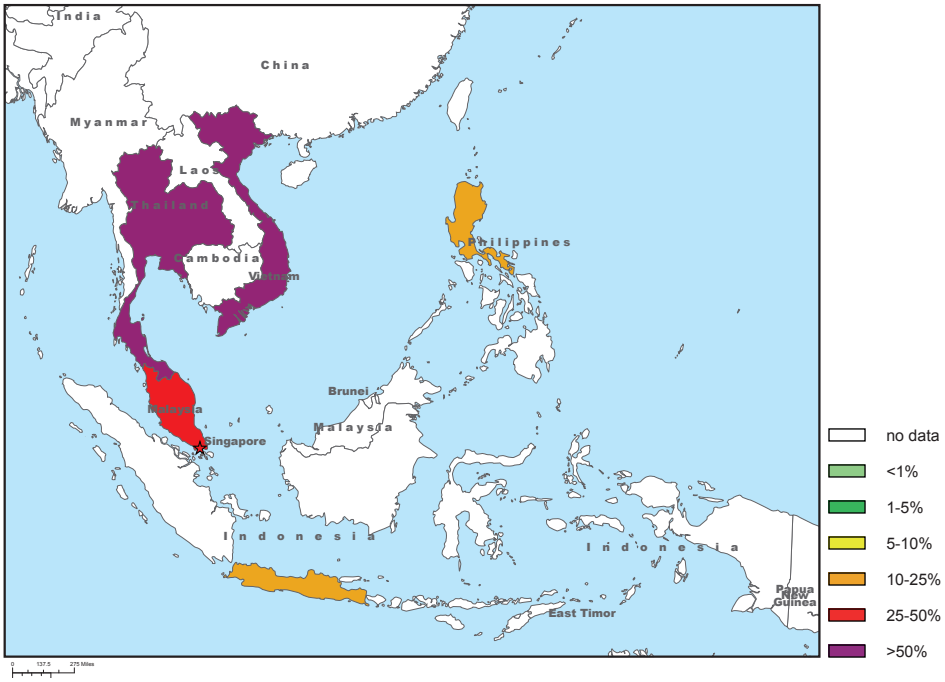

Figure 1. Prevalence of penicillin-non-susceptible *S. pneumoniae* (PNSP) in SE Asian countries, 1995-2007.

rates of PNSP were more than 50% in the 90s: 61% in 1996, 53% in 1997, and 50% in 1998. In 2001, a strikingly high resistance rate of 92% was found by an ANSORP study (71). In 1996, 21% of *S. pneumoniae* from sputum cultures in Jakarta, Indonesia, were PNSP. In the Philippines, the PNSP prevalence was 21% in 2000 (Figure 2a). Compared to many European countries, the prevalence of PNSP in SE Asia is high. In the UK, Denmark, Norway, Sweden, and the Netherlands, prevalences are between 1-5%, and in Iceland between 5-10% (www.rivm.nl/earss/database).

Erythromycin resistance among *S. pneumoniae* isolates from Malaysia was only 3% in 1996, but 35% in 1999 (Figure 2b). In Vietnam, the level of erythromycin resistance increased from 59% in 1995 to over 65% in 1996 and thereafter. In Thailand, erythromycin resistance rates among *S. pneumoniae* ranged from 16% to 52%. In Singapore, erythromycin resistance was increasing as well (Figure 2b). The resistance rate in Indonesia was 36% in 1996 and in the Philippines 18% in 2000 (Figure 2b).

Resistance to tetracycline increased over the years in Singapore and Vietnam (Figure 2c). In 1995, tetracycline resistance was 46% in Indonesia and 27% in Malaysia. In 1996, 52% of Thai isolates were resistant to this agent (Figure 2c).

In Singapore, Thailand, and Vietnam, trimethoprim-sulfamethoxazole resistance rates were more than 30% (Figure 2d). In Indonesia and Malaysia, trimethoprim-sulfamethoxazole levels of resistance in 1996 were 14% and 15%, respectively (Figure 2d).

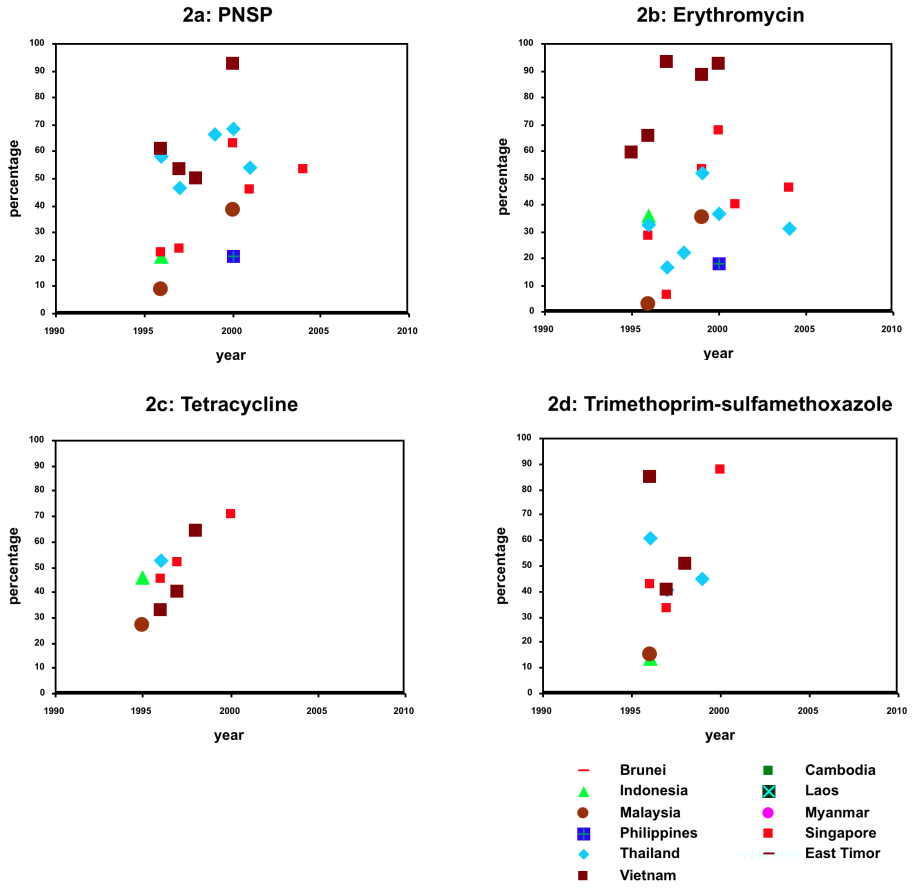

Figure 2. Resistance among *S. pneumoniae* from SE Asia.

Additional data on the antimicrobial resistance among *S. pneumoniae* are presented in Supplementary Table S3.

Enterococcus spp.

In general, enterococci are regarded as low grade pathogens, but in the hospital setting these bacteria have emerged as an important cause of nosocomial infections. Enterococci are intrinsically resistant to a large number of antibiotics, and can easily acquire new mechanisms of resistance. Ampicillin is the antibiotic of choice for the treatment of enterococcal infections, and vancomycin is the alternative agent. Ciprofloxacin, erythromycin, tetracycline, and chloramphenicol may exhibit *in vitro* activity, but clinical success is limited. Among *Enterococcus* spp. from diabetic feet in Malaysia, the resistance rate was 50% to erythromycin, 25% to trimethoprim-sulfamethoxazole, 17% to ampicillin, 8% to imipenem and 0% to penicillin and vancomycin (63). In a multicenter study in Thailand in 2002-2003, resistance found among *Enterococcus*

spp. from community- and hospital-acquired infections was 36% to ampicillin, 47% to gentamicin, 4% to vancomycin, 69% to ciprofloxacin, and 76% to erythromycin (18). Vancomycin-resistant enterococci, first described in the late 1980s in Europe and now a major problem in hospitals in the USA, have been documented in case reports from Singapore in 1996 (1) and in 1997 (12), and from Malaysia in 2005 (63), but the full extent of the phenomenon in SE Asia is unknown.

Staphylococcus aureus

S. aureus is a major cause of both hospital- and community-acquired infections, in developed as well as developing countries (4, 54). A study in Laos showed that *S. aureus* was the second most common cause of bacteremia, and was associated with a mortality rate of 17% (62). Treatment of *S. aureus* infections is becoming increasingly more complicated due to the emergence of various types of antimicrobial resistance worldwide. Methicillin-resistant *S. aureus* (MRSA) strains are of most concern since these are resistant to all β-lactam antibiotics and in many cases to other groups of antimicrobials as well, especially in the hospital setting. Data on prevalences of MRSA in SE Asia are available from Laos (62), Malaysia (11, 63, 66), the Philippines (6, 14), and Singapore (6, 14, 78). Overall, resistance rates of MRSA ranged from 0% in Laos and 7% in the Philippines to 25% in Malaysia and 39% in Singapore (Figure 3 and Supplementary Table S4).

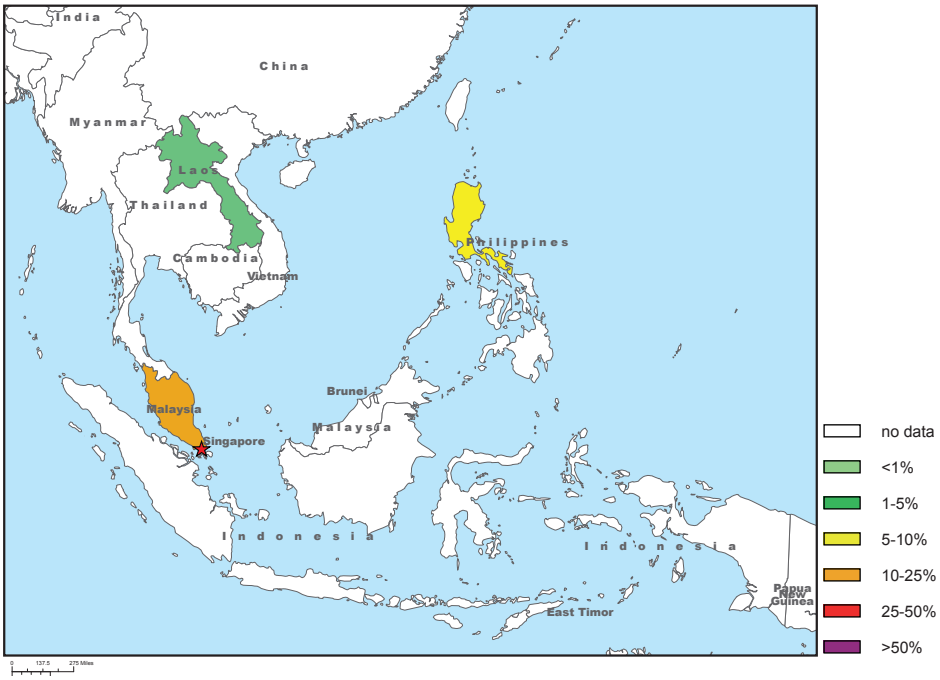

Figure 3. Prevalence of methicillin-resistant *S. aureus* (MRSA) in SE Asian countries, 1995-2007.

In Malaysia, MRSA rate was 0% among *S. aureus* from a variety of specimens from nonhospitalized patients in 1992, 40% among *S. aureus* from various clinical specimens in 1996 and 16% among *S. aureus* from diabetic foot cultures in 2004. In Laos, no MRSA was found among *S. aureus* blood culture isolates from patients with a community-acquired bacteremia.

SENTRY is an international antimicrobial surveillance program that documents resistance patterns in bacteria isolated from predominantly hospitalized patients. Between April 1998 and December 1999, MRSA accounted for 5% of all *S. aureus* isolates from the Philippines and 62% of isolates from Singapore in this program. During the period 1999 to 2001, these rates were 8% and 52% for the Philippines and Singapore, respectively (Figure 4a). In another study from Singapore among *S. aureus* from skin infections in 1995-1996, 7% was MRSA.

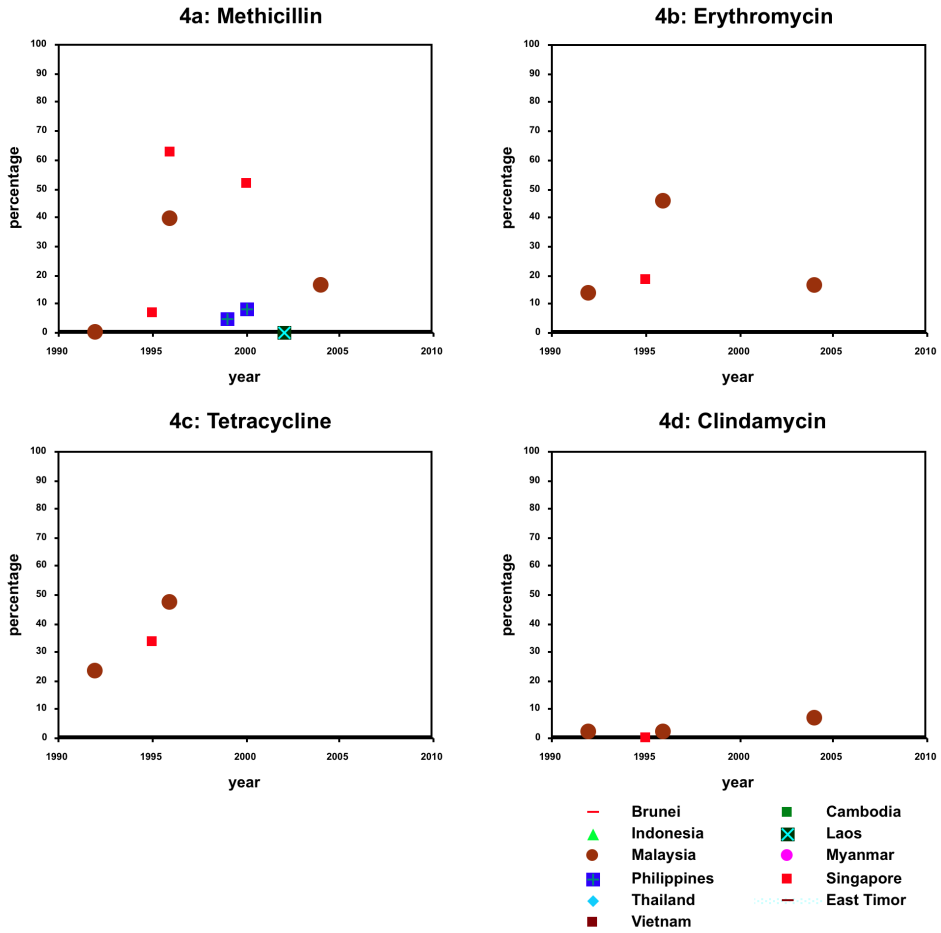

Figure 4. Resistance among *S. aureus* from SE Asia.

Vancomycin, a glycopeptide, is the cornerstone for treating invasive MRSA infections. In 1997, the first clinical isolate of *S. aureus* with reduced susceptibility to vancomycin was reported from Japan (10). Subsequently, vancomycin intermediately susceptible *S. aureus* (VISA) and heterogenous resistance to vancomycin (hVISA) have been identified in many parts of the world. In a study by Song et al. (70) heterointermediate resistance to vancomycin was found among MRSA isolates from the Philippines (4%), Vietnam (2%), Singapore (2%), Thailand (2%), but not among MRSA strains from Indonesia (70) and Malaysia (63, 66). In the Malaysian studies, however, disk diffusion was used, which is an inaccurate method for the assessment of intermediate resistance to vancomycin.

In Malaysia, overall resistance to tetracycline, erythromycin, and clindamycin among *S. aureus* was 39%, 33%, and 2%, respectively. Tetracycline resistance varied from 23% in 1992 to 47% in 1996 (Figure 4c). Erythromycin resistance rates varied from 13% in 1992 to 46% in 1996 and 16% in 2004 (Figure 4b). Clindamycin resistance was 2% in 1992 and 1996, and 7% in 2004 (Figure 4d), suggesting an increase over the years. In 1995, in Singapore, 19% of *S. aureus* was resistant to erythromycin, 34% to tetracycline, and 0% to clindamycin. However, in none of these studies an induction test was performed to check for inducible resistance, thus resistance rates for clindamycin may be higher. Additional data on resistance among *S. aureus* is presented in Supplementary Table S4. From many countries in SE Asia information on resistance data of *S. aureus* is lacking.

Coagulase-negative staphylococci (CoNS)

CoNS are the most frequently reported pathogens in nosocomial bloodstream infections (33, 39). Patients with CoNS infections are usually immunocompromised, with indwelling or implanted foreign bodies. Resistance to antibiotics in CoNS is of concern, especially methicillin resistance encoded by the *mecA* gene, because there is evidence of horizontal transfer of the *mecA* containing staphylococcal cassette chromosome between staphylococcal species (27). From SE Asia, only data from Singapore and Thailand are available. In a university hospital in Singapore, CoNS showed a high incidence of resistance to gentamicin (38%), erythromycin (38%), trimethoprim-sulfamethoxazole (29%), methicillin (25%), and fusidic acid (22%), but vancomycin resistance was not found (45). In Thailand, resistance rates were 36% for gentamicin, 43% for erythromycin, 40% for trimethoprim-sulfamethoxazole, 57% for oxacillin, and 1% for vancomycin (18). Resistance to methicillin is much more prevalent in the USA (78%) and other parts of the world (74%) (36, 37). Similarly, erythromycin resistance was found in 71% of CoNS from the USA and 65% from other parts of the world (36).

Resistance among clinical isolates: Gram-negative bacteria

Haemophilus influenzae

Accurate data on the susceptibility of *H. influenzae* in SE Asia are available from Singapore and Thailand. Results of 318 isolates, obtained in 1993-1994 in Singapore, were reported by Tee et al. (82). Resistance to ampicillin and trimethoprim-sulfamethoxazole was prevalent: 41% and 38%, respectively. Resistance to chloramphenicol, cefuroxime, and ceftriaxone was 11%, 2%, and 0%, respectively. In Thailand, 305 isolates from respiratory samples were studied. β-lactamase production was present in 45% of isolates, with 135 of these being ampicillin-resistant. Resistance to trimethoprim-sulfamethoxazole was prevalent as well (50%). All isolates were susceptible to amoxicillin-clavulanic acid, cefuroxime, ceftriaxone, azithromycin, and levofloxacin (17, 82).

The production of β-lactamase is the most common mechanism of ampicillin resistance expressed by *H. influenzae*, with wide geographical variation (86). One international surveillance study of almost 3,000 strains from 1999 to 2000 showed an overall prevalence of 17% β-lactamase-positive strains, ranging from as low as 3% in Germany to as high as 65% in South Korea (28). Compared to European countries, but also to North and South America, ampicillin resistance rates in Singapore and Thailand were high. Resistance to trimethoprim-sulfamethoxazole is common worldwide (28).

Neisseria gonorrhoeae

Gonorrhea is among the most prevalent sexually transmitted diseases throughout much of the world (79, 80, 98). Complications of urogenital infection include pelvic inflammatory disease in women, leading to infertility, chronic pelvic pain, or later, ectopic pregnancy, and conjunctivitis in the newborn of infected mothers. Effective antibiotic treatment is an essential element in approaches to control the disease. The epidemiology of antimicrobial resistance guides decisions about gonococcal treatment recommendations. Data on antimicrobial resistance in *N. gonorrhoeae* have been reported from Indonesia, Thailand, the Philippines, Brunei, Laos, Malaysia, Singapore and Vietnam. The latter six countries participate in the WHO Western Pacific Region Gonococcal Antimicrobial Surveillance Programme (WPR GASP) that has monitored resistance in gonococci since 1992. Resistance to penicillins, which may be the result of penicillinase production (PPNG) or aggregation of a number of chromosomally mediated mechanisms (CMRNG), is widespread and at high levels in SE Asia. The PPNG prevalences are depicted in Figure 5 and Figure 6a. Although the PPNG prevalence in Malaysia was 39%, the total rate of resistance to penicillin was 48%. In Brunei, the overall rate of resistance to penicillin was 64% (Supplementary Table S5). There was considerable regional variation in the distribution of high-level

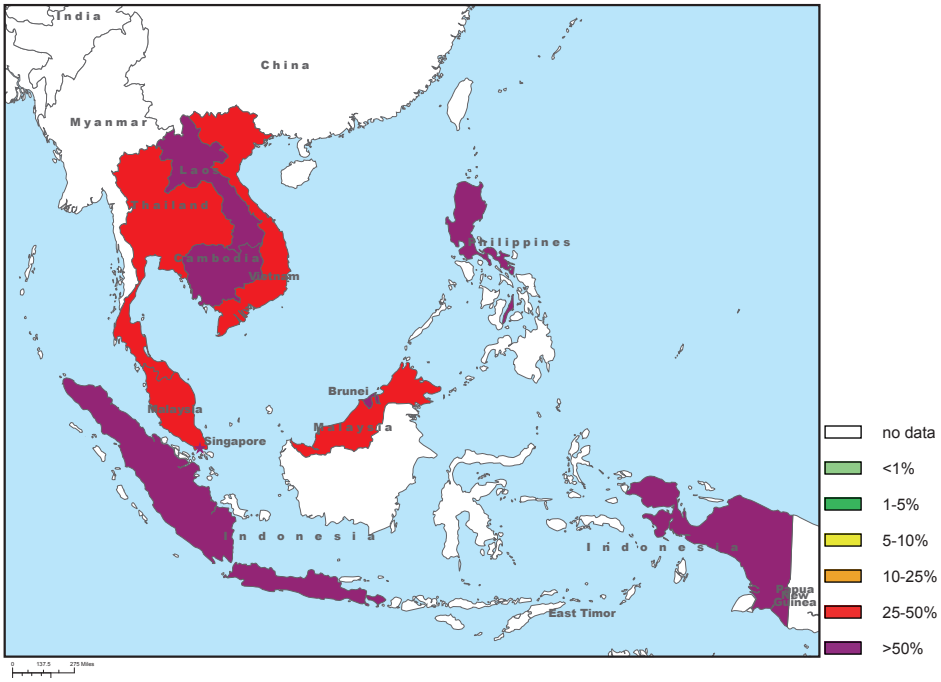

Figure 5. Prevalence of penicillinase-producing *N. gonorrhoeae* (PPNG) in SE Asian countries, 1995-2007.

plasmid-mediated tetracycline resistance (TRNG) (Figure 6b). Rates ranged from 6% in Malaysia (2005) to 100% in Bali and East-Java, Indonesia (2004) (21, 77, 96). In the Philippines, an increase in tetracycline resistance was observed from 8% in 1994 to 30% in 2005 (40, 96). Quinolone resistance in gonococci, which is the result of chromosomal changes in *gyrA* or *parC* genes, has been emerging in SE Asia since 1993, as shown by the WHO WPR GASP data. First, strains "less sensitive" to quinolones were observed. Infections with these strains could be treated with an increased dose of the fluoroquinolone. Subsequently, strains with higher MICs were detected and these were not amenable to therapy even with higher-dose regimens. In 2005, more than 50% of isolates were resistant or less susceptible to quinolones in Brunei, Laos, Singapore, and Vietnam (96). Other countries in SE Asia with high rates of decreased susceptibility to quinolones, but not part of the WHO WPR GASP study, include Indonesia (50% in 2004) and Thailand (22% in 1994-1995) (Figure 6c) (21, 41, 77). Resistance to penicillin, tetracycline, and quinolones are now so widespread in most SE Asian countries that these have become unreliable as a first-line treatment for gonococcal disease. Alternatives include third-generation cephalosporins, spectinomycin (injectible), and azithromycin. Strains with a decrease in susceptibility to third-generation cephalosporins have been detected in recent WHO WPR GASP surveys, but exact prevalences were not reported. Spectinomycin resistance is only

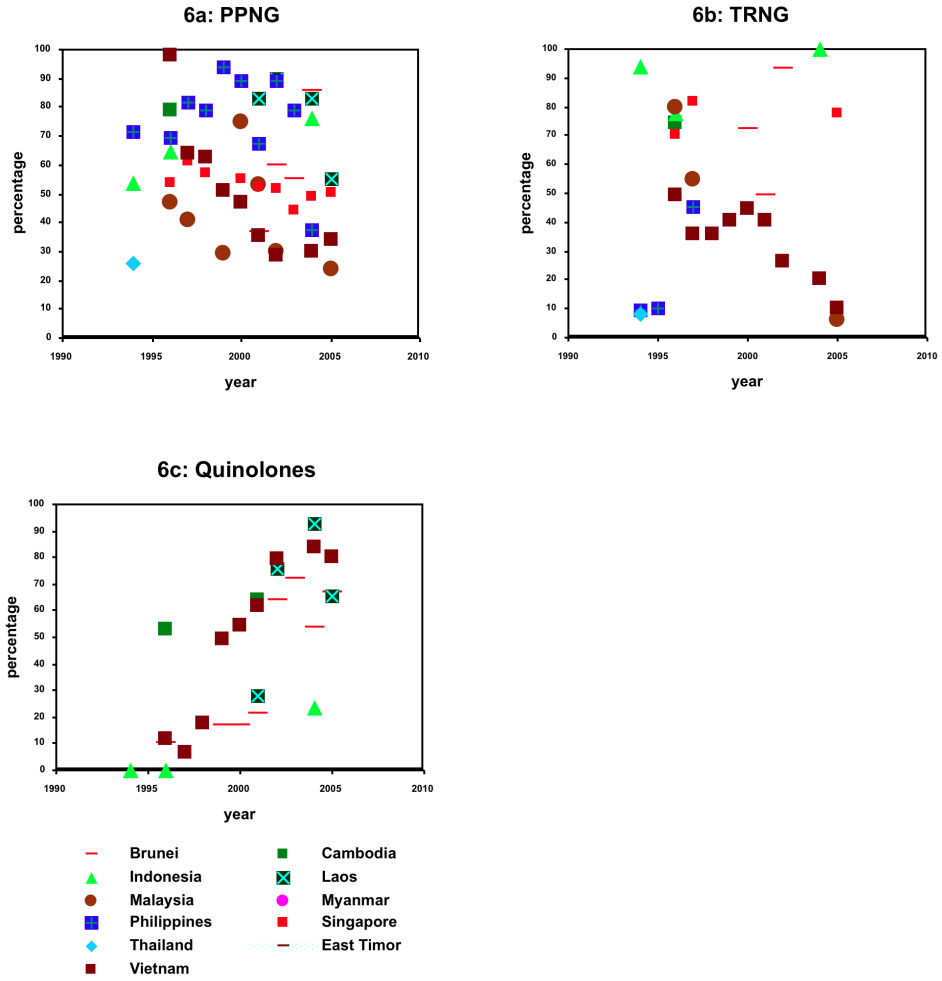

Figure 6. Resistance among *N. gonorrhoeae* from SE Asia.

rarely observed. In Jakarta, this antibiotic is now used as a first-line therapy. However, when extensively used, resistance emerges quickly, as occurred in the mid-80s. Data on azithromycin resistance from SE Asia are scarce. In 2004, no resistance to this macrolide was observed in isolates from Denpasar, Indonesia (21, 77).

Enterobacteriaceae

Escherichia coli

E. coli isolates exist as normal flora in the gut of humans and animals and were originally susceptible to many antimicrobial agents. However, selective pressure by repeated exposure to antibiotics has led to the development of resistance (97). From

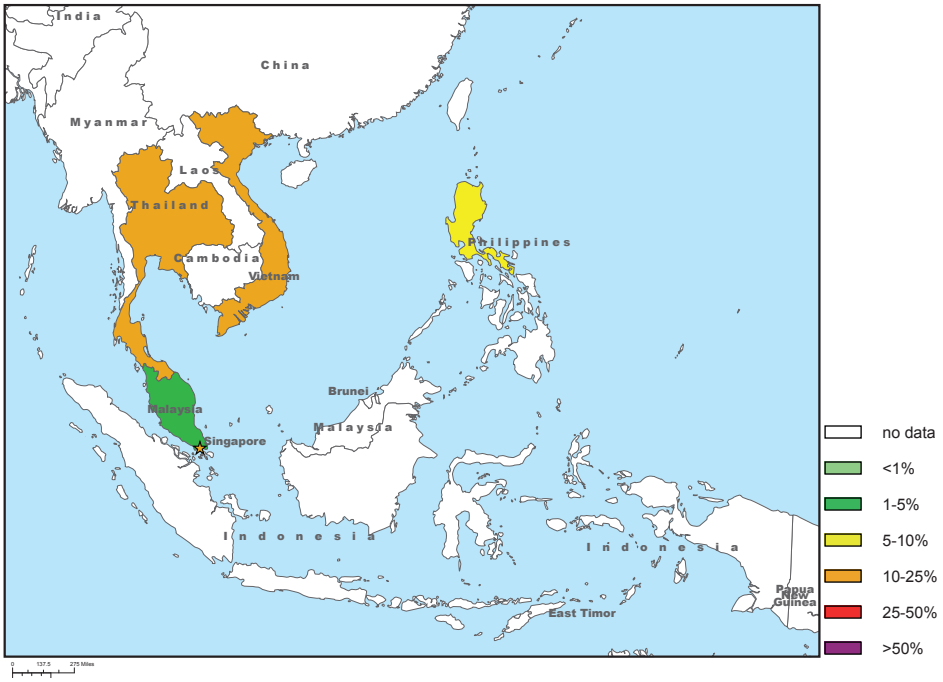

Figure 7. Prevalence of ESBL-producing *E. coli* in SE Asian countries, 1995-2007.

SE Asia, there are many studies that have assessed antimicrobial resistance of *E. coli* (Figure 7 and Figure 8). The overall prevalence of ampicillin resistance was ≥50%, which is much higher than found in other countries in the world (22). The emergence of fluoroquinolone resistance among Gram-negative rods is, however, a phenomenon seen worldwide. In Malaysia, ciprofloxacin resistance increased from 0% in 1992 (11) to 29% in 2004 (63). An increase in ciprofloxacin resistance was also observed in Singapore and Thailand (Figure 8b). In the Philippines, the resistance rate to ciprofloxacin was the highest among all SE Asian countries, 54% in 1998, but in this analysis only extended spectrum β-lactamase (ESBL)-positive *E. coli* isolates were included. The association between ESBL production and quinolone resistance has been recognized worldwide (7).

The aminoglycosides, such as gentamicin, are important antibiotics in the empirical treatment of severe infections suspected to be caused by aerobic Gram-negative rods. In Malaysia, gentamicin resistance increased from 1% in 1992 to 30% in 2004. In Singapore, gentamicin resistance rate was ≤10% in 1992 and 1994, but more recent data are not available. Again, the highest resistance rate described in SE Asian reports was among ESBL-positive isolates from the Philippines (46% in 1998). No resistance to gentamicin was found among *E. coli* from blood from HIV-positive patients in Thailand in 1997 (Figure 8c). However, resistance to trimethoprim-sulfamethoxazole

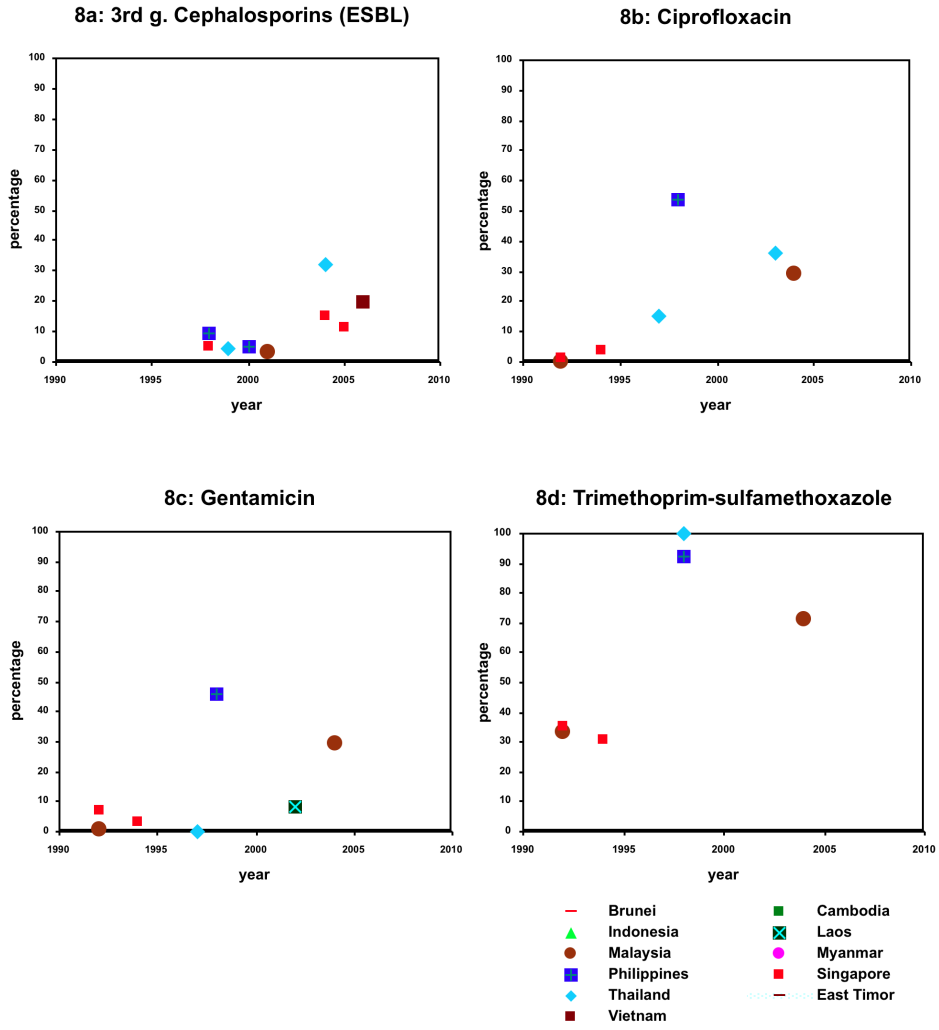

Figure 8. Resistance among *E. coli* from SE Asia.

was high in this collection (100%). Among ESBL-positive isolates from the Philippines, the resistance rate was 92%. In Figure 8d it is shown that resistance to trimethoprim-sulfamethoxazole in Malaysia increased from 34% in 1992 to 71% in 2004. This is much higher than prevalences described in Europe or the Americas (22).

Additional resistance data are presented in Supplementary Table S6. No data were available from Brunei, Cambodia, Laos, Myanmar, and Vietnam.

Enterotoxigenic *E. coli* (ETEC)

The antimicrobial resistance among ETEC, an important cause of diarrhea in developing countries, particularly among young children, has been studied in three SE

Asian countries: Indonesia (76), Thailand (31) and Vietnam (31). The prevalence of resistance to tetracycline was 81%, 43%, and 65%; to chloramphenicol 61%, 13%, and 17%; to trimethoprim-sulfamethoxazole 63%, 51%, and 63%; and to ampicillin 78%, 54%, and 67% in Indonesia, Thailand and Vietnam, respectively. Resistance to ciprofloxacin, azithromycin, and nalidixic acid was less than 5% (Supplementary Table S7).

Klebsiella pneumoniae

Studies on the prevalence of antimicrobial resistance among *K. pneumoniae* are available from Malaysia (63), Laos (62), the Philippines (7), Singapore (7), and Thailand (46) (Figure 9 and Figure 10). It should be noted that the latter three studies only involved ESBL-positive isolates. Not surprisingly, ciprofloxacin resistance was prevalent in the Philippines (62%), Thailand (29%), and Singapore (22%). Among *K. pneumoniae* from diabetic feet in Malaysia, the resistance rate to ciprofloxacin was 9%, to nalidixic acid 17%, and to trimethoprim-sulfamethoxazole 26%. Resistance to aminoglycosides also often exists in ESBL-positive isolates. Indeed, 26% of Thai and 6% of Philippine isolates were resistant to amikacin (Figure 10d).

Although there is a rapid global spread of carbapenemase-producing *K. pneumoniae*, imipenem resistance was not present in Malaysia, the Philippines, Singapore, and Thailand (Figure 10c).

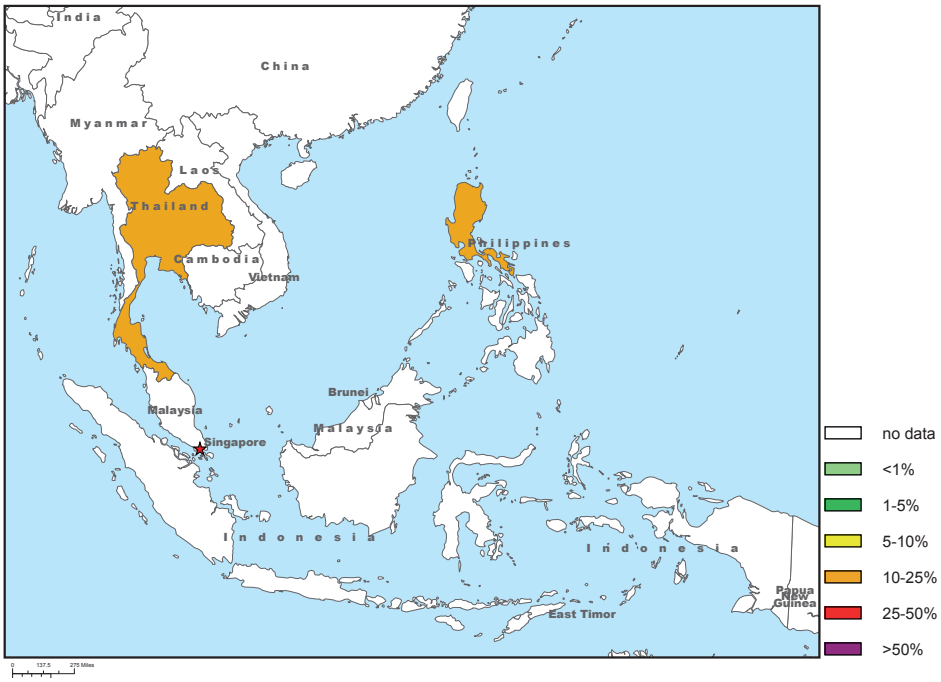

Figure 9. Prevalence of ESBL-producing *K. pneumoniae* in SE Asian countries, 1995-2007.

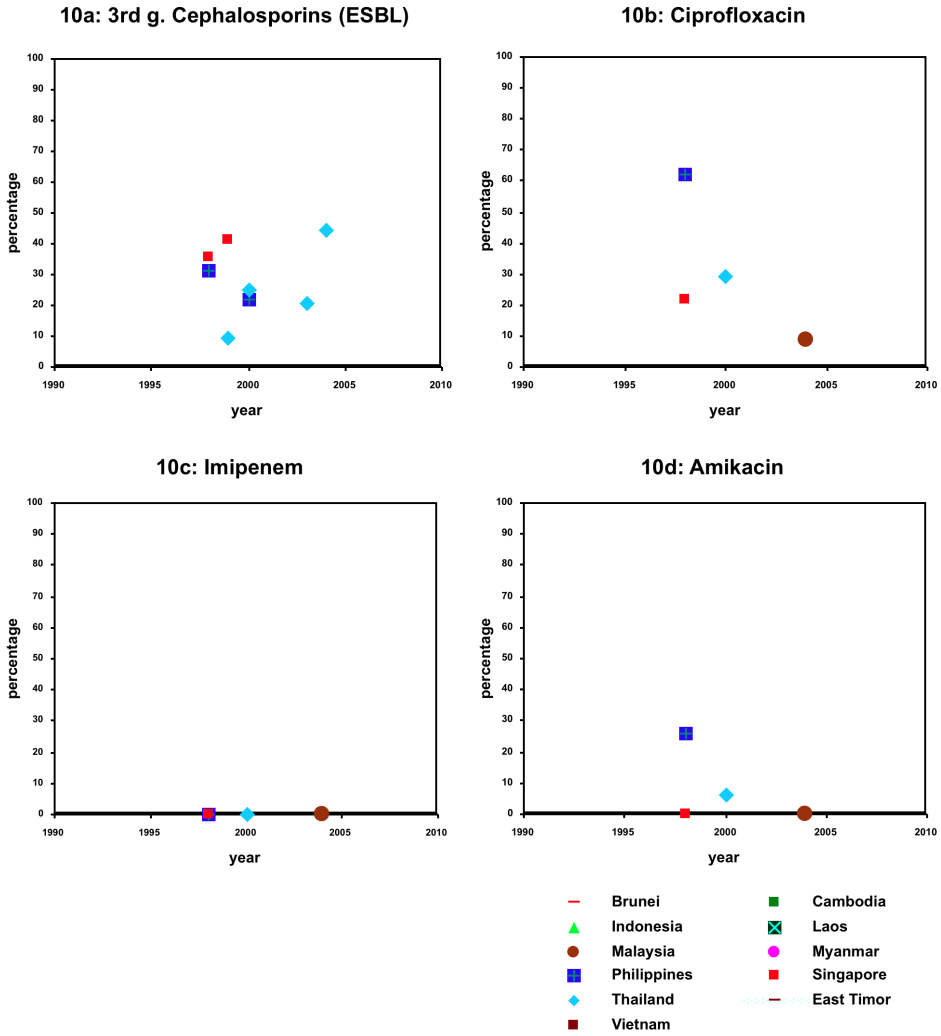

Figure 10. Resistance among *K. pneumoniae* from SE Asia.

Additional resistance data are presented in Supplementary Table S8.

ESBL-producing Enterobacteriaceae

ESBL-producing Enterobacteriaceae are an increasing problem worldwide. Therefore, this emerging resistance phenomenon is discussed separately. Reliable detection of ESBL-producing bacteria includes a screening test followed by a confirmation test. This is, however, not straightforward, because different ESBL enzymes hydrolyze oxyimino-cephalosporins at different rates and other mechanisms of resistance may interfere with the tests. Furthermore, methods for screening and phenotypic confirmatory testing of bacteria other than *E. coli*, *K. pneumoniae*, and *K. oxytoca*, have not yet been

determined by the CLSI. Accurate information on ESBLs from SE Asia is available from Singapore (7, 101, 103), the Philippines (7, 103), Thailand (46, 100, 102, 104), Malaysia (97), and Vietnam (105) (Figure 7, Figure 8a, Figure 9, and Figure 10a). In 1998-1999, confirmed ESBL-producing isolates were present in SE Asia as reported by the Asia-Pacific group of the SENTRY Antimicrobial Surveillance Programme (7). Among clinical strains of *E. coli* rates were 5% and 9%, respectively in Singapore and the Philippines. For *K. pneumoniae*, these rates were much higher, 42% and 32%. In the same period, 11% of *E. coli*, 36% of *K. pneumoniae*, and 39% of *K. oxytoca* were ESBL-positive in Singapore. A steep increase was observed for *K. pneumoniae* in Thailand: from 10% in 1999 in Bangkok to 21% in Pratumthani (central Thailand) in 2003 and 44% in Songkhla Province (south Thailand) in 2004. The latter high prevalence is from isolates from blood cultures only, which is of concern since ESBL-producing strains are associated with poor patient outcome probably due to inappropriate first-line treatment. In Kuala Lumpur, Malaysia, and in Ho Chi Minh City, Vietnam, 3% and 19% of clinical *E. coli* were ESBL-producing, respectively. Overall, the prevalence of ESBLs in SE Asia is higher in *K. pneumoniae* than in *E. coli*, which is consistent with findings elsewhere in the world.

Molecular characterization of clinical strains producing ESBLs has been reported from Malaysia, Vietnam, Singapore, and Thailand. However, this is beyond the scope of this review.

Salmonella spp.

Antimicrobial therapy is not recommended for uncomplicated salmonellosis, but appropriate antibiotics are crucial for patients with invasive infections. The fluoroquinolones are the most optimal choice for the treatment of typhoid fever, but the emergence of resistance to fluoroquinolones suggests that their use should be restricted. Then, traditional first-line drugs should be considered (tetracycline, chloramphenicol, ampicillin, trimethoprim-sulfamethoxazole), or the more expensive parenteral ceftriaxone in severe infections. Trends in tetracycline, chloramphenicol, ciprofloxacin, and ampicillin resistance among all *Salmonella* spp. are depicted in Figures 11a to 11d.

Resistance to tetracycline among *Salmonella* spp. in Thailand was 58% in 1998 (31) and 100% in 2003 (2). In Malaysia, Vietnam, and Indonesia, tetracycline resistance rates were much lower: 11% in 1995 (Malaysia), 7% in 1998 (Vietnam), 21-20% in 1998-1999 (Indonesia) (Figure 11a).

Chloramphenicol resistance among Thai *Salmonella* spp. decreased slightly over the years from 35% in 1993 to 28% in 1998 and 24% in 2003. In Indonesia, the resistance rate remained stable: 16% in 1998 and 13% in 1999. In Laos, Malaysia and Vietnam, the resistance rate was 12% (2002), 7% (1995), and 0% (1998), respectively (Figure 11b).

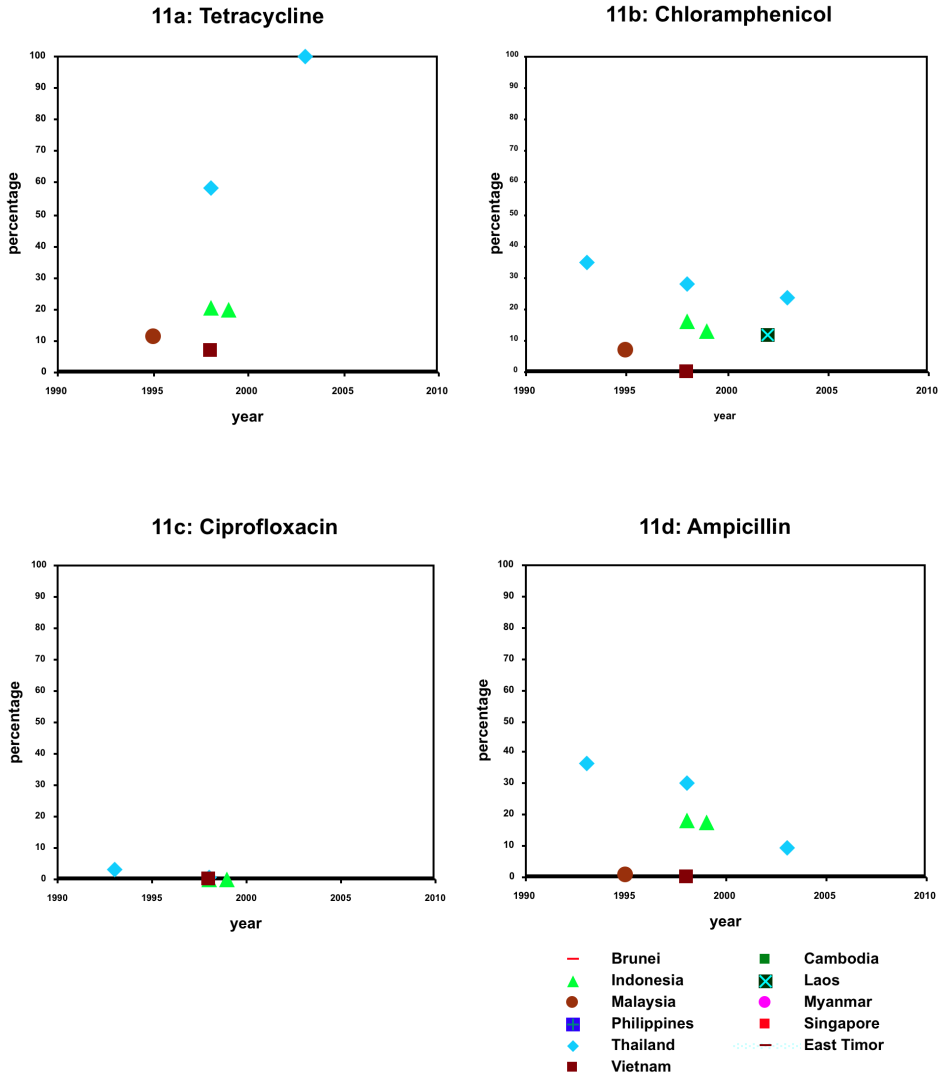

Figure 11. Resistance among *Salmonella* spp. from SE Asia.

Resistance rates to ciprofloxacin were lower than to the other antibiotics tested in this species (range 0 – 0.5%) (Figure 11c). However, in almost all studies included in our analysis, antimicrobial susceptibility testing was performed using disk diffusion. This method does not detect reduced susceptibility to ciprofloxacin (MIC ≥0.25 µg/mL). In a Finnish study on *Salmonella* isolates from travelers returning from abroad, reduced fluoroquinolone susceptibility was particularly seen among isolates from travelers returning from SE Asia (Thailand, Indonesia, Malaysia) (25). In all these isolates, a point mutation in the quinolone-resistance determining region (QRDR) of *gyrA* was present. Such isolates are important to identify, since infections with these

should not be treated with standard fluoroquinolone therapy. The CLSI currently recommends disk diffusion testing of nalidixic acid as a marker for the detection of reduced susceptibility of *Salmonella* spp. to fluoroquinolones, which was indeed performed in the studies by Oyofo et al. and Isenbarger et al. In these studies, resistance to nalidixic acid was frequent among Thai isolates (up to 33%), but not present at all among Indonesian isolates.

In Thailand, ampicillin resistance decreased from 37% in 1996 to 30% in 1998 and 10% in 2003. In Indonesia, the rates of resistance remained stable in the late 1990s: 19% in 1998 and 18% in 1999. Lower prevalences of resistance were observed in Malaysia and Vietnam, 1% in 1995 and 0% in 1998, respectively (Figure 11d). Overall resistance rates are presented in Supplementary Table S9.

In Indonesia, *S.* Typhi isolates were universally susceptible to commonly used antimicrobials (59, 60, 85), but in Laos, resistance rates to ampicillin, chloramphenicol, and trimethoprim-sulfamethoxazole were 12%, 12%, and 11%, respectively (62).

A collection of non-Typhi *Salmonella* was studied in Malaysia with low resistance rates found to tetracycline (11%), chloramphenicol (7%), trimethoprim-sulfamethoxazole (5%), streptomycin (4%) and ampicillin (1%). There was no resistance against ciprofloxacin and kanamycin (49).

Overall, although resistance to quinolones seems to be emerging in SE Asia, resistance to traditional first-line antibiotics is decreasing.

Shigella spp.

Worldwide it is estimated that shigellosis is responsible for some 600,000 deaths each year, two-thirds of which are in children aged under 10 years. The incidence of shigellosis is the highest in developing countries where general standard of living and sanitary conditions are usually poor (3). *S. flexneri* and *S. sonnei* are the predominant species in developing countries. Antimicrobial treatment can reduce morbidity, mortality, and transmission. The antibiotics commonly used are trimethoprim-sulfamethoxazole, tetracycline, ciprofloxacin, chloramphenicol, and ampicillin.

In 1984 and 1985, no resistance to trimethoprim-sulfamethoxazole was observed among *Shigella* spp. in Myanmar, but in 1989 48% and in 1993 63% of isolates was resistant (Figure 12a) (58). In Vietnam, resistance patterns of *Shigella* spp. isolates collected between 1989 and 1998 were studied by the National Program for Surveillance of Antimicrobial Resistance (NPSAR) (3). Resistance to trimethoprim-sulfamethoxazole increased from 25% in 1989 to 94% in 1994 and remained >70% thereafter. Trimethoprim-sulfamethoxazole resistance rates were also high in Indonesia (54% in 1998, 73% in 1999, and 67% in 2000) and in Thailand (91% in the period 1996 to 1999) (31) (Figure 12a).

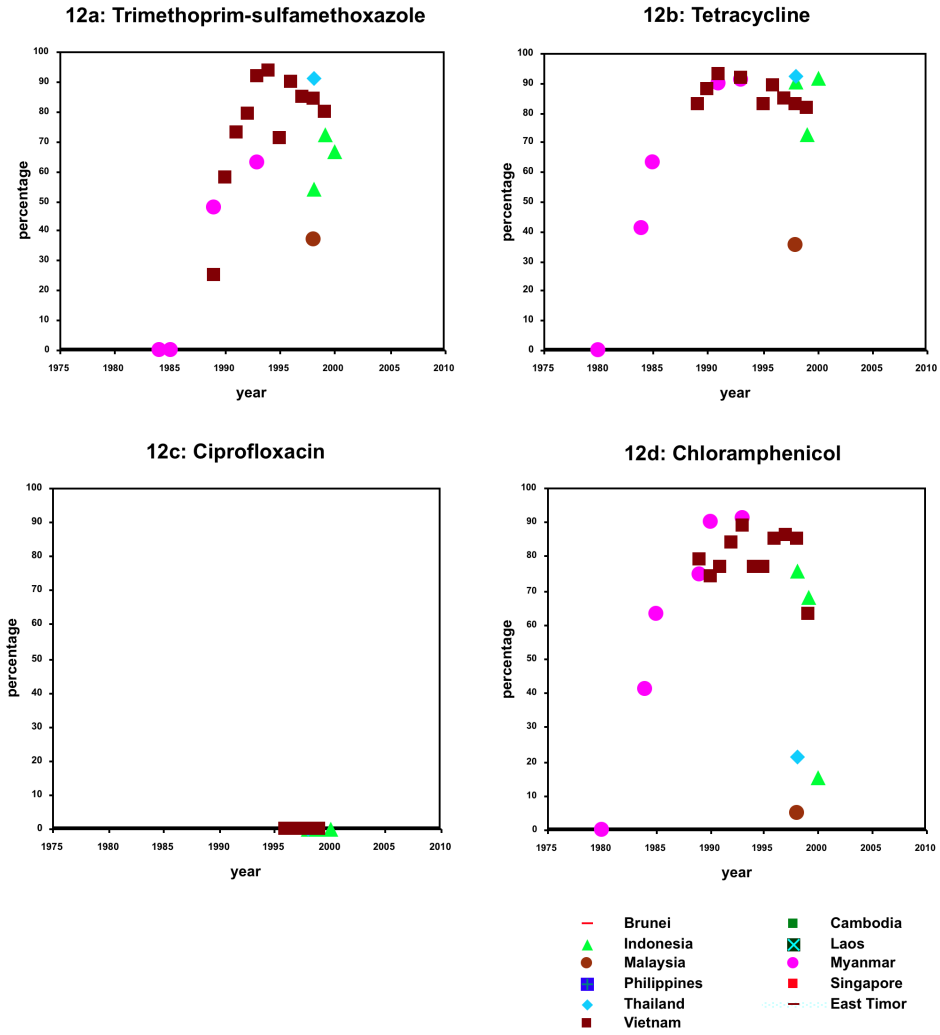

Figure 12. Resistance among *Shigella* spp. from SE Asia.

In many countries, tetracycline is not used anymore as empirical therapy for shigellosis due to the high resistance rates. High resistance rates are also observed in SE Asia. In Myanmar, tetracycline resistance among *Shigella* spp. increased over the years from 0% in 1980 to 41% in 1984, 63% in 1985, 90% in 1991 and 91% in 1993. In Vietnam, prevalences of resistance have been ≥80% since 1990. In Indonesia, resistance to tetracycline was studied by Oyofo et al. (59, 60) and Tjaniadi et al. (85). Resistance was 90%, 73% and 92% in 1998, 1999 and 2000, respectively. In Thailand, an even higher rate of resistance to tetracycline was observed compared with Malaysia and Indonesia in the same period (Figure 12b).

Ciprofloxacin resistance among *Shigella* spp. was not found in Vietnam and Indonesia (Figure 12c).

For chloramphenicol, there was a steady increase in resistance rates over the years in Myanmar: from 0% in 1980 to 41% in 1984, 63% in 1985, 75% in 1989, 90% in 1990 and 91% in 1993. In Vietnam, the resistance rates were over 60% (range 63% - 89%). In Indonesia, the prevalence of resistance decreased from 76% in 1998 to 68% in 1999 and 15% in 2000. In Thailand and Malaysia, resistance rates were 21% and 5%, respectively, in 1998 (Figure 12d).

In Supplementary Table S10 and Supplementary Table S11 additional data are provided for *S. flexneri* and *S. sonnei*. Overall, prevalences of resistance were high for ampicillin, tetracycline, chloramphenicol, and trimethoprim-sulfamethoxazole in *S. flexneri* and for tetracycline, chloramphenicol, and streptomycin in *S. sonnei*, which is in agreement with trends observed worldwide (57).

Other glucose fermenting Gram-negative rods

Microorganisms belonging to the genera *Proteus*, *Enterobacter*, *Serratia*, and *Citrobacter* are members of the family Enterobacteriaceae, of which the latter three rarely cause infections in healthy hosts, but are common nosocomial pathogens. Most information on antimicrobial resistance among these four bacterial species in SE Asia are from Biedenbach and Jones, together with the Philippines, Thailand, or Indonesia Antimicrobial Resistance Study Group (9, 34, 51). They evaluated the *in vitro* activity of broad-spectrum β-lactam antibiotics against isolates of clinical bacteria from the Philippines, Thailand, and Indonesia, collected in 1998. However, infections caused by *Enterobacter*, *Serratia*, and *Citrobacter* should not be treated with third-generation cephalosporins or piperacillin-tazobactam, due to their inducible AmpC enzyme, and therefore, results for these antibiotics from this study are of limited use. Furthermore, the exact origin of the isolates in these studies, i.e. community-acquired or hospital-acquired, from an intensive care unit or a general surgery ward, was not indicated. This information is of utmost importance for the proper interpretation of the results.

Proteus spp.

Proteus spp. are common causes of urinary tract infections, occasionally in normal hosts and very commonly in patients with indwelling catheters. They have been studied for antimicrobial susceptibility in Malaysia (63), Singapore (45), the Philippines (34), and Thailand (9). In Malaysia, *Proteus* spp. from diabetic foot infections were analyzed. Of the 42 isolates, 62% were resistant to ampicillin, 33% to trimethoprim-sulfamethoxazole, 27% to ciprofloxacin, 19% to amoxicillin-clavulanic acid, and 10% to gentamicin (63). All isolates remained susceptible to imipenem and amikacin. In Singapore, resistance rates among *Proteus* spp. from various clinical specimens to am-

picillin, trimethoprim-sulfamethoxazole, nitrofurantoin and ciprofloxacin were 60%, 45%, 40%, and 0%, respectively. In the Philippines and Thailand, isolates remained highly susceptible to broad-spectrum β-lactams, except for ceftazidime, to which 20% of the indole-positive Thai isolates were resistant (Supplementary Table S12).

Enterobacter spp.

Data from antimicrobial resistance in *Enterobacter* spp. are available from Indonesia (51), the Philippines (7, 34), Singapore (7, 44, 45), and Thailand (9).

The SENTRY Antimicrobial Surveillance Program (1998-99) found a presumptive ESBL prevalence among *Enterobacter* spp. of 11% in Singapore, and 2% in the Philippines (7). In the period thereafter, the prevalence increased to 44% in Singapore and 35% in the Philippines, for *E. cloacae* (8). Strains with an ESBL phenotype had high rates of resistance to other antibiotics, such as ciprofloxacin, gentamicin, and trimethoprim-sulfamethoxazole: 50%, 58%, and 83%, respectively, in Singapore, and 33%, 48%, and 91%, respectively, in the Philippines. In two other reports from these two countries, imipenem resistance was not detected (34, 44). In addition, resistance to the aminoglycosides was low among isolates collected from a university hospital in Singapore in 1994 to 1995: 8% for amikacin and 3% for gentamicin, but resistance to ciprofloxacin was 11% and to trimethoprim-sulfamethoxazole 24%.

In Malaysia, *Enterobacter cloacae* from diabetic foot infections were highly susceptible to ciprofloxacin, imipenem and gentamicin, but resistance to trimethoprim-sulfamethoxazole was prevalent (31%) (63).

Among strains from Indonesia and Thailand, imipenem resistance was not detected, but resistance to cefepime, a fourth-generation cephalosporin, was 4% in both countries (9, 51). Non-β-lactams were not tested. Additional information on the resistance among *Enterobacter* spp. is shown in Supplementary Table S13.

Serratia spp.

Antimicrobial resistance among *Serratia* spp. was studied in Indonesia (51), the Philippines (34), and Thailand (9). Resistance to cefepime was 2% among isolates from Thailand, but not observed in isolates from the Philippines and Indonesia. A worrisome observation was the prevalence of imipenem resistance among Indonesian isolates (7%). Imipenem resistance was not found in isolates from the Philippines and Thailand (Supplementary Table S14).

Citrobacter spp.

Citrobacter spp. were studied in 1998 in the Philippines (34) and Thailand (9). The resistance to cefepime was 0% and 6% and to imipenem 0% and 2%, respectively, in

the Philippines and Thailand. No data are available on the susceptibility of *Citrobacter* spp. to non-β-lactam antibiotics.

Other (glucose-non-fermenting) bacteria

Campylobacter jejuni

Infection with *Campylobacter jejuni* has been recognized worldwide as the most frequent cause of bacterial gastroenteritis. Treatment of presumed *Campylobacter* gastroenteritis in otherwise healthy individuals is symptomatic and does not include antibiotics. When antibiotic therapy is indicated, macrolides, quinolones, and tetracycline are most often used. Antimicrobial resistance among *Campylobacter jejuni* has been studied in Thailand, Vietnam, and Indonesia (31, 59, 60, 85). In 1997, resistance to ciprofloxacin was 75% and 1%, respectively, among Thai and Vietnamese isolates, but resistance to azithromycin was low in Thailand (2%) and absent in Vietnam (31). From Indonesia, there are many data on *Campylobacter*. All report essentially similar trends, which are the emergence of ciprofloxacin-resistant strains (up to 43% in 2000), stably high prevalences of tetracycline resistance (>30%), and no resistance to macrolides (Supplementary Table S15).

Pseudomonas aeruginosa

P. aeruginosa strains exhibit intrinsic resistance to several β-lactam antibiotics and may acquire additional resistance mechanisms, such as broad-spectrum β-lactamases, that further reduce their susceptibility to antimicrobial drugs. For example, the Vietnamese extended-spectrum β-lactamase (VEB)-1, first described in a strain from Vietnam, was present in 23% of *P. aeruginosa* from Thailand, 1999, causing complete resistance to the oxymino-cephalosporins (23). The total resistance to ceftazidime in Thailand increased from 27% in 1999, to 52% in 2001 and 40% in 2002 (Figure 13b).

In one Malaysian report with isolates collected in 1992, strains were highly susceptible to various antibiotic classes, including ceftazidime, but from several surveillance studies conducted after 1992, it was shown that ceftazidime resistance was over 10% in the Philippines, Vietnam, and Malaysia. In Indonesia, resistance to ceftazidime was 7% in 1998 (51).

Except for the Malaysian strains collected in 1992, ciprofloxacin resistance rates among *Pseudomonas* spp. were over 10% in Malaysia (after 1992), Vietnam, Thailand and Singapore. In Malaysia, resistance increased from 1% in 1992 to 11% in 2005. The highest resistance rate was found in Vietnam, 82% in 2000, among isolates causing surgical site infections (Figure 13a).

Imipenem resistance was already detected in Malaysian *Pseudomonas* strains in 1992 (1%). Resistance rates of less than 10% were also reported in Malaysia (2004),

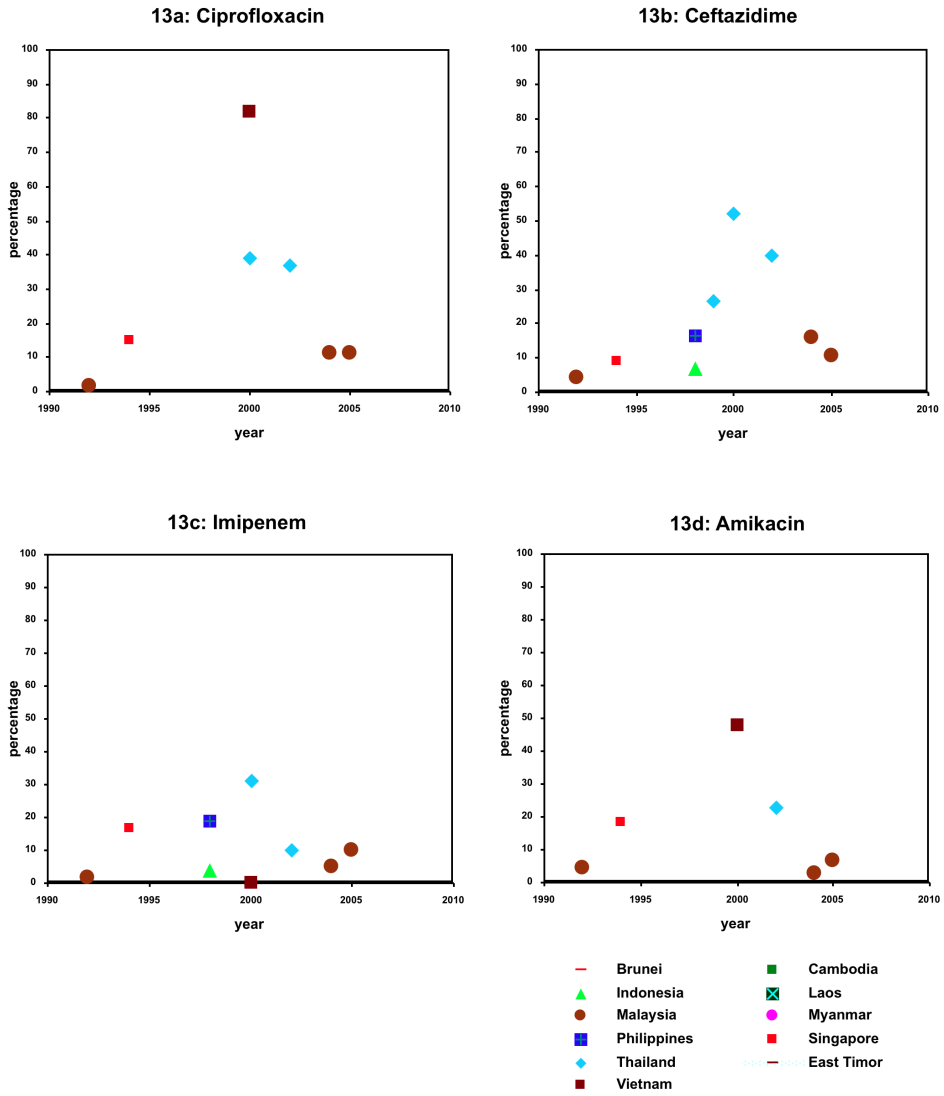

Figure 13. Resistance among *Pseudomonas aeruginosa* from SE Asia.

Indonesia, and Vietnam, but in Singapore, the Philippines and Thailand, rates were 10% or more (Figure 13c).

Amikacin resistance in Malaysia was less than 10% (range 3% - 7%). In Singapore, Thailand, and Vietnam, *Pseudomonas* isolates were more frequently resistant to amikacin. Again, the highest resistance rate was found in Vietnam (48% in 2000) (Figure 13d). None of the reports on resistance among *Pseudomonas* isolates presented prevalences of multi-drug resistance, i.e. resistance to three antibiotic classes or more, which occurrence is a matter of concern in many countries.

Acinetobacter spp.

Acinetobacter spp. – principally *A. baumannii* – are opportunistic pathogens of greatest concern, especially in intensive cares and as invaders of burn wounds. *A. baumannii* has shown a remarkable propensity to develop resistance to virtually every antibiotic class (52). Multidrug-resistant (MDR) *A. baumannii* and extensively-resistant *A. baumannii* (XDR) are reported with increasing frequency from around the world, but recent data from SE Asia are lacking (20). In a study from the National University Hospital in Singapore of 165 *Acinetobacter* spp. collected in 1991, resistance to ceftazidime was almost 50%, to the aminoglycosides ranged from 34-54%, to the quinolones from 4-21%, and to imipenem 5% (45). In another study from the same hospital, but three years later, it was shown that *Acinetobacter* spp. were one of the most commonly isolated Gram-negative bacilli from blood cultures (44). The authors reported moderate prevalences of resistance to ceftazidime (23%), piperacillin (26%), gentamicin (27%), ciprofloxacin (26%), and trimethoprim-sulfamethoxazole (15%). Resistance to imipenem and amikacin was less frequent: 7% and 8%, respectively (44). Among Thai isolates collected in 2002 from 24 hospitals across Thailand, resistance rates were much higher: 56% to ceftazidime, 24% to imipenem, 52% to amikacin, 56% to ciprofloxacin, and 66% to trimethoprim-sulfamethoxazole, but whether MDR isolates were present remains unknown. In this study, the isolates from community-acquired infections were generally more sensitive to the antibiotics than those from hospital-acquired infections (18). The data of resistance rates to several broad-spectrum β-lactam antibiotics from Singapore, Thailand, Indonesia, and the Philippines, which are mainly extracted from the reports by Biedenbach, Jones, and colleagues, are shown in Figures 14a to 14c. Remarkably, already in 1998 the imipenem resistance was 10% and 13%, respectively, in Indonesia and the Philippines (Figure 14b and Supplementary Table S16).

Burkholderia pseudomallei

B. pseudomallei, which is the causative microorganism of melioidosis, an endemic disease in most of SE Asia, has been studied in Laos, Malaysia, and Thailand. In Laos, *B. pseudomallei* was isolated from blood cultures of 14 out of 4,460 patients, but the mortality in these patients was as high as 60%. The resistance rate to chloramphenicol was 1% and to trimethoprim-sulfamethoxazole 0% (62). In Malaysia, resistance to cefoperazon, a third-generation cephalosporin, was 14%, but to cefoperazon-sulbactam 0% (42). In Thailand, resistance rate of trimethoprim-sulfamethoxazole was 13% by E-test and 71% by disk diffusion (99).

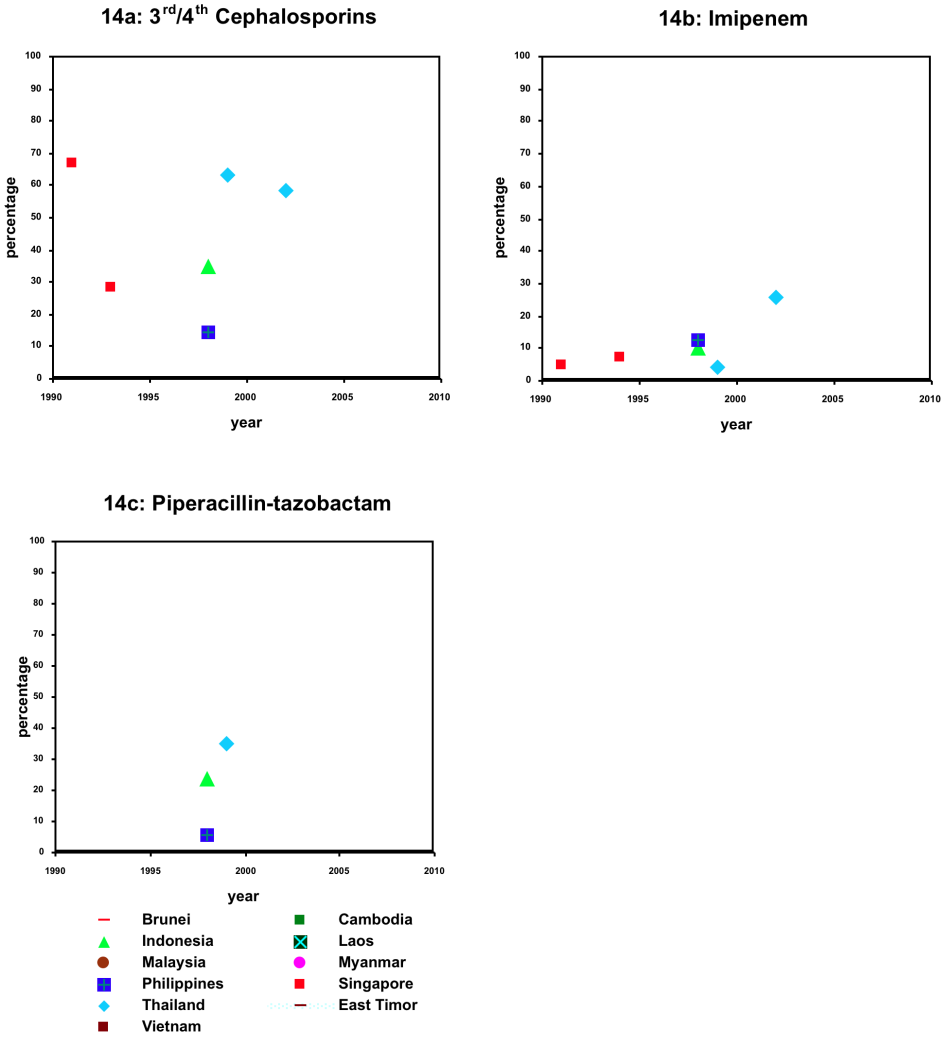

Figure 14. Resistance among *Acinetobacter* spp. from SE Asia.

Resistance among commensals

Carriage strains of *S. pneumoniae* have been studied in Malaysia, the Philippines, Singapore, Thailand, and Vietnam and commensal *E. coli* in the Philippines and Thailand.

Commensal *E. coli*

The fecal flora of the general population represents a potentially large reservoir of antimicrobial-resistant bacteria and mobile genetic elements with resistance genes. Therefore, commensal *E. coli* can be used as an indicator organism for resistance. In Thailand, the antimicrobial resistance profiles of *E. coli* from swine and chicken farm workers were determined. The prevalence of resistance for tetracycline, chloram-

phenicol, ampicillin, and nalidixic acid were 67%, 56%, 44%, and 33%, respectively. All strains were tested susceptible to ciprofloxacin and ceftriaxone (26). Nys et al. studied fecal *E. coli* from healthy adult volunteers from the Philippines. High resistance rates were found for all antibiotics tested, including tetracycline (90%), trimethoprim (84%), ampicillin (81%), chloramphenicol (64%), ciprofloxacin (49%), gentamicin (33%), and cefazolin (21%) (55).

Carriage isolates of *S. pneumoniae*

Knowledge of the prevalence of carriage of resistant pneumococci in children is likely to have predictive value in defining the status of resistance in a certain region. The

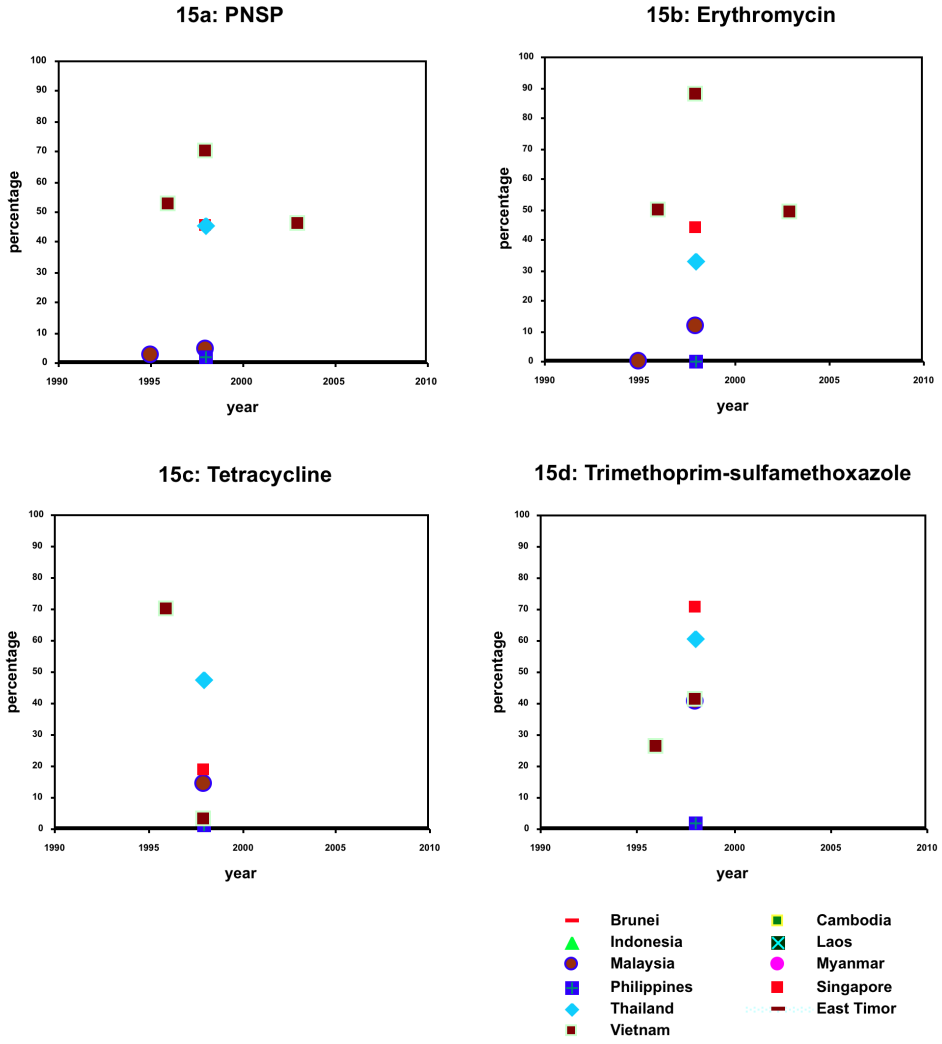

Figure 15. Resistance among *S. pneumoniae* from SE Asia (from nonclinical material).

general trend observed among the different collections studied is that the resistance to penicillin and erythromycin has reached very high levels. In Malaysia, the PNSP rate increased from 3% in 1995 to 5% in 1998. In Vietnam, the prevalence of PNSP increased from 53% in 1996 to 70% in 1998 and decreased thereafter to 46% in 2003. In 1998, the PNSP rate was only 2% in the Philippines, but 46% in Thailand and Singapore (Figure 15a).

Erythromycin resistance in *S. pneumoniae* isolates was found in Malaysia, Vietnam, Singapore, Thailand, and the Philippines, but some geographical variation existed. In Malaysia, resistance to erythromycin was 0% in 1995 and 11% in 1998. In Vietnam, levels of resistance were much higher: 50% in 1996, 88% in 1998 and 49% in 2003. In 1998, resistance rates to erythromycin were 44%, 33% and 0%, respectively, in Singapore, Thailand and the Philippines (Figure 15b).

For tetracycline, the highest prevalence of resistance was again found in Vietnamese pneumococci (70% in 1996). The lowest prevalence was observed in strains from the Philippines (1% in 1998) (Figure 15c). Similarly, prevalences of resistance to trimethoprim-sulfamethoxazole in the SE Asian countries ranged from 2% to 71% and are depicted in Figure 15d. Thus, the increasing levels of resistance to penicillin and other antimicrobials in carriage isolates of *S. pneumoniae* are consistent with increasing drug resistance in clinical isolates.

CONCLUSIONS AND FINAL REMARKS

Antimicrobial resistance to pathogenic bacteria has been and still is on the rise in SE Asia. The high prevalences of resistance to β-lactam and non-β-lactam antibiotics in *S. pneumoniae* and *N. gonorrhoeae* is of great concern. Pathogens causing diarrheal diseases are now often resistant to inexpensive, older antibiotics. Among Enterobacteriaceae and nonfermenting Gram-negative bacteria, resistance to virtually all antibiotic classes have been reported, but whether MDR Gram-negatives are a problem is unknown. The picture for MRSA is not fully clear yet as well, but in some countries, such as Singapore, MRSA is endemic in the health care system. However, there is still much unknown. In the period between January 1, 1995 to January 1, 2007, 97 reports were published with accurate data on the resistance patterns among the major pathogens. None of these reports contained data from East Timor. From Brunei and Cambodia, only data on *N. gonorrhoeae* were available. From Myanmar, a single report on *Shigella* was included. Thailand was the country where from most published data were found. Most SE Asian countries have been parcipitating in one of the large international surveillance programmes. The Philippines and Singapore are, for example, part of the Asia-Pacific group of the SENTRY Antimicrobial Surveil-

lance Programme. In 1996, the Asian Network for Surveillance of Resistant Pathogens (ANSORP) was initiated, and as for 2008, six SE Asian countries are participating. These large international surveys have shown large-scale secular trends, often with high-quality microbiological data. However, from resistance data from other parts of the world, we know that there is great variation in resistance rates among different hospital and patient types wihtin countries. Especially in a large country such as Indonesia with more than 17,000 islands, it is likely that the resistance rates differ between one region and another. Therefore, good local data are essential, and for this, good laboratory facilities and qualified personnel.

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Supplementary Table S1. References available per species of organisms isolated from clinical material in Southeast Asian countries.

Organisms	Brunei	Cambodia	Indonesia	Laos	Malaysia	Myanmar	Philippines	Singapore	Thailand	East Timor	Vietnam
Streptococcus pneumoniae			(72)		(48, 69, 71, 72)		(48, 71)	(14, 43, 48 68, 69, 71, 72)	(13, 17, 35, 48, 69, 71, 72, 129)		(48, 69, 71, 72, 88)
Enterococcus spp.					(63, 64)			(12)	(18)		
Staphylococcus aureus			(70)	(62)	(11, 63, 66)		(6, 14, 70)	(6, 14, 70)	(70)		(70)
CoNS								(45)	(18)		
Haemophilus influenzae								(82)	(17)		
Neisseria gonnorrhoeae	(81, 88, 91-96)	(88)	(19, 21, 30, 50, 75, 77)	(92-96)	(81, 88-93, 96)		(38, 40, 81, 88-96)	(81, 88-96)	(41)		(81, 88-93, 95, 96)
Escherichia coli			(51)	(62)	(11, 63, 97)		(7, 14, 103)	(7, 44, 45, 101, 103)	(9, 18, 73, 100, 102, 104)		(105)
Enterotoxigenic *E. coli* (ETEC)			(76)						(31)		(31)
Klebsiella pneumoniae				(62)	(63)		(7, 103)	(7, 103)	(46, 100, 102, 104)		
Klebsiella spp.			(51)				(34)	(44, 45)	(9,)		
Salmonella spp.			(59, 60)	(62)	(49)		(34)		(2, 31, 73, 74)		
Shigella spp.			(58-60, 85)		(29)	(58)			(31)		(31, 32)
Proteus spp.					(63)		(34)	(45)	(9)		
Enterobacter spp.			(51)				(7, 34)	(7, 44, 45)	(9)		
Serratia spp.			(51)				(34)		(9)		
Citrobacter spp.							(34)		(9)		
Campylobacter jejuni			(59, 60, 85)						(31)		(31)

Organisms	Brunei	Cambodia	Indonesia	Laos	Malaysia	Myanmar	Philippines	Singapore	Thailand	East Timor	Vietnam
Pseudomonas aeruginosa			(51)		(11, 63, 65)		(14, 34)	(44, 45)	(9, 73, 84)		(47)
Acinetobacter spp.			(51)				(34)	(44, 45)	(9, 18)		
Burkholderia pseudomallei				(62)	(42)				(99)		

Supplementary Table S2. References available for species of potentially pathogens organisms isolated by screening in Southeast Asian countries.

Organisms	Brunei	Cambodia	Indonesia	Laos	Malaysia	Myanmar	Philippines	Singapore	Thailand	East Timor	Vietnam
Streptococcus pneumoniae					(48, 53)		(48)	(48)	(48)		(48, 61, 67)
Escherichia coli							(55)		(26)		

Supplementary Table S3. Overall resistance rates among *S. pneumoniae* from clinical samples.

Antibiotic	Indonesia (% R)	Malaysia (% R)	Philippines (% R)	Singapore (% R)	Thailand (% R)	Vietnam (% R)
Penicillin I[a]	3	8	21	14	29	26
Penicillin	18	18	0	36	30	41
Erythromycin	36	26	18	45	29	76
Tetracycline	46	27		59	52	52
Chloramphenicol	6	9		28	18	87
Ciprofloxacin		5	9	6	4	5
Trimethoprim-sulfamethoxazole	42	15		59	50	61
Cefepime						6
Ceftriaxone		2	0	0	5	2
Imipenem					0	0
Clindamycin				11		
Azithromycin					45	61
Cefuroxime	3	30	0	23	48	74
Cefotaxime	3			8	21	17
Clarithromycin					55	
Moxalactam		0	0	0	0	0
Gatifloxacin		0	0	0	0	0
Levofloxacin		0	0	0	0	0
Amoxicillin					0	
Ceftibuten					42	
Roxithromycin					30	

[a] I, intermediately susceptible.

Supplementary Table S4. Overall resistance rates among *S. aureus* from clinical samples.

Antibiotic	Indonesia (% R)	Laos (% R)	Malaysia (% R)	Philippines (% R)	Singapore (% R)	Thailand (% R)	Vietnam (% R)
Methicillin		0	25	7	39		
Penicillin		88	91		94		
Erythromycin		59	33		0		
Tetracycline			39				
Fucidic acid			7		7		
Chloramphenicol		21	3				
Ciprofloxacin				60	97		
hVISA[a]	0		0	4	2	2	2
Rifampicin			0				
Trimethoprim-sulfamethoxazole		22	5		55		
Cefepime	0					0	
Cefpirome	0					0	

Ceftazidime	14				7			6	
Ceftriaxone	0							0	
Imipenem	0								
Clindamycin				2					
Telithromycin	3								
Amikacin							0		
Gentamicin		2							

[a] hVISA, heterogenous vancomycin intermediately susceptible *S. aureus*.

Supplementary Table S5. Overall resistance rates among *N. gonorrhoeae* from clinical samples.

Antibiotic	Brunei (% R)	Laos (%R)	Cambodia (% R)	Indonesia (% R)	Malaysia (%R)	Philippines (% R)	Singapore (% R)	Thailand (% R)	Vietnam (% R)
Penicillin	64	98	79	66	48	84	58		29
Ciprofloxacin				30.6					
Trimethoprim-sulfamethox-azole				0					
Ceftriaxone				0					
Ofloxacin				0					
Norfloxacin				0					
PPNG[a]	65	80	79	68	39	76	54	26	47
TRNG[a]	68	98	74	86	61	14	74	8	33
Spectinomycin	0		0	0	0	0	0	0	
Kanamycin				0				22	
Norfloxacin				0					
Thiamphenicol				2.7					
Cefotaxime				0					
Cefixime				0					
Cefoxitin				0					
Azithromycin				0					
QRNG[a]	45	50	56	23	13	55	24		51

[a] PPNG, penicillinase-producing *N. gonorrhoeae*; TRNG, high-level plasmid-mediated tetracycline-resistant *N. gonorrhoeae*; QRNG, quinolone-resistant *N. gonorrhoeae*.

Supplementary Table S6. Overall resistance rates among *E. coli* from clinical samples.

Antibiotic	Indonesia (% R)	Laos (% R)	Malaysia (% R)	Philippines (% R)	Singapore (% R)	Thailand (% R)
Tetracycline			62	92		
Chloramphenicol		38	25			40
Ciprofloxacin			2	54	2	36
Gentamicin		8	2	46	7	0
Trimethoprim-sulfamethoxazole			36	92	35	100
Cefepime	3					0

Cefpirome	0			1		0
Ceftazidime	10	64	0	4	3	14
Ceftriaxone	3	8	0	2	2	1
Imipenem	0		0	0	2	0
Pefloxacin			2			
Amikacin			0	8	0	9
Netilmicin			0			0
Nitrofurantoin			3		10	
Ampicillin		75	53		50	85
Ampicillin-sulbactam			23		24	64
Amoxicillin-clavulanic acid			18		23	58
Cefazolin						33
Cefuroxime			0			
Cefotaxime			0			8
Kanamycin			15			
Piperacillin	7		41		24	0
Piperacillin-tazobactam			2	2		
Ofloxacin			2			
Cephalothin			17			
Tobramycin			1	77		
Nalidixic acid		13	2			
Aztreonam			3			
Carbenicillin			46			
Cefoperazone			7			
Cefoperazone-sulbactam					0	
Ticarcillin			48			
Trimethoprim			37			
Cefalexin					47	

Supplementary Table S7. Overall resistance rates of Enterotoxigenic *E. coli* (ETEC) from clinical samples.

Antibiotic	Indonesia (% R)	Thailand (% R)	Vietnam (% R)
Tetracycline	88	43	65
Chloramphenicol	61	13	17
Ciprofloxacin	0	2	0
Trimethoprim-sulfamethoxazole	63	51	63
Ceftriaxone	0		
Azithromycin		4	3
Ampicillin	78	54	67
Norfloxacin	0		
Cephalothin	87		
Nalidixic acid	0	3	0

Supplementary Table S8. Overall resistance rates among *K. pneumoniae* from clinical samples.

Antibiotic	Laos (%R)	Malaysia (% R)	Philippines (% R)	Singapore (% R)	Thailand (% R)
Tetracycline			53	30	
Ciprofloxacin		9	62	22	31
Gentamicin					47
Trimethoprim-sulfamethoxazole	29	26	89	48	53
Chloramphenicol	33				
Cefepime					100
Ceftazidime		9			100
Ceftriaxone		9			100
Imipenem		0	0	0	0
Amikacin		0	26	0	6
Nitrofurantoin					42
Ampicillin	94	17			
Cefazolin					100
Cefuroxime		13			
Piperacillin		4	24	7	34
Tobramycin			73	78	81
Aztreonam					0
Cefoperazone		13			
Levofloxacin					31
Cefotetan					100
Nalidicid acid	17				

Supplementary Table S9. Overall resistance rates among *Salmonella* spp. from clinical samples.

Antibiotic	Indonesia (% R)	Laos (%R)	Malaysia (%R)	Phillippine (% R)	Thailand (% R)	Vietnam (% R)
Ampicillin	7	12	1		33	0
Amoxicillin					9	
Ciprofloxacin	0				1	0
Chloramphenicol	15	12	7		30	0
Tetracycline	20		11		59	7
Gentamicin			2		2	
Trimethoprim-sulfamethoxazole	10	11	5		31	7
Cefepime				0		
Ceftazidime				0	0	
Ceftriaxone	2	0	0	0	2	
Imipenem				0	0	
Azithromycin					6	7
Amoxicillin-clavulanic acid					4	
Pefloxacin					7	

Amikacin				0	
Netilmicin				46	
Ampicillin-sulbactam				10	
Cefazolin				0	
Cefuroxime				0	
Cefotaxime				1	
Ofloxacin				2	
Kanamycin	10	0			
Piperacillin-tazobactam			0		
Norfloxacin	1			0	
Cephalothin	7				
Nalidixic acid	0			31	0
Colistin	7				
Neomycin	3	4			
Streptomycin				100	
Sulfamethoxazole				100	

Supplementary Table S10. Overall resistance rates among *Shigella flexneri* from clinical samples.

Antibiotic	Indonesia (% R)	Thailand (% R)	Vietnam (% R)
Tetracycline	89	96	87
Chloramphenicol	81	61	76
Ciprofloxacin		0	0
Trimethoprim-sulfamethoxazole	66	86	52
Azithromycin		0	5
Ceftriaxone	0		
Neomycin	1		
Ampicillin	83	82	82
Colistin	2		
Kanamycin	0		
Norfloxacin	0		
Cephalothin	12		
Nalidixic acid	0	0	0

Supplementary Table S11. Overall resistance rates among *Shigella sonnei* from clinical samples.

Antibiotic	Indonesia (% R)	Malaysia (% R)	Thailand (% R)	Vietnam (% R)
Tetracycline	36	35	92	60
Chloramphenicol	5	5	3	36
Ciprofloxacin	0		0	0
Trimethoprim-sulfamethoxazole	37	37	97	67
Azithromycin			2	28
Ceftriaxone	0			

Neomycin	0			
Ampicillin	30	7	4	62
Colistin	0			
Kanamycin	0	0		
Norfloxacin	0			
Cephalothin	10			
Nalidixic acid	0		0	0
Streptomycin	63	63		

Supplementary Table S12. Overall resistance rates among *Proteus* spp. from clinical samples.

Antibiotic	Malaysia (% R)	Philippines (% R)	Thailand (% R)	Singapore (% R)
Ciprofloxacin	7			1
Trimethoprim-sulfamethoxazole	33			47
Cefepime		0	0	
Cefpirome		0	6	
Ceftazidime	0	2	20	2
Ceftriaxone	2	0	2	
Imipenem	1	4	0	
Amikacin	0			1
Ampicillin	62			60
Ampicillin- sulbactam	12			20
Cefuroxime	5			9
Piperacillin				19
Piperacillin-tazobactam	0	0	0	
Cefoperazone	0			9
Aztreonam				2
Amoxicillin-clavulanic acid				21
Gentamicin				10
Netilmicin				3
Cephalexin				40
Cefuroxime				9
Cefotiam				10
Pefloxacin				2
Norfloxacin				0
Ofloxacin				3
Nalidixic acid				4
Nitrofurantoin				45

Supplementary Table S13. Overall resistance rates among *Enterobacter* spp. from clinical samples.

Antibiotic	Indonesia (% R)	Philippines (% R)	Singapore (% R)	Thailand (% R)
Cefepime	4	0		4
Cefpirome	0	1		14
Ceftazidime	20	29	8	42
Ceftriaxone	16	9	8	28
Imipenem	0	0	1	0
Piperacillin			21	
Piperacillin- tazobactam	16	5		17
Gentamicin			9	
Ciprofloxacin			2	
Trimethoprim-sulfamethoxazole			43	
Amoxicillin-clavulanic acid			22	
Pefloxacin			2	
Amikacin			3	
Netilmicin			3	
Nitrofurantoin			41	
Ampicillin			60	
Ampicillin- sulbactam			20	
Cefuroxime			20	
Ofloxacin			2	
Norfloxacin			0	
Nalidixic acid			3	
Aztreonam			2	
Cefoperazone			11	
Cefoperazone- sulbactam			0	
Cephalexin			37	
Cefotiam			10	

Supplementary Table S14. Overall resistance rates among *Serratia* spp. from clinical samples.

Antibiotic	Indonesia (% R)	Philippines (% R)	Thailand (% R)
Cefepime	0	0	2
Cefpirome	0	0	2
Ceftazidime	7	9	22
Ceftriaxone	7	4	11
Imipenem	7	0	0
Piperacillin- tazobactam	0	0	2

Supplementary Table S15. Overall resistance rates among *Campylobacter jejuni* from clinical samples.

Antibiotic	Indonesia (% R)	Thailand (% R)	Vietnam (% R)
Tetracycline	39		
Chloramphenicol	1		
Ciprofloxacin	31	75	1
Trimethoprim-sulfamethoxazole	88		
Ceftriaxone	37		
Azithromycin	0	2	0
Ampicillin	52		
Kanamycin	29		
Norfloxacin	30		
Nalidixic acid		72	1

Supplementary Table S16. Overall resistance rates among *Acinetobacter* spp. from clinical samples.

Antibiotic	Indonesia (% R)	Philippines (% R)	Singapore (% R)	Thailand (% R)
Ciprofloxacin			11.1	58
Trimethoprim-sulfamethoxazole			40	
Cefepime	19	12	51	
Cefpirome	29	9	60	
Ceftazidime	29	14	41	59
Ceftriaxone	29	21	52	73
Imipenem	10	13	6	24
Pefloxacin		20		
Amikacin			24	54
Netilmicin			32	
Nitrofurantoin			95	
Ampicillin			93	
Ampicillin- sulbactam			6	
Cefuroxim			92	
Ofloxacin			14	
Piperacillin			40	
Piperacillin-tazobactam	24	5		35
Norfloxacin			39	
Nalidixic acid			38	
Cefoperazone			68	
Cefoperazone- sulbactam			0	
Cephalexin			88	
Cefotiam			97	

Chapter 3

Rationale and design of the AMRIN study

I GENERAL DESCRIPTION

I.1 Introduction

Antimicrobial drug resistance is widely recognized as a global public health threat because it endangers the effectiveness of treatment of infectious diseases. The emergence and spread of resistant microorganisms are the result of the selective pressure exerted by use of antimicrobial agents and the transmission of resistant microorganisms (Figure 1). Antimicrobial use targets susceptible pathogens but always implies selection of strains, either at the site of infection and/or in the commensal microflora, that are less susceptible or completely resistant to the therapy given. When given the opportunity to spread beyond the individual given therapy, these resistant clones may expand and cause small outbreaks, epidemics and, finally, attain pandemic levels. Clearly, prudent use of antibiotics, i.e. antibiotic control, and measures to prevent the spread of resistant clones, i.e. infection control, are the critical determinants in managing the emergence of antimicrobial resistance (Figure 1).

Resistance emerges both in health care settings and in the community since antibiotics are frequently used in both societal strata. Community use of antimicrobial agents involves humans as well as animals, especially those animals that are raised in animal husbandry. In addition, antimicrobial agents are applied in agriculture to protect crops from microbial pests. Microorganisms, including resistant bacteria, may

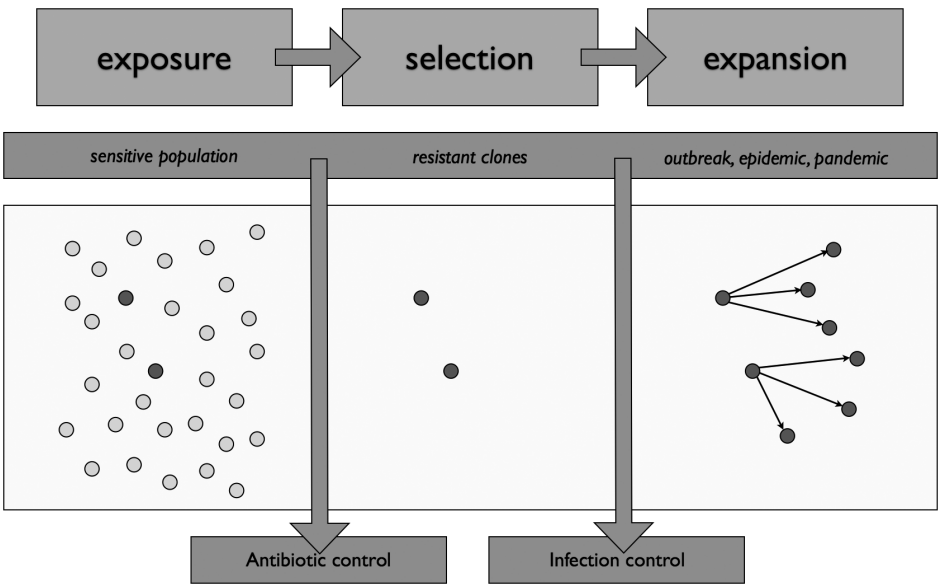

Figure 1. Model for the emergence and spread of resistant microorganisms as the result of the selective pressure exerted by use of antimicrobial agents and the transmission of resistant microorganisms. Antibiotic control and infection control are measures to stop this process.

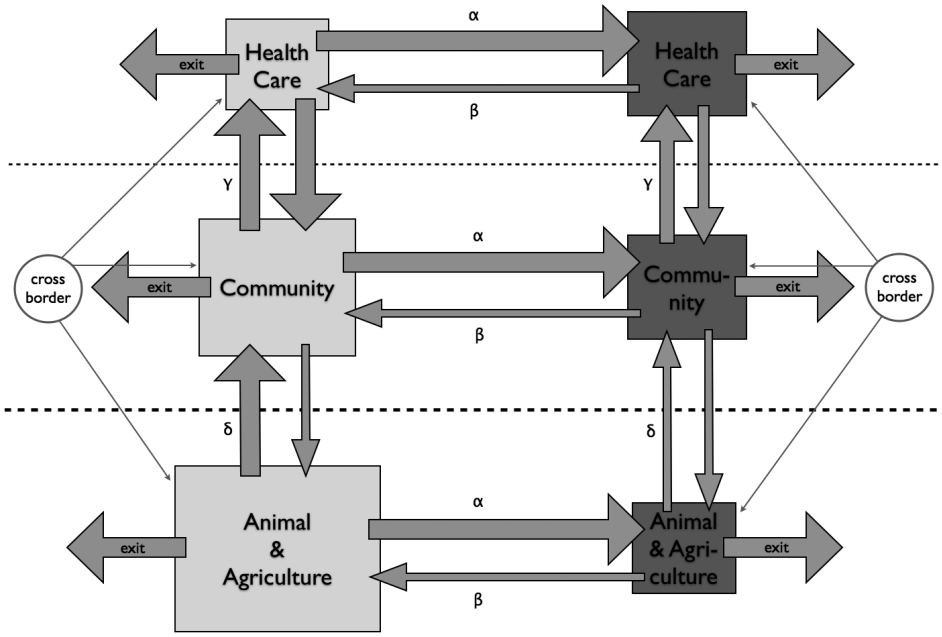

Figure 2. Model for the emergence and spread of resistant microorganisms through different sectors of society. Light-grey boxes represent the susceptible populations of microorganisms, whereas the dark-grey boxes represent their resistant mutants. The Greek letters represent the rates of transmission between the various microbial populations. Exit arrows indicate that microorganisms are eradicated from each of the microbial populations, whereas cross border trafficking implies that resistant and susceptible strains may 'travel' across man-made borders.

spread as part of the (commensal) microflora of humans and animals, but may also do so via innate objects and produce. Thus, there are no natural barriers between the various societal strata that will prevent resistant microorganisms to spread beyond their site of origin. Antimicrobial resistant pathogens arisen in health care may spread to the community via patients discharged from hospitals and long term care facilities; in addition, hospital personnel may become colonized with resistant pathogens while at work and carry them to their households and beyond. Importantly, resistant bacteria emerging in animal husbandry as a consequence of antibiotic use in that sector of society may cause zoonotic infections in humans. Through international trade of livestock, meat and agricultural produce, and through travel resistant microorganisms can spread and cause multinational outbreaks or even pandemic expansion of resistant pathogens (Figure 2).

Since limited data were available on the area of antimicrobial usage, resistance, and infection control in Indonesia, the fourth most populous country in the world, a large population-based study was designed in 1999 to investigate these items on Java, the most densely populated island of Indonesia.

I.2 Rationale

Antimicrobial resistance of bacteria is a worldwide and ever growing problem. Resistant bacteria emerge under the selective pressure of antibiotics. In hospitals where large-scale usage of antibiotics is common, bacteria resistant to several antibiotics frequently occur and generate major problems in the treatment of patients with infections caused by these microorganisms. A lack of hospital infection control measures may facilitate the spread of such bacteria from patient to patient. Outside hospitals, antimicrobial resistance is increasing as well, especially in countries where antimicrobial drugs are used frequently, including antibiotics sold over the counter, i.e. without a doctor's prescription.

Antimicrobial resistance has been recognized as a major threat to the health of people since the early nineteen's of the previous century. The World Health Assembly Resolution of the World Health Organization (WHO) of 1998 urged Member States to develop measures to improve practices to prevent the spread of infection and thereby the spread of resistant pathogens. In 2001, WHO released the *Global Strategy for Containment of Antimicrobial Resistance*, which included 14 priority interventions and 67 recommendations. The report stated that, to reduce the problem of antimicrobial resistance, action should be taken along two tracks: promotion of the prudent use of antibiotics and prevention of the spread of resistant bacteria. Data on the prevalence of antimicrobial resistance in Indonesia were limited at that time, but the sparse data available suggested that Indonesia was no exception to other countries in the world.

The "Antimicrobial resistance in Indonesia: prevalence and prevention" (AMRIN) study was therefore started in 2000 to determine the prevalence and genetic basis of antimicrobial resistance in the Indonesian population inside and outside hospitals, the level and the quality of antibiotic usage and the application of infection control measures in Indonesian hospitals. Interventions to improve antibiotic usage and infection control measures would be developed, implemented and their effects evaluated. The study would result in a scientifically based, efficient, and standardized program for the assessment of antimicrobial resistance, antibiotic usage, infection control measures, and execution of interventions in Indonesian hospitals. This assessment program could be the starting point for nationwide surveillance of antibiotic resistance, antibiotic usage, and infection control measures.

I.3 Study questions

The AMRIN proposal was to investigate the following research questions:

1. What is the prevalence and genetic basis of antibiotic resistance among bacteria in the Indonesian population inside and outside hospitals?
2. What is the level and quality of antibiotic usage in the Indonesian population inside and outside hospitals?

3. What is the correlation between antibiotic use and the development of antimicrobial resistance?
4. Does the introduction of guidelines for antimicrobial usage, e.g. prophylaxis, improve the use of antimicrobial drugs in Indonesian hospitals?
5. Which time-proven measures for the prevention of the spread of bacteria and nosocomial infections are implemented in Indonesian hospitals?
6. Which preventive measures should be given priority in order to optimize infection control in Indonesian hospitals and does introduction of preventive measures improve infection control?

I.4 Goals of the present thesis

The main questions to be answered in this thesis are derived from AMRIN study questions 1 and 3 and include:

1. Is the agar diffusion method using antibiotic impregnated disks an appropriate method for antimicrobial susceptibility testing in a surveillance study in Indonesia?
2. What is the prevalence of resistance among commensal *Staphylococcus aureus* and *Escherichia coli* isolates carried in the Indonesian population inside and outside hospitals?
3. What are the determinants of carriage of resistant *S. aureus* and *E. coli* in the Indonesian population inside and outside hospitals?
4. What are the genetic characteristics of the *S. aureus* isolates carried in the Indonesian population?
5. What is the genetic basis of resistance towards ciprofloxacin and cefotaxime, two worldwide emerging phenomenons, in (commensal) *E. coli* from Indonesia?

AMRIN study questions regarding the quantity and quality of antibiotic usage and infection control measures are addressed separately in the theses of dr. U. Hadi and dr. D.O. Duerink, respectively, of the Leiden University Medical Center, Leiden, the Netherlands.

II DESIGN

II.1 Study population

Most resistance data are obtained from routine susceptibility testing of strains isolated from clinical cultures, since these data are readily available at little additional costs. This kind of information, however, has limited predictability beyond the setting from which the strains originate. E.g. antimicrobial resistance patterns observed among

Figure 3. Map of Indonesia and Java.

isolates from intensive care patients do not predict the level of resistance encountered elsewhere in the same hospital, let alone in populations in the community outside the hospital. Resistance levels among bacteria present in the community at large can be monitored only by screening individuals from the community. In this case, culturing the commensal microflora of a random sample of the population in a well-defined geographic area is the preferable approach. Such a population-based design was chosen for the AMRIN project.

The AMRIN study was carried out in two urban areas on the island of Java. Two class A government hospitals, the Dr. Soetomo Hospital in Surabaya, East Java, and the Dr. Kariadi Hospital in Semarang, Central Java, Indonesia, as well as three so-called "puskesmas" or primary health centers (two in Surabaya and one in Semarang) were selected for this study (Figure 3). The Dr. Soetomo Hospital has 1,432 beds and approximately 41,000 admissions per year. The Dr. Kariadi Hospital has 900 beds and reports approximately 21,000 admissions per year.

The carriage of resistant bacteria and the use of antibiotics were investigated in four population groups (Table 1). The first group consisted of patients being admitted to the hospital (admission group), the second group were patients being discharged from the hospital after a hospitalization of five or more days (discharge group),

Table 1. Overview of the four population groups studied in the AMRIN project.

Population group	Investigations	Data
Admission group	- Nose and rectum swab for culture - Interview: antibiotic usage during the four weeks preceding admission	- Prevalence of resistance - Prevalence of antibiotic usage
Discharge group	- Nose and rectum swab for culture - Examination of patient records for antibiotic usage during hospital stay	- Prevalence of resistance - Prevalence of antibiotic usage - Quality of antibiotic treatment
PHC group	- Nose and rectum swab for culture - Interview: antibiotic usage during the preceding four weeks	- Prevalence of resistance - Prevalence of antibiotic usage
Relatives group	- Nose and rectum swab for culture - Interview: antibiotic usage during the preceding four weeks	- Prevalence of resistance - Prevalence of antibiotic usage

the third group contained persons visiting primary health centers for vaccination or consultation (PHC group), and the fourth group consisted of household members or relatives of patients being admitted to the hospital (relatives group). The discharge group was considered to represent a "hospital" population, whereas the other three groups were considered to represent "community" populations. Patients in the admission group were included within the first 24 hours of admission. Patients at admission and discharge were selected from four hospital departments: Internal Medicine, Surgery, Obstetrics and Gynecology, and Pediatrics. Patients from the discharge group were included on specific study days twice weekly in Surabaya and once weekly in Semarang. In the relatives group, only one relative or household member was included per admitted patient. Individuals were excluded from the study if they were transferred from another hospital, if they were not accompanied by a family member (admission group), or if they had been admitted to a hospital within the previous 3 months (admission group, PHC group and relatives group). Written informed consent was obtained from all participants and caretakers of children before enrollment.

II.2 Target microorganisms and niche

Not all bacterial species that can cause disease in man need to be included in a surveillance study for antimicrobial resistance. For practical purposes a selection has to be made. The two major selection criteria are the social relevance of the bacterial species (i.e. its prevalence and impact on health of the population), and the probability of yielding data with predictive value for similar resistance in other, related, species (sentinel function). For Gram-positive species the shortlist includes in decreasing order of importance: *Staphylococcus aureus, Streptococcus pneumoniae, Mycobacterium tuberculosis complex, Streptococcus pyogenes, Streptococcus agalactiae*. For Gram-negative species the shortlist prioritizes: *Escherichia coli*, other species of Enterobacteriaceae (*Klebsiella*, *Enterobacter*, *Salmonella*, *Shigella*, *Serratia*, *Citrobacter*), *Campylobacter jejuni, Neisseria gonnorhoeae, Pseudomonas aeruginosa,*

Stenotrophomonas maltophilia, Burkholderia cepacia, Haemophilus influenzae, and *Helicobacter pylori*. In the AMRIN study, only *S. aureus* and *E. coli* were included. Other species of Enterobacteriaceae were collected as well for specific purposes. For *S. aureus*, the moist squamous epithelium of the anterior nares acts as the primary ecological niche. *E. coli* and most other species of Enterobacteriaceae are common constituents of the gastrointestinal flora. Therefore, both a nasal swab and a rectal swab were obtained from each individual included in this study. For an accurate estimate of the level of resistance minimally 300 isolates per species per group needed to be tested. Given an estimated carriage prevalence of 25-30% of *S. aureus* and 90% of *E. coli*, 1,000 individuals had to be included per group. The aim was to include 4,000 individuals in total; 500 individuals per group per city, whereby each department was equally represented. The specimens were collected from July to October 2001 in Surabaya and from January to May 2002 in Semarang.

II.3 Selection of antibacterial agents

For each bacterial species included in the surveillance, an a priori well-defined selection of antimicrobial agents should be tested. The agents to be included are selected on the basis of natural susceptibility of the species, the actual use of the agent in the hospital or area under surveillance, and the availability of a validated test method for that particular combination of "bug-drug". Not all antibiotics need to be tested, not even when they are frequently prescribed in the population under the study. Within each class of antibiotics one can select one or a few agents for surveillance purposes as being predictive for the whole class. For example, oxacillin resistance predicts resistance to all β-lactam antibiotics in *S. aureus* and erythromycin may be used to monitor macrolide resistance in this pathogen. In Table 2 the "bug-drug" combinations as recommended by the Dutch Working Party on Antibiotic Policy (SWAB) are shown. In the AMRIN study, *S. aureus* isolates were tested for their susceptibility to oxacillin, tetracycline, gentamicin, erythromycin, chloramphenicol, and trimethoprim-sulfamethoxazole. *E. coli* and other Enterobacteriaceae were tested against ampicillin, cefotaxime, gentamicin, chloramphenicol, trimethoprim-sulfamethoxazole, and ciprofloxacin. Chloramphenicol, although fallen out of favour in western countries, is still frequently used in Indonesia.

II.4 Microbiological techniques

II.4.1 Sampling

Nasal and rectal samples were taken with sterile, dry cotton-tipped swabs, and, after sampling, these swabs were placed in Amies transport medium (Copan, Brescia, Italy)

Table 2. "Bug-drug" combinations as recommended by the Dutch Working Party on Antibiotic Policy (slightly modified).

Antimicrobial agent	*S. epidermidis*	*S. aureus*	Haemolytic strep. A & B	*S. pneumoniae*	*E. faecalis / E. faecium*	Enterobacteriaceae	*P. aeruginosa / Acinetobacter*	*C. jejuni*	*H. influenzae / Neisseria /Moraxella*	*H. pylori*
Penicillin										
Amoxicillin					+	+			+	+
Amoxicillin+clavulate					+	+			+	
Penicillin	+	+	+	+						
Cloxacillin	+	+								
Piperacillin						+	+			
Piperacillin+tazobactam							+			
Cephalosporin										
Cefuroxime (2nd)				+		+			+	
Cefotaxime (3rd)			+	+		+				
Ceftazidime (3rd)						+	+			
Other beta-lactams										
Imipenem				+		+	+			
Aminoglycoside										
Streptomycin					+					
Gentamicin	+	+			+	+	+			
Amikacin							+			
Chloramphenicol			+	+	+				+	
Tetracycline										
Doxycycline	+	+		+	+	+			+	+
Macrolide										
Erythromycin	+	+	+	+	+			+	+	
Clarithromycin										+
Lincosamid										
Clindamycin	+	+	+	+						
Glycopeptide										
Vancomycin	+	+	+		+					
Teicoplanin	+									
Sulfonamide										
Trimethoprim						+				
Co-trimoxazole	+	+	+			+	+	+		
Quinolone										
Ciprofloxacin	+	+	+		+	+	+	+	+	
Nitrofurantoin					+	+				
Metronidazole										+
Rifampicin	+	+		+						

and transported in closed boxes at ambient temperature to the laboratory on the same day. All swabs were cultured within 24 hours after sampling.

II.4.2 Isolation and identification

Nasal swabs were cultured by using phenol red mannitol agar (Becton Dickinson, Heidelberg, Germany) on which *S. aureus* typically produces yellow colonies due to its ability to ferment mannitol. All yellow colonies were stored in duplicate in trypticase soy agar medium. One tube was sent to Rotterdam, the Netherlands, and the other tube was stored in Indonesia. At both locations tubes were stored at room temperature. In the Netherlands, all suspect *S. aureus* isolates were re-cultured and identified by an agglutination test (Slidex Staph Plus, bioMérieux, Marcy l' Etoile, France) and the Vitek® 2 system (bioMérieux, Marcy l'Etoile, France). In case of doubt, an *S. aureus*-specific DNA hybridization test (Accuprobe, Genprobe inc., San Diego, CA, USA) was performed. Isolates that had been re-cultured in the Netherlands were subsequently stored at -80°C in glycerol containing broth.

Rectal swabs were cultured on CHROMagar Orientation (Becton Dickinson, Heidelberg, Germany). Chromogenic substrates are incorporated into this media to detect certain bacterial enzymes so that isolates can easily be identified based on colony color. *E. coli* produces pink colonies and *Klebsiella*, *Enterobacter* and *Serratia* and some other Gram-negative bacilli appear as blue colonies. After 18-24 h of incubation at 37°C, two colonies representing the dominant growth in the fecal flora were collected from each swab. Bacteria were stored in duplicate in trypticase soy agar medium. One tube was sent to Rotterdam, the Netherlands, and the other tube was stored in Indonesia. At both locations tubes were stored at room temperature. Pink colonies were used for susceptibility testing in Indonesia without additional identification testing. Gram-negative bacteria that were investigated by molecular methods to unravel their mechanism of resistance and genetic relatedness were identified using the Vitek® 2 system in the Netherlands. Isolates that had been re-cultured in the Netherlands were stored at -80°C in glycerol containing broth.

II.4.3 Antimicrobial susceptibility testing

There are several methods that can be used for susceptibility testing, but in the AMRIN study the disk diffusion test on Mueller Hinton agar as described by the National Committee on Clinical Laboratory Standards (NCCLS) / Clinical and Laboratory Standards Institute (CLSI) was employed, since it is less costly compared to other methods, and has a long history of reliable use around the world.

Reference strains with known resistance patterns should always be included, and results should be recorded and checked for stability over time. In the AMRIN study,

S. aureus American Type Culture Collection (ATCC) 25923 and *E. coli* ATCC 25922 were included twice weekly.

III QUESTIONNAIRE AND COLLECTION OF DATA ON ANTIBIOTIC USAGE

Demographic and socioeconomic data and, for community patients, data on health complaints and consumption of antibiotics in the month preceding the study were collected by semi-structured interviews, performed by pairs of trained Indonesian and Dutch data collectors (researchers, residents, and medical students). For children (<17 years), a caregiver (usually the mother) was interviewed. For the admission group, diagnosis on admission, and for discharge group patients, data on antibiotic consumption during hospitalization and diagnosis on discharge were collected from medical records.

Origin (Surabaya or Semarang), sex, age, ethnicity and living area (urban or rural) were the selected demographic variables. Health insurance, income, education, employment, and number of individuals sharing a household were the chosen socioeconomic variables. Group, department, nursing ward, nursing class (I, II, or III, with class I being the most expensive class) and length of stay in hospital were studied as health care-related variables. Only the last ward of admission prior to discharge was recorded; transfers were not recorded. For community patients, clinical signs and symptoms in the month preceding the study (fever, diarrhoea, respiratory symptoms, other symptoms, or no symptoms) were the disease-related variables and for patients upon admission and discharge whether or not an infection was diagnosed.

With the information on antibiotic usage and prevalence of resistance among each group, the correlation between antibiotic use and the development of antimicrobial resistance could be investigated. Demographic, socioeconomic, health care- and disease-related variables were used to study determinants of resistance.

IV ANTIBIOTIC USAGE

In a subpopulation of the discharge group patients, the amount of antibiotics used, i.e. the quantity, and the appropriateness of the choice and dosage of antibiotics and the duration of therapy, i.e. the quality of use, were examined.

V INFECTION CONTROL

Infection control in the hospital was assessed by surveillance of a restricted number of health care-associated infections among discharge group patients, an inquiry into the knowledge, attitude, and behaviour of health care workers concerning infection control, and evaluation of the infrastructure of infection control.

VI ORGANIZATION

The AMRIN study was a collaborative research project between five universities: Leiden University Medical Center, Leiden; Erasmus University Medical Center, Rotterdam; Radboud University Medical Center, Nijmegen, all in the Netherlands; Airlangga University / Dr. Soetomo Hospital, Surabaya, and Diponegoro University / Dr. Kariadi Hospital, Semarang, both located on the island of Java, Indonesia.

The project was run by a joint Indonesian-Dutch Steering Committee, which consisted of four Dutch investigators and three Indonesian investigators. In each of the two university hospitals a local committee was responsible for day-to-day execution of the AMRIN project (Figure 4). In the local committees at least one clinician, one medical microbiologist and one hospital pharmacist would participate. The hospital pharmacist would especially participate in the evaluation of antibiotic usage in the clinical setting. The actual field work was done by two Indonesian PhD fellows, one

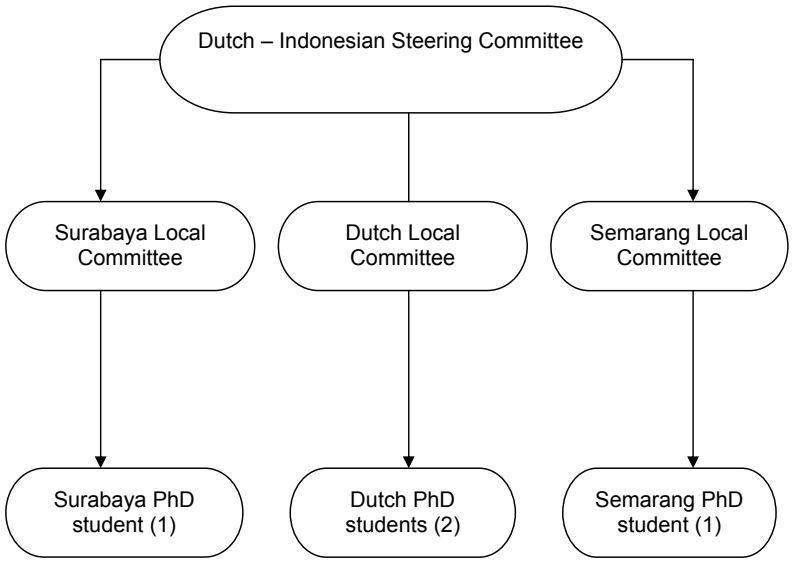

Figure 4. Organization of the AMRIN study.

in each hospital, and one Dutch PhD fellow. The local committees and PhD fellows were assisted by Dutch medical students during the periods of data collection in Indonesia. One of the two Indonesian PhD fellows was assisted by Dutch technicians and another Dutch PhD fellow who was responsible for the analysis of mechanisms of resistance performed in the Netherlands.

Before the AMRIN study was started, the members of the local Indonesian committees came to the Netherlands for a period of one month. In this period they were trained in the principles of antibiotic policy and infection control by visiting Dutch antibiotic policy and infection control committees, the Dutch Workingparty on Infection Prevention (WIP) and the Dutch Working Party on Antibiotic Policy (SWAB). Further training was individualized to the needs of the investigators. Medical microbiologists were trained in laboratory methods and quality control, clinicians were trained in clinical infectious diseases and antibiotic treatment and pharmacists were trained in controlling the use of medical drugs. The execution of the project was discussed and prepared in detail with the Indonesian researchers. The Indonesian microbiology laboratories in both hospitals were visited by the Dutch medical microbiologist of the Steering Committee. The local methods of cultures and susceptibility testing were evaluated as required for the project. These visits also served to finalize the technical protocols for isolation, identification, storage, and transport of the microbial isolates, and antimicrobial susceptibility testing. The medical ethics committees of the hospitals approved the study protocol (ethical clearance Nos. Panke.KKE / 2001 (Surabaya) and 11 / EC/ FK /RSDK/ 2001 (Semarang)).

VII FUNDING

The AMRIN study was one of the first Scientific Programme Indonesia – Netherlands (SPIN) projects (Research Programme No. 99-MED-03). SPIN is a programme for bilateral scientific research cooperation between Indonesia and the Netherlands, executed by the Royal Netherlands Academy of Arts and Sciences (KNAW), which has been running successfully since 2000. SPIN is based on agreements between the Dutch and Indonesian governments. The Programme adheres to the principle of mutual benefit and aims to stimulate the establishment of long-term scientific cooperation between Indonesian and Dutch research groups. The applicant of the project was Prof.dr. P.J. van den Broek from the Leiden University Medical Center, and his Indonesian counterpart was Prof.dr. Widjoseno Gardjito from the Airlangga University / Dr. Soetomo Hospital.

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Part II

Prevalence of antimicrobial resistance in Indonesia

Chapter 4

Comparison of the accuracy of disk diffusion zone diameters obtained by manual zone measurements to that by automated zone measurements to determine antimicrobial susceptibility

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ABSTRACT

Although a variety of techniques are available for antimicrobial susceptibility testing, disk diffusion methods remain the most widely used. We compared the accuracy of disk diffusion zone diameters as obtained by manual zone measurements in a low resource country (Indonesia) to that by automated zone measurements (Oxoid aura image system) in a high resource setting (the Netherlands) to determine susceptibility categories (sensitive, intermediate susceptible, or resistant). A total of 683 isolates were studied, including 294 *Staphylococcus aureus,* 195 *Escherichia coli*, and 194 other Enterobacteriaceae. Antimicrobial agents included tetracycline, oxacillin, gentamicin, erythromycin, trimethoprim-sulfamethoxazole, and chloramphenicol for *S. aureus* and ampicillin, gentamicin, cefotaxime, ciprofloxacin, trimethoprim-sulfamethoxazole, and chloramphenicol for *E. coli* and other Enterobacteriaceae. Of the 4,098 drug-organism combinations, overall category agreement (CA), major discrepancy (MD), and minor discrepancy (mD) between the two methods were 82.4% (3,379/4,098), 6.0% (244/4,098), and 11.6% (475/4,098), respectively. One hundred and sixty three of 244 MDs were resolved using reference broth microdilution method. Overall very major error (VME), major error (ME), and minor error (mE) of manual zone measurement were 28.8%, 45.4%, and 4.9%, respectively and for the aura image system 4.9%, 16.0%, and 4.9%, respectively.

The results of this study indicate that the disk diffusion method with manual zone measurement in Indonesia is reliable for susceptibility testing. The use of an automated zone reader, such as the aura image system, will reduce the number of errors, and thus improve the accuracy of susceptibility test results for medically relevant bacteria.

INTRODUCTION

Antimicrobial susceptibility testing (AST) is one of the most important tasks performed by clinical microbiology laboratories. It is important for the choice of an antimicrobial drug for the treatment of a patient and for epidemiological monitoring. Although a variety of methodologies are available for detecting resistance to antimicrobials, disk diffusion techniques remain the most widely used (7). Whenever these are performed according to a standard such as the Clinical and Laboratory Standards Institute (CLSI; formerly the NCCLS) reference procedure, they are considered reliable methods. Furthermore, the disk diffusion method is cost effective, highly reproducible, and drug combinations can be changed easily (4). However, measurement of zone sizes is tedious, time-consuming, and prone to transcription errors (20).

In recent years, a variety of automated instruments for AST have been introduced. The advantages of automation include a higher degree of standardization resulting in an increased accuracy, improved data management with a concomitant reduction in transcription errors, earlier availability of results, and the possibility for the use of so-called "expert" software (3, 9, 12, 20). Unfortunately, most automated systems may be too expensive for some laboratories in the Western world and for most laboratories in low resource countries, including Indonesia.

In medical microbiology laboratories in Indonesia the AST is still performed using the disk diffusion method and zones of inhibition are measured manually with a ruler or caliper. The aura image system (Oxoid, Basingstoke, UK) combines the advantages of disk diffusion and automation. By automatically performing zone measurements and giving interpretations against the user's chosen reference database it also obviates the aforementioned limitations of the disk diffusion method.

The present study was conducted in the course of a large population-based study of antibiotic resistance among bacteria from patients and healthy individuals in Indonesia (13). The purpose of our study was to compare the accuracy of disk diffusion zone diameters as obtained by manual zone measurements in Indonesia to that by automated zone measurements (Oxoid aura image system) in the Netherlands to determine interpretative categories. Discrepancies were analyzed using broth microdilution, a CLSI reference method.

MATERIALS AND METHODS

Bacterial isolates

A total of 683 isolates were studied, including 294 *Staphylococcus aureus,* 195 *Escherichia coli*, and 194 other Enterobacteriaceae. The collection of other Entero-

bacteriaceae consisted of 152 *Klebsiella pneumoniae*, 19 *Enterobacter cloacae*, 15 *Klebsiella ozaenae*, 4 *Enterobacter amnigenus*, 2 *Enterobacter aerogenes*, 1 *Citrobacter koseri*, and 1 *Citrobacter youngae*. These isolates were collected in the framework of a population-based study aimed at determining the prevalence of antimicrobial resistance in Indonesia (13). Strains were collected from healthy individuals and patients in two major urban centers: Surabaya (East Java) and Semarang (Central Java), Indonesia. Strains were isolated from nose and rectal swabs of individuals from July to October 2001 in Surabaya and from January to May 2002 in Semarang. Strains were stored in duplicate in trypticase soy agar medium. One tube was sent to Rotterdam, the Netherlands, and the other tube was stored in Indonesia. At both locations, strains were stored at room temperature. Phenol red mannitol agar (Becton Dickinson, Heidelberg, Germany) was used for the isolation of *S. aureus*. All putative *S. aureus* isolates were further identified in the Netherlands by the Vitek® 2 system (bioMérieux, Marcy l'Etoile, France) and, in case of a doubtful Vitek® 2 result, confirmed by an *S. aureus* specific DNA hybridization test (AccuProbe, Gen-Probe Inc., San Diego, CA, USA). Rectal swabs were cultured by using CHROMagar Orientation (Becton Dickinson, Heidelberg, Germany) for the isolation of *E. coli* and other Enterobacteriaceae (8). The identity of these strains was also confirmed by the Vitek® 2 system.

Inoculum preparation

In Indonesia, bacterial isolates were suspended in sterile saline so that the visual turbidity was equivalent to a 0.5 McFarland standard. This bacterial suspension was used directly for the disk diffusion test. In Rotterdam, the inoculum was prepared by suspending bacteria in 0.45% sterile saline to the equivalent of a 0.5 McFarland turbidity standard using a photometric device. This suspension was used for susceptibility testing by the Oxoid aura image system.

Disk diffusion method with manual zone diameter readings

In Indonesia, the disk diffusion method was performed as recommended by the CLSI (19). Briefly, the bacterial suspension was spread on the surface of a Mueller-Hinton agar (Becton Dickinson, Heidelberg, Germany). Antibiotic disks (Oxoid, Basingstoke,

Table 1. Antibiotic disks used in the study.

Organism	Antibiotic disk content[a]					
S. aureus	TET 30	OXA 1	GEN 10	ERY 15	SXT 1.25/23.75	C 30
E. coli	AMP 10	GEN 10	CTX 30	CIP 5	SXT 1.25/23.75	C 30
Enterobacteriaceae	AMP 10	GEN 10	CTX 30	CIP 5	SXT 1.25/23.75	C 30

[a] TET, tetracycline; OXA, oxacillin; GEN, gentamicin; ERY, erythromycin; SXT, trimethoprim-sulfamethoxazole; AMP, ampicillin; CTX, cefotaxime; CIP, ciprofloxacin; C, chloramphenicol. Numbers indicate disk load in micrograms.

UK) were placed on the agar surface by using a multi-disk dispenser (Table 1). After overnight incubation at 35°C in air, inhibition zones were measured with a ruler or caliper and the zones were recorded in millimeter. The susceptibility category (sensitive, intermediate susceptible, or resistant) was determined by comparing the zone of inhibition with the zone diameter breakpoint as recommended by the CLSI (19).

Disk diffusion method with automated zone measurements by the aura image system

The bacterial suspension was spread on the surface of a Mueller-Hinton agar (Becton Dickinson, Heidelberg, Germany). A bar code label was applied to the side of the test Petri dish. Using a hand-held bar code reader this code was automatically entered into the corresponding organism database. Disks containing antibiotics (Oxoid, Basingstoke, UK) were distributed onto the plate by using a disk dispenser (Table 1). After incubation at 35°C for 18-24 h, the agar plate was placed onto the sliding tray of the aura system, and the zone size of inhibition was measured automatically. A clear image of the plate appeared on the screen together with all the corresponding organism data. As recommended by the manufacturer, zone sizes were adjusted when the image analysis showed incorrect zone sizes. The diameter sizes (in millimeter), the adjustments and the interpretative category (sensitive, intermediate, and resistant) were shown in the result screen of the machine. In a clinical setting these data can be linked to the corresponding patient files (2). This feature was not used in the present study.

Broth microdilution

Broth microdilution testing was carried out according to CLSI guidelines in an independent laboratory and results were interpreted using CLSI breakpoints (17, 18).

Quality control

The performance of the susceptibility testing was monitored twice weekly by using the quality control strains *S. aureus* ATCC 25923 and *E. coli* ATCC 25922.

Analysis of data

The interpretative categorization results (sensitive, intermediate susceptible, resistant) from disk diffusion with manual measurement of zones of inhibition and from disk diffusion with automated measurements using the aura image system were compared. Category agreements (CA) and discrepancies were determined. Discrepancies were recorded as major discrepancy (MD) when disk diffusion with manual measurement indicated resistance and the aura image system indicated susceptibility and vice versa, and minor discrepancy (mD) when disk diffusion with manual measurement indicated intermediate susceptibility and the aura image system indicated susceptibility or resistance or when disk diffusion with manual measurement indicated susceptibility

or resistance and the aura image system indicated intermediate susceptibility. Major discrepancies from this comparison were further analyzed using broth microdilution (BMD). A very major error (VME) was defined as a BMD interpretation of resistant and a disk diffusion interpretation of sensitive; major error (ME) when the reference method indicated sensitive and disk diffusion tests indicated resistance and minor error (mE) when BMD indicated intermediate susceptibility and disk diffusion tests indicated sensitive or resistance or when BMD indicated sensitive or resistance and disk diffusion tests indicated intermediate susceptibility (3, 11, 14, 20). The correlation of zone diameters obtained using the aura image system and those measured manually was determined using SPSS version 11.5 (SPSS Inc., Chicago, IL, USA).

RESULTS

Of the 4,098 drug-organism combinations, overall CA, MD, and mD between the two methods, manual zone measurement and aura image system, were 82.4% (3,379/4,098), 6.0% (244/4,098), and 11.6% (475/4,098), respectively. The coefficient of correlation between the test results from manual measurements and those from the aura image system was 0.63 ($P<0.001$).

Table 2 shows the performance of the disk diffusion method with manual zone measurement compared to the aura image system for AST of *S. aureus, E. coli*, and other Enterobacteriaceae. MDs were more frequently observed in Surabaya than in Semarang for susceptibility testing of *S. aureus*, *E. coli* and other Enterobacteriaceae. In Surabaya, for a total of 2,154 drug combination, MDs among *S. aureus, E. coli*, and Enterobacteriaceae were 2.9% (63/2,154), 2.2% (48/2,154), and 2.9% (62/2,154), respectively. In Semarang, for 1,944 drug combination, MDs among *S. aureus*, *E. coli*, and Enterobacteriaceae were 1.4% (27/1,944), 0.5% (9/1,944), and 1.5% (30/1,944) respectively.

MDs were mainly found in case of trimethoprim-sulfamethoxazole testing of *S. aureus* isolates from Surabaya and tetracycline testing of *S. aureus* from both Semarang and Surabaya. Erythromycin susceptibility of *S. aureus* yielded a high rate of mDs resulting in a relatively low category agreement for this agent in both Surabaya and Semarang. In Surabaya, a high rate of mDs was found among the cefotaxime susceptibility testing of Enterobacteriaceae compared to Semarang.

From 244 drug combinations of MD, only 163 drug-organism combinations could be analyzed, because 79 drug-organism combinations were not available for analysis. In total, 163 drug-organism combinations were compared with BMD (Table 3). Overall VME, ME, and mE of manual zone measurement were 28.8%, 45.4%, and 4.9%, respectively and for the aura image system 4.9%, 16.0%, and 4.9%, respectively.

Table 2. Performance of the disk diffusion with manual zone measurements compared to automated zone measurements with aura image system.

	Manual[a]			Aura[b]			CA (%)	MD (n)	mD (n)
	S	I	R	S	I	R			
S. aureus Surabaya (n=167)									
Tetracycline	114	2	51	128	1	38	86.2	20	3
Oxacillin	149	0	18	155	10	2	86.2	15	8
Gentamicin	167	0	0	166	1	0	99.4	0	1
Erythromycin	144	18	5	89	76	2	52.7	2	77
SXT[c]	145	1	21	165	2	0	85.6	21	3
Chloramphenicol	149	4	14	140	15	12	86.2	5	18
S. aureus Semarang (n=127)									
Tetracycline	94	16	17	94	1	32	79.5	11	15
Oxacillin	122	2	3	113	11	3	86.6	6	11
Gentamicin	125	0	2	124	1	2	96.9	3	1
Erythromycin	117	6	4	66	60	1	51.2	0	62
SXT	126	0	1	125	1	1	97.6	2	1
Chloramphenicol	112	1	14	96	17	14	81.9	5	18
E. coli Surabaya (n=95)									
Ampicillin	52	5	38	22	20	53	64.2	13	21
Gentamicin	90	1	4	76	10	9	83.2	5	11
Cefotaxime	88	5	2	67	21	7	75.8	3	20
Ciprofloxacin	90	0	5	83	6	6	91.6	2	6
SXT	62	1	32	49	2	44	81.1	15	3
Chloramphenicol	64	6	95	61	6	28	78.9	10	10
E. coli Semarang (n=100)									
Ampicillin	53	8	39	39	20	41	73.0	6	21
Gentamicin	100	0	0	96	3	1	96.0	1	3
Cefotaxime	97	2	1	93	7	0	91.0	0	9
Ciprofloxacin	97	1	2	97	1	2	98.0	0	2
SXT	61	0	39	63	0	37	98.0	2	0
Chloramphenicol	84	0	16	79	5	16	95.0	0	5
Other Enterobacteriaceae Surabaya (n=97)									
Ampicillin	25	2	70	3	2	92	72.2	23	4
Gentamicin	90	1	6	75	12	10	78.4	8	13
Cefotaxime	87	6	4	53	37	7	51.5	6	41
Ciprofloxacin	93	1	3	87	7	3	88.7	3	8
SXT	81	2	14	71	10	16	75.3	12	12
Chloramphenicol	80	4	13	70	11	16	74.2	10	15
Other Enterobacteriaceae Semarang (n=97)									
Ampicillin	5	0	92	3	1	93	94.8	4	1
Gentamicin	81	4	12	79	8	10	82.5	5	12
Cefotaxime	79	9	9	67	15	15	75.3	4	20
Ciprofloxacin	90	2	5	86	4	7	91.8	2	6
SXT	66	5	26	68	6	23	77.3	11	11
Chloramphenicol	76	0	21	78	3	16	87.6	9	3

[a] Manual zone measurements.

[b] Automated zone measurements by the aura image system.

[c] SXT, trimethoprim-sulfamethoxazole.

Table 3. Discrepancy analysis of manual zone measurements and aura image system compared to the reference method.

Microorganisms and antimicrobial agents	MD	Reference method			Manual measurement				Aura image system			
						No. of errors				No. of errors		
		S	I	R	CA[a]	VME	ME	mE	CA[b]	VME	ME	mE
***S. aureus* Surabaya**												
Tetracycline	20	16	2	2	2	1	15	2	16	1	1	2
Oxacillin	15	12	0	3	2	1	12	0	13	2	0	0
Erythromycin	2	2	0	0	0	0	2	0	2	0	0	0
SXT[c]	21	21	0	0	0	0	21	0	21	0	0	0
Chloramphenicol	5	3	0	2	0	2	3	0	5	0	0	0
***S. aureus* Semarang**												
Tetracycline	9	5	1	3	2	3	3	1	6	2	0	1
Oxacillin	5	3	0	2	1	2	2	0	4	0	1	0
Gentamicin	2	2	0	0	1	0	1	0	1	0	1	0
SXT	2	2	0	0	1	0	1	0	1	0	1	0
Chloramphenicol	3	2	0	1	0	1	2	0	3	0	0	0
***E. coli* Surabaya**												
Ampicillin	11	2	0	9	2	9	0	0	9	0	2	0
Gentamicin	5	4	0	1	4	1	0	0	1	0	4	0
Cefotaxime	2	2	0	0	2	0	0	0	0	0	2	0
SXT	5	2	0	3	2	3	0	0	3	2	0	0
Chloramphenicol	9	3	0	6	0	6	3	0	9	0	0	0
***E. coli* Semarang**												
Ampicillin	3	3	0	0	3	0	0	0	0	0	3	0
Other Enterobacteriaceae Surabaya												
Ampicillin	20	2	4	14	3	13	0	4	13	1	2	4
Gentamicin	6	5	0	1	4	1	1	0	2	0	4	0
Cefotaxime	4	3	1	0	1	0	2	1	2	0	1	1
Ciprofloxacin	1	1	0	0	1	0	0	0	0	0	1	0
SXT	2	2	0	0	1	0	1	0	1	0	1	0
Chloramphenicol	9	5	0	4	2	4	3	0	7	0	2	0
Other Enterobacteriaceae Semarang												
SXT	1	1	0	0	0	0	1	0	1	0	0	0
Chloramphenicol	1	1	0	0	0	0	1	0	1	0	0	0
Total[d]	163	104	8	51	34	47	74	8	121	8	26	8
					(20.9)	(28.8)	(45.4)	(4.9)	(74.2)	(4.9)	(16.0)	(4.9)

[a] Category agreement between results from the reference method and disk diffusion with manual zone measurements.

[b] Category agreement between results from the reference method and disk diffusion with automated zone measurements.

[c] SXT, trimethoprim-sulfamethoxazole.

[d] The numbers between brackets are percentages.

DISCUSSION

Automated image analysis systems for the reading of disk diffusion zone diameters have been evaluated in several countries (9, 12, 16, 20). In this study, we compared the accuracy of disk diffusion zone diameters as obtained by manual zone measurements in Indonesia to that by automated zone measurements (Oxoid aura image system) in the Netherlands for antimicrobial susceptibility testing of 4,098 drug-organism combinations. The overall CA was 82.4%. A discrepancy analysis of the MDs using reference broth microdilution showed that more automated zone measurements were correct than manual measurements. Since the MD rates were much lower for disk diffusion testing in Semarang, we assume that the visual reading, interpretation, or recording of inhibition zones in Surabaya has met with inconsistencies and can be improved. Upon further analysis it became clear that the large majority of MDs in Surabaya were generated in one of the three participating laboratories in that city. In that particular laboratory testing had not been assigned to a single person but, rather, was performed by many relatively inexperienced personnel. A high rate of mDs in erythromycin testing of *S. aureus*, which may be explained by the so-called "beach effect", caused a relatively low category agreement for this agent in both Surabaya and Semarang, and resulting CAs overall (16). The "beach effect" is a failure to detect light growth at the margins of the inhibition zones.

A significant correlation between manual and aura image system measurements was found (r=0.63). In the previous study on the aura image system by Andrews et al., much higher correlation coefficients were reported, ranging from 0.986 to 0.998 (2). However, in the report by Andrews et al., a single inoculum was prepared for both manual and automated disk diffusion testing methods performed at a single site by the same dedicated staff, while in the present study the inoculum preparation and tests were performed by different technicians in different laboratories at five different sites in Indonesia and the Netherlands, a situation resembling proficiency testing. Several previous reports on proficiency testing showed that antimicrobial susceptibility testing is still a challenging task for microbiology laboratories worldwide (1, 5, 6, 10, 15, 23). In the United States, categorical accuracies for Gram-positive bacteria tested by disk diffusion were between 89.6-100% and for Gram-negative strains between 88.0-100% (21). Another proficiency testing survey showed that antimicrobial susceptibility testing for low-level penicillin resistance in a strain of *Streptococcus pneumoniae* was suboptimal in many laboratories in the United States (6).

In recent years, there has been a growing interest in the use of instrumentation for the improvement of reading disk diffusion tests (7). Several systems are available that use a camera or scanner for the automated reading of zone sizes, including the OSIRIS video reader system, the BIOMIC video reader system, and the Sirscan automated

zone reader (4, 9, 12, 16, 20, 22). The different systems appear to perform reasonably well in evaluation studies comparing the automated readings to manual measurements of inhibition zones performed in one laboratory by experienced technicians. The estimated accuracy of the disk diffusion method with manual zone measurements in Indonesia in the present study is at least 83.3% (3,413/4,098), with automated zone measurements at least 85.4% (3,500/4,098).

In conclusion, the results of this study indicate that the disk diffusion method with manual zone measurement in Indonesia is reliable for susceptibility testing. The use of an automated zone reader, such as the aura image system, will reduce the number of errors, and thus improve the accuracy of susceptibility test results for medically relevant bacteria.

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Chapter 5

Antimicrobial resistance among commensal isolates of *Escherichia coli* and *Staphylococcus aureus* in the Indonesian population inside and outside hospitals

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ABSTRACT

The prevalence of antimicrobial resistance among the commensal microflora was examined in the Indonesian population inside and outside hospitals. A total of 3,995 individuals were screened in two major urban centers. Among *Escherichia coli* from rectal samples (n=3,284), the prevalence of resistance to ciprofloxacin and other classes of antibiotics was remarkably high, especially in individuals at the time of discharge from hospital. *Staphylococcus aureus* isolates (n=361) were often resistant to tetracycline (24.9%), but this was not associated with hospital stay. Two *S. aureus* isolates harboured the *mecA* gene. Regional differences in resistance rates exist, suggesting regional differences in selection pressure, i.e. antibiotic usage patterns. The results show that antimicrobial resistance among commensal *E. coli* and *S. aureus* has emerged in Indonesia.

INTRODUCTION

Antimicrobial resistance has become a major health problem worldwide, both in hospitals and the community (3, 7, 8, 10, 34). The emergence of antimicrobial resistance is correlated with selective pressure from the use, often inappropriate, of antimicrobial agents (3, 7, 8, 10, 34) and results in increased mortality, morbidity, and health care costs (8).

Data on the epidemiology of antimicrobial resistance is important and can be drawn from three general sources: surveillance, outbreak investigations, and prospective studies (8). Resistance data may be gathered either from clinical isolates or from isolates cultured from the commensal microflora of individuals in the absence of infection (1, 2, 5, 39).

Surveillance of antimicrobial resistance has been reported in many countries, including developing countries in the Far East (4, 6, 11, 12, 14, 15, 21-23, 29, 30, 33, 35, 38). However, resistance data from Indonesia are limited to bacterial pathogens isolated from patients with infections, especially diarrheal disease (21, 22, 30, 33, 35). No data exist on the presence of resistance among potential pathogens in the commensal flora of Indonesian people. Such data are relevant, as they should provide a basis for the selection of empiric use of antimicrobial agents, either for therapy or for prophylaxis. Therefore, the aim of this study was to investigate antimicrobial resistance rates among potential pathogens in the commensal microflora of representative cohorts of people in two major urban centers in Indonesia.

MATERIALS AND METHODS

Population

Two university hospitals, Dr. Soetomo Hospital in Surabaya, East Java, and Dr. Kariadi Hospital in Semarang, Central Java, Indonesia, and three primary health centers (two in Surabaya and one in Semarang) were selected for this study. Four thousand individuals were targeted to be screened constituting four different populations in each of the two cities (500 individuals per population group per city). Group 1 consisted of patients being admitted to the hospital (admission group), group 2 were patients being discharged from the hospital (discharge group), group 3 contained ambulatory patients visiting primary health centers (puskesmas group), and group 4 consisted of relatives of patients being admitted to the hospital in each of the two cities (relatives group). Patients at admission and discharge were selected from four hospital departments: Internal Medicine, Surgery, Obstetrics and Gynecology, and Pediatrics. Individuals were excluded from the study if they were transferred

from another hospital, if they were not accompanied by a family member (admission group), or if they had been admitted to a hospital within the previous three months (admission group, puskesmas group, and relatives group). Approval of the Medical Ethics Committees was obtained before the start of the study. Only patients who had given their informed consent were included in the study.

Bacterial isolation

Rectal and nasal samples were taken with sterile, cotton-tipped swabs, and these were put in Amies transport medium (Copan, Brescia, Italy) and transported in closed boxes at ambient temperature to the laboratory on the same day. All swabs were cultured within 24 hours. Rectal swabs were cultured on CHROMagar Orientation (Becton Dickinson, Heidelberg, Germany) for isolation of *Escherichia coli* (9). From each swab, two colonies representing the dominant growth in the fecal flora were collected. Pink colonies were assumed to be *E. coli* and were used for susceptibility testing without additional identification testing. Nasal swabs were cultured by using phenol red mannitol agar (Becton Dickinson, Heidelberg, Germany) for isolation of *S. aureus*. In the Netherlands, all putative *S. aureus* isolates were speciated with an agglutination test (Slidex Staph Plus, bioMérieux, Marcy l' Etoile, France) and the Vitek® 2 system (bioMérieux, Marcy, l'Etoile, France). In case of doubt an *S. aureus* specific DNA hybridization test (Accuprobe, Genprobe inc., San Diego, USA) was performed.

Antimicrobial susceptibility testing

Antimicrobial susceptibility testing of the isolates was performed in Indonesia by disk diffusion as recommended by the Clinical and Laboratory Standards Institute (CLSI; formerly the NCCLS) (28). Disks loaded with the following antimicrobial agents (Oxoid, Basingstoke, UK) were used for susceptibility testing of *E. coli*: gentamicin, chloramphenicol, trimethoprim-sulfamethoxazole, ampicillin, cefotaxime, and ciprofloxacin. One *E. coli* isolate per patient was included in the analysis. Tetracycline, oxacillin, gentamicin, erythromycin, chloramphenicol, and trimethoprim-sulfamethoxazole disks were used for susceptibility testing of *S. aureus*. The performance of the susceptibility testing was monitored twice weekly by the quality control strains *E. coli* ATCC 25922 and *S. aureus* ATCC 25923.

PCR for *mecA* gene detection

Polymerase chain reaction (PCR) for *mecA* gene detection was performed with primers MEC1 (5'- AAA ATC GAT GGT AAA GGT TGG C- 3') and MEC2 (5'- AGT TCT GCA GTA CCG GAT TTG C- 3'), which generate a 533-bp product as described previously (27). Bacterial DNA was isolated on a MagNA Pure LC™ with MagNA Pure LC DNA Isolation Kit III for bacteria and fungi (Roche Molecular Biochemicals, Mannheim,

Germany). DNA concentration was assessed spectrophotometrically. PCR program was performed under the following conditions: predenaturation for 4 min at 94°C; 25 cycles of 45 s at 94°C, 45 s at 55°C and 45 s at 72°C, and a final extension step of 5 min at 72° C. PCR products were resolved in a 1% agarose gel in 0.5 Tris-borate + ethylenediaminetetraacetate (TBE) stained with ethidium bromide and visualized under ultraviolet (UV) light. A positive and a negative control were included in each PCR run.

Statistical analysis

Statistical comparisons of antibiotic resistance between the strains collected from the four different groups and between similar groups in the two cities were made by using the Chi-square analyses or Fisher's exact test (two-tailed). Data were analyzed by using the statistical software package SPSS version 11.5 for Windows (SPSS Inc., Chicago, IL, USA). A P value <0.05 was considered significant.

RESULTS

A total of 3,995 individuals were screened between July and October 2001 in Surabaya and between January and May 2002 in Semarang. We collected 5,535 *E. coli* strains from 3,284 individuals and 362 *S. aureus* isolates from as many individuals.

Resistance among *E. coli*

E. coli isolates (n=3,284) showed considerable levels of resistance against a number of commonly used antibiotics (Table 1). For most antimicrobials, the lowest resistance rates were measured in *E. coli* isolated from the relatives group, whereas the highest rates were measured in the patients in the discharge group. For all antimicrobials, resistance rates on discharge were higher compared with those in patients on admission, relatives, and patients visiting a primary health center. Substantial resistance rates towards chloramphenicol, trimethoprim-sulfamethoxazole, and ampicillin were observed among *E. coli* cultured from patients visiting primary health centers compared with *E. coli* from the relatives. Resistance towards gentamicin, cefotaxime, and ciprofloxacin was low on admission to the hospital. However, high rates of resistance against these latter antibiotics were found among *E. coli* cultured from patients at the time of discharge from the hospital.

Resistance among *S. aureus*

The rate of nasal carriage of *S. aureus* was 362 out of 3,995 (9.1%) individuals. One strain of *S. aureus* did not grow on the Mueller Hinton agar plate for antimicrobial

Table 1. Antimicrobial resistance rates (n [%]) of *E. coli* and *S. aureus* from different cohorts in Indonesia.

	Admission	Discharge	Relatives	Primary health center (Puskesmas)	Total
E. coli	(n=822)	(n=784)	(n=815)	(n=863)	(n=3,284)
Gentamicin	32 (3.9)[a]	141 (18.0)[d,e]	11 (1.3)[c]	18 (2.1)[b]	202 (6.2)
Chloramphenicol	210 (25.5)[a]	335 (42.7)[d,e]	64 (7.9)[c]	95 (11.0)[b,f]	704 (21.4)
Trimethoprim-sulfamethoxazole	342 (41.7)[a]	435 (55.5)[d,e]	164 (20.1)[c]	209 (24.2)[b,f]	1,150 (35.0)
Ampicillin	416 (50.6)[a]	571 (72.8)[d,e]	162 (19.9)[c]	271 (31.4)[b,f]	1,420 (43.2)
Cefotaxime	17 (2.1)[a]	98 (12.5)[d,e]	6 (0.7)[c]	8 (0.9)	129 (3.9)
Ciprofloxacine	48 (5.8)[a]	173 (22.1)[d,e]	17 (2.1)[c]	17 (2.0)[b]	255 (7.8)
S. aureus	(n=84)	(n=98)	(n=82)	(n=97)	(n=361)
Tetracycline	29 (34.5)	24 (24.5)	18 (22.0)	19 (19.6)[b]	90 (24.9)
Oxacillin	0 (0)	2 (0.6)	0 (0)	0 (0)	2 (0.6)
Gentamicin	2 (2.4)	1 (1.0)	0 (0)	1 (1.0)	4 (1.1)
Erythromycin	4 (4.8)	5 (5.1)	1 (1.2)	2 (2.1)	12 (3.3)
Chloramphenicol	12 (14.3)	9 (9.2)	7 (8.5)	6 (6.2)	34 (9.4)
Trimethoprim-sulfamethoxazole	10 (11.9)	7 (7.1)[d]	7 (8.5)	0 [b,f]	24 (6.6)

Resistance rates were compared in defined pairs (see a-f), using the Chi-square test or Fisher's exact test as applicable ($P<0.05$ was considered significant).

[a] significant difference for admission versus discharge

[b] significant difference for admission versus puskesmas

[c] significant difference for admission versus relatives

[d] significant difference for discharge versus puskesmas

[e] significant difference for discharge versus relatives

[f] significant difference for puskesmas versus relatives

susceptibility testing. Overall, 90/361 (24.9%) *S. aureus* strains were resistant to tetracycline and 2/361 (0.6%) were resistant to oxacillin (Table 1). The two oxacillin-resistant strains of *S. aureus* harboured the *mecA* gene. Resistance rates against tetracycline among patients on admission were higher compared with rates found in the patients visiting the primary health care centers (P=0.022). For trimethoprim-sulfamethoxazole, the resistance rates among isolates from patients on admission, patients at discharge, and from relatives of patients on admission were higher compared with the rates among *S. aureus* isolates from patients visiting primary health centers (P<0.001, P=0.013, and P=0.007, respectively).

Geographical differences

When comparing resistance data of *E. coli* and *S. aureus* isolated in Semarang with those isolated in Surabaya, the overall trends observed were similar. However, some striking differences became obvious. For *E. coli*, the resistance rates among patients at discharge in Semarang were higher than those observed in Surabaya for all antibiotics except for gentamicin (data not shown). Also, more resistance among *E. coli* was

observed in Semarang compared with Surabaya with regard to ampicillin (792/1,707 [46.4%] in Semarang versus 628/1,577 [39.8%] in Surabaya; $P<0.001$). In contrast, chloramphenicol resistance was higher among *E. coli* isolated from Surabaya patients on admission (117/389 [30.1%] in Surabaya versus 93/433 [21.5%] in Semarang; $P=0.005$) and likewise when comparing isolates from their relatives (38/383 [9.9%] in Surabaya versus 26/432 [6.0%] in Semarang; $P=0.05$).

With regard to *S. aureus*, the rates of resistance to trimethoprim-sulfamethoxazole in the admission and relatives groups and tetracycline resistance in the discharge group in Surabaya were much higher than in Semarang (data not shown).

DISCUSSION

This study is the first population-based study of antimicrobial resistance among common pathogenic bacteria in the commensal microflora of several groups of individuals in Indonesia. We stress the fact that the strains examined in the present study were not isolated from clinical materials as in previous studies but from the normal flora of the anterior nares and from the rectum of individuals with a variety of diseases as well as from healthy persons. We targeted our surveillance of antimicrobial resistance towards *E. coli* and *S. aureus,* since they can be considered sentinel species of microorganisms for the emergence of resistance (1, 2, 5, 39).

We showed that antibiotic resistance rates among *E. coli* isolated from patients on discharge from the hospital are consistently higher than previously described. An earlier study in Indonesia by US Naval Medical Research Unit #2 (NAMRU- 2) (33) showed that resistance rates for ampicillin, chloramphenicol, and trimethoprim-sulfamethoxazole among clinical isolates of enterotoxigenic *E. coli* (ETEC) were higher than in this study. In contrast, our study yielded higher rates of ciprofloxacin resistance compared with that study. There are no earlier data about resistance rates of *E. coli* against gentamicin and cefotaxime in Indonesia. However, in a survey performed in Indonesian hospitals in the late 1990s, 23.3% of *E. coli* had an extended-spectrum β-lactamase phenotype (23).

From a series of studies that were conducted through the 1980s and 1990s in Taiwan, 100% of clinical isolates of *E. coli* proved susceptible to fluoroquinolones (norfloxacin, ofloxacin, and ciprofloxacin) until 1996. By 1996 – 1997, 20% of *E. coli* had become resistant to ciprofloxacin, and 48% of patients with ciprofloxacin-resistant *E. coli* reportedly had not used any fluoroquinolones (31). Another study from Taiwan reported that ciprofloxacin resistance rate among *E. coli* was 11.3% between August and December 1998, (25) 0% in 1998-1999 by the SENTRY surveillance program, (4) and 46% in 1999 by the National Taiwan University Hospital study

(NTUH) (13). Data of antimicrobial resistance of clinical isolates of *E. coli* against ciprofloxacin from other countries have also been reported by the SENTRY global antimicrobial surveillance program: 61% in Hong Kong, 93% in mainland China, 75% in Singapore, and 54% in the Philippines (4). In Korea, the Korean Nationwide Surveillance of Antimicrobial Resistance study (KONSAR) reported a rate of 28%, (20) and the Taiwan Surveillance of Antimicrobial Resistance (TSAR) reported 12% in Taiwan (18). Although in all studies clinical isolates of *E. coli* were used, it is clear that fluoroquinolone resistance is now prevalent in most countries in Asia, including Indonesia. The genetic backgrounds and virulence characteristics of the Indonesian fluoroquinolone-resistant *E. coli* from the present study have recently been described by Kuntaman et al. (17).

Multiple studies have reported high frequencies of methicillin-resistant *S. aureus* (MRSA) among clinical isolates of *S. aureus* in Asia. The rate of MRSA among clinical *S. aureus* isolates was more than 50% in Korea in 1997 (6, 19) and more than 60% in Taiwan between 1993 and 1999 (11, 37). In Japan, it was 35.8% in 1987, and has increased to 67.3% in 1989 (36). Another study in Japan showed that the frequency of MRSA was 22-64% in 1988, 22-69% in 1989 and 29-76% in 1990, and increasing yearly (29). Furthermore, *S. aureus* with reduced susceptibility to vancomycin has been found in hospitals in several Asian countries (32).

In this study, we found that methicillin resistance in *S. aureus* isolates from Indonesia was 2/361(0.6%). These oxacillin-resistant strains of *S. aureus* harboured the *mecA* gene. This finding suggests that MRSA has not yet gained a strong foothold in the Indonesian archipelago. Only two of 3,995 (0.05%) persons screened harboured MRSA, which compares favorably with the 0.84% MRSA carrier rate found in the same period in a population-based screening study in the USA (24). However, as we did not include clinical strains causing nosocomial infections in this study, we cannot exclude the possibility that MRSA has emerged in some hospital settings in Indonesia. Of note, the overall rate of *S. aureus* nasal carriage in our study was only 9.1%, i.e. much lower than the 20% or higher levels generally found among populations in Western countries (16). Low carriage rates of *S. aureus* among the Indonesian population may, by itself, reduce or interfere with the spread and emergence of MRSA variants.

There are no prior data about the prevalence of antibiotic resistance of commensal *S. aureus* against tetracycline, gentamicin, erythromycin, chloramphenicol and trimethoprim-sulfamethoxazole in Indonesia. When we compare our findings with recent data from Taiwan, (26) the prevalence of antimicrobial resistance among clinical isolates of *S. aureus* was higher in Taiwan than in Indonesia. The resistance rates for tetracycline were 71% and 24.9%, for oxacillin 60% and 0.6%, for gentamicin 51% and 1.1%, for erythromycin 73% and 3.3%, for chloramphenicol 29% and 9.4%, and for trimethoprim-sulfamethoxazole 42% and 6.6% in *S. aureus* from Taiwan and

from Indonesia (this study), respectively. Antimicrobial resistance profiles of clinical isolates of *S. aureus* have also been reported from Korea, (14) showing that the resistance rates of methicillin-sensitive *S. aureus* (MSSA) and MRSA against tetracycline were 22% and 90%, against gentamicin 28% and 95%, against erythromycin 37% and 98%, and against trimethoprim-sulfamethoxazole 1% and 9% in MSSA and MRSA, respectively. In contrast to our study, the Taiwanese and Korean surveillance systems were based on clinical samples and not on sampling of commensal flora, as was done in this study. Thus, direct comparison is not valid, as resistance rates among clinical isolates of *S. aureus* may well be higher in Indonesia also.

In conclusion, we show that antimicrobial resistance among *E. coli* and *S. aureus* present in the commensal microflora of people has emerged in Indonesia. Among *E. coli*, the prevalence of resistance to ciprofloxacin and other antibiotics is remarkably high, especially in individuals after hospitalization. Although the prevalence of MRSA is low, tetracycline resistance is common among *S. aureus* and not associated with hospital stay. Within Indonesia, regional differences in resistance rates exist, suggesting regional differences in selection pressure, i.e. antibiotic usage patterns. Moreover, for *S. aureus*, resistance rates of isolates from patients at discharge from the hospital were not higher than on admission, whereas these rates in *E. coli* were significantly higher on discharge. Therefore, resistance in *E. coli* appeared to be both community and hospital associated, whereas resistance in *S. aureus* seems to be mainly community derived. This is an important finding for future strategies that target the containment of antimicrobial resistance in Indonesia.

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Part III

Focus on *Staphylococcus* spp.

Chapter 6

Determinants of carriage of resistant *Staphylococcus aureus* among *S. aureus* carriers in the Indonesian population inside and outside hospitals

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ABSTRACT

Objectives: To identify determinants of carriage of resistant *Staphylococcus aureus* in both hospitalized patients and individuals from the community in two urban centers in Indonesia.

Methods: *S. aureus* cultures and data on recent antibiotic use, demographic, socioeconomic, disease-related and healthcare-related data were collected from 3,995 community-dwellers and hospitalized persons. Nasal *S. aureus* carriage was found in 362 persons (9.1%). Logistic regression analysis was performed to identify which variables were independently associated with carriage of resistant *S. aureus*.

Results: The penicillins were the most frequently used antibiotics both in the community and in hospitalized patients. In the community, admission to a hospital was associated with carriage of *S. aureus* resistant to any of the tested antibiotics (odds ratio [OR] 2.5, 95% confidence interval [95% CI] 1.3-4.9) and any tetracycline resistance (OR 2.4, 95% CI 1.1-5.1). Having no symptoms was associated with less carriage of *S. aureus* with resistance to any of the tested antibiotics (OR 0.5, 95% CI 0.3-0.9) and any tetracycline resistance (OR 0.5, 95% CI 0.3-0.9). Crowding (OR 4.5, 95% CI 1.2-4.9) and low income (OR 8.9, 95% CI 1.8-43.9) were associated with multidrug resistance. In hospitalized patients, the use of penicillins was associated with resistance to any of the tested antibiotics (OR 3.9, 95% CI 1.4-11.6) and any tetracycline resistance (OR 3.7, 95% CI 1.1-12.0).

Conclusions: Antibiotic policies including proper diagnosis, treatment, and drug delivery process should be made by healthcare providers in Indonesia to help limit the emergence of antibiotic resistance.

INTRODUCTION

Antimicrobial resistance of bacteria is a growing problem worldwide. In low-resource countries, where extensive empirical use of antibiotics is common, the extent and the impact of this phenomenon tends to be even larger than in industrialized countries (20, 21). In general, the use of antibiotics is the key mechanism that drives the emergence of resistant microorganisms (3, 4). However, the epidemiology of such organisms is far from straightforward, and may even differ between carriage and disease. For example, for carriage of resistant *Staphylococcus aureus*, several determinants have been established, in addition to individual antibiotic use (9, 12). This is important knowledge since nasal carriage of *S. aureus* is the strongest risk factor for subsequent infection with this pathogen (22). In 2001-2002, 9,929 individuals were examined for *S. aureus* carriage in the United States. In a multivariate model, methicillin-resistant *S. aureus* (MRSA) carriage was associated with age 60 years or more and female sex (9). In a survey of nasal carriage in a rural American Indian reservation in 2001, MRSA was associated with antimicrobial use in the previous year and residence in a household of more than 7 individuals (12). Reported risk factors for MRSA infections vary by population. Among patients with *S. aureus* infections (whether MRSA or not) it includes exposure to healthcare settings, while among community dwellers, risk factors include intravenous drug use, diabetes, socioeconomic status and crowding (2, 15, 16). These determinants and risk factors, however, have only been established in studies in industrialized countries. Also, little is known about determinants that are associated with resistance patterns other than MRSA in *S. aureus*.

The 'Antimicrobial Resistance in Indonesia: Prevalence and Prevention' (AMRIN) study group investigated rectal and nasal carriage of resistant bacteria among inhabitants of two cities on the island of Java, Indonesia (3, 10, 13). The aim of the present study is to identify whether recent antibiotic use as well as demographic, socioeconomic, healthcare- related and disease-related variables are determinants for nasal carriage of resistant *S. aureus*.

MATERIALS AND METHODS

The AMRIN study surveillance was performed in two university hospitals, *viz.* Dr. Soetomo Hospital in Surabaya, East Java, and Dr. Kariadi Hospital in Semarang, Central Java, Indonesia, and three primary health centers (PHC, two in Surabaya and one in Semarang). Four population groups were studied for carriage of resistant microorganisms: patients upon admission to hospital within the first 24 hours of admission (group A), relatives of patients upon admission to the hospital at a rate of

one contact per patient (group B), patients visiting a PHC for consultation or vaccination (group C), and patients upon discharge from the hospital after hospitalization for five or more days (group D). Group C patients were included on specific study days twice weekly in Surabaya and once weekly in Semarang. Individuals in group A, B, and D were recruited from four departments: Internal Medicine, Surgery, Obstetrics and Gynecology and Pediatrics. The aim was to include 4,000 individuals; 500 individuals per group per city, whereby each department was equally represented. Individuals were excluded from the study if they had been transferred from another hospital, if they were not accompanied by a family member (group A), or if they had been admitted to a hospital during the previous three months (group A, B, and C). Approval of the Medical Ethics Committees was obtained before the start of the study (ethical clearance No/Panke.KKE/2001 [Surabaya] and 11/EC/FK/RSDK/2001 [Semarang]). Only patients who had given their informed consent were included in the study. For the purpose of analysis, individuals who had not been hospitalized (group A, B, and C) were combined into a community population, whereas group D patients formed the hospital population. Demographic data, socioeconomic data and, for community patients, data on health complaints and consumption of antibiotics in the month preceding the study were collected by semi-structured interviews, performed by specifically trained personnel (Indonesian and Dutch data collectors including researchers, residents, and medical students). For group A, diagnosis on admission and for group D, data on antibiotic consumption during hospitalization and diagnosis on discharge were collected from medical records.

Isolation of *S. aureus*

Nasal samples were taken with sterile, cotton-tipped swabs, which were put in Amies transport medium (Copan, Brescia, Italy) and transported in closed boxes at ambient temperature to the laboratory on the same day. All swabs were streaked on phenol red mannitol agar (Becton Dickinson, Heidelberg, Germany) within 24 hours. All colonies suspected of being *S. aureus* were tested with an agglutination test (Slidex Staph Plus, bioMérieux, Marcy l' Etoile, France) and the Vitek® 2 system (bioMérieux, Marcy l'Etoile, France) for identification. In case of doubt an *S. aureus* specific DNA hybridization test (Accuprobe, Genprobe Inc., San Diego, USA) was performed.

Antimicrobial susceptibility testing

Antimicrobial susceptibility testing of *S. aureus* was performed by disk diffusion according to the criteria of the Clinical and Laboratory Standards Institute (CLSI; formerly the NCCLS) (18). For susceptibility testing, disks were used loaded with the following antimicrobial agents (Oxoid, Basingstoke, UK): tetracycline (30 µg), oxacillin (1 µg), gentamicin (10 µg), erythromycin (15 µg), chloramphenicol (30 µg),

and trimethoprim-sulfamethoxazole (1.25/23.75 µg). Isolates that were intermediate susceptible according to the CLSI criteria were categorized as susceptible for the purpose of analysis. Oxacillin-resistant isolates were further subjected to confirmatory testing of methicillin resistance by *mecA* polymerase chain reaction (PCR) (17).

Variables

Origin (Surabaya or Semarang), sex, age (newborn to 16 years of age versus more than 16 years of age in accordance with the age limit for the Department of Pediatrics, and children less than two years old versus people of more than two years of age in accordance with approximate pre- and post-weaning periods), ethnicity and living area (urban versus rural) were selected demographic variables. Health insurance, income level (below or above poverty line),(1) education (primary school not completed versus primary school education and higher), employment, and crowding (one through eight versus nine or more individuals living together) were selected socioeconomic variables. Group (A, B, C, or D), Department (Internal Medicine, Surgery, Obstetrics and Gynecology, or Pediatrics), nursing class (first, second, or third with first class being the most expensive class) and length of stay in hospital (5 through 8 versus 9 or more days) were studied as healthcare-related variables. Only the last ward of admission was recorded; transfers were not recorded. For community patients, clinical signs and symptoms in the month preceding enrollment in the study (fever, diarrhoea, respiratory symptoms, other symptoms, or no symptoms) were the diseases-related variables and for patients upon admission and discharge whether or not an infection was diagnosed.

Antibiotic usage was recorded according to the nomenclature and subcategory definitions of the WHO ATC Classification code, subgroup antibacterials for systemic use (23). We analyzed any antibiotic use, i.e. whether or not a patient took any antibiotic in the preceding month or during hospitalization; use of an antibiotic from a specific ATC class, combined or not combined with an antibiotic from a different class; and single antibiotic use, i.e. use of an antibiotic from a specific ATC class not combined with an antibiotic from a different class. Combined use was defined as either simultaneous or successive use of antibiotics from different ATC classes.

Statistical analysis

Statistical analysis to identify determinants of resistance in *S. aureus* was carried out using SPSS version 13.0 for Windows (SPSS Inc., Chicago, IL, USA).

Individuals carrying resistant strains were compared with individuals carrying strains susceptible or intermediate to all tested antibiotics. Resistance as an outcome variable for each of the different antibiotics was explored in two different ways:

(i) Resistance of *S. aureus* to any of the tested antibiotics, irrespective of whether this was resistance to the specific antibiotic considered, or whether the resistance to the antibiotic of interest was part of a pattern of resistance to multiple antibiotics, was taken as the outcome (dependent) variable, and possible determinants for this variable identified.
(ii) Carriage of *S. aureus* resistant to the specific antibiotic of interest was taken as the outcome variable, and determinants for this outcome variable identified. This approach was only pursued when at least 10 isolates with the relevant resistance pattern were available.

To identify determinants for any of these outcome variables, logistic regression analysis with backward selection of variables was used. In view of the large number of inter-related candidate determinants, some of which were sparse (i.e. most individuals had the same value for this variable), each of the analyses was performed using a two-step procedure. First, candidate variables were selected by performing logistic regression on four partially overlapping sets of co-variables (Table S1).

(i) any antibiotic use, combined with all demographic, socioeconomic, healthcare-related and disease-related determinants,
(ii) demographic determinants,
(iii) socioeconomic determinants,
(iv) healthcare-related and disease-related determinants.

In the final step, logistic regression analysis was performed with all variables that were significantly associated with antibiotic resistance in any of these four analyses. The variables that were significantly associated with resistance in this final analysis were presumed to be independently associated with resistance. Use of antibiotics from specific antibiotic classes and single use of specific antibiotic classes were analyzed as separate sets of variables. When logistic regression could not be performed because of sparse data, variables with very small dispersion were excluded from the analyses.

RESULTS

A total of 3,995 individuals were enrolled between the period of July to October 2001 in Surabaya and January to May 2002 in Semarang. Their nasal carriage rate of *S. aureus* was 9.1% (362/3,995). One strain did not grow on Mueller Hinton agar when antimicrobial susceptibility testing was performed. Thus, complete data for analysis was available for 361 *S. aureus* carriers, 98 from the hospital and 263 from the community population. Data that were not suitable for analysis are shown in Figure 1 and Table S2. No significant differences in demographic, socioeconomic, disease-related and healthcare-related variables were observed in the community and hospital popu-

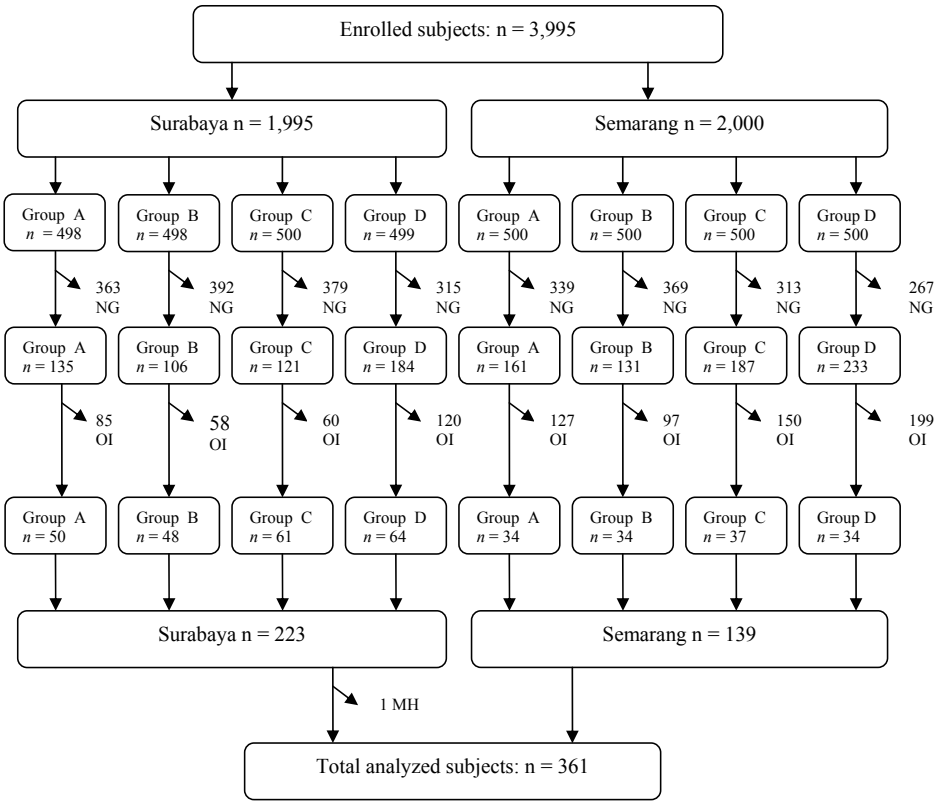

Figure 1. Flow chart with numbers of enrolled and analyzed subjects.
Reasons for exclusion of enrolled subjects from analysis: NG, no growth on agar plate; OI, other identification (Table S2), MH: no growth on Muller Hinton agar plate.

lations (Table 1). Additional information regarding population characteristics can be found in Table S3 and in Table S4.

Antimicrobial resistance

Of the 361 *S. aureus*, 245 (67.9%) were susceptible to all antibiotics tested, 116 (32.1%) were resistant to one or more antimicrobial agents, 78 (21.6%) were resistant to one antimicrobial agent and 38 (10.5%) were resistant to two or more antimicrobials (Table 2). Single resistance to oxacillin or gentamicin was not present in any of the isolates.

In the community, resistance rates to tetracycline, oxacillin, gentamicin, erythromycin, chloramphenicol, and trimethoprim-sulfamethoxazole (n=263) were 66 (25.1%), 0 (0%), 3 (1.1%), 7 (2.7%), 25 (9.5%), and 17 (6.5%), respectively. Single resistance rates to tetracycline, erythromycin, chloramphenicol, and trimethoprim-sulfamethoxazole were 42 (16%), 3 (1.1%), 9 (3.4%), 3 (1.1%), respectively.

Table 1. Demographic characteristics of community and hospital populations.

	Community n=263		Hospital n=98		*P*
Surabaya	158	(60.1)	64	(65.3)	NS
Group A (admission)	84	(31.9)	-		-
Group B (relatives)	82	(31.2)	-		-
Group C (PHC)	97	(36.9)	-		-
Group D (discharge)	-		98	(100)	-
Internal Medicine	10[a]	(11.9)	17	(17.3)	NS
Surgery	25[a]	(29.8)	19	(19.4)	NS
Obstetrics and Gynaecology	34[a]	(40.5)	46	(46.9)	NS
Paediatrics	15[a]	(17.9)	16	(16.3)	NS
Age above 16	207	(78.7)	73	(74.5)	NS
Female sex	188	(71.5)	65	(66.3)	NS
Javanese ethnicity	250	(95.1)	89	(90.8)	NS
Urban provenance	180	(68.4)	66	(67.3)	NS
Health insurance	77	(29.3)	28	(28.6)	NS
Low income	109	(41.4)	42	(42.9)	NS
Primary school completed	146	(55.5)	51	(52.0)	NS
Employment	143	(70.1)	50	(71.4)	NS
Crowding > 8 persons per household	16	(6.1)	8	(8.2)	NS
Nursing class III	65[a]	(77.4)	73	(74.5)	NS
Length of stay > 8 days	-		45	(45.9)	-
Clinical sign and infection	181	(68.8)	-		-
Infection diagnosis in hospital	11[a]	(13.1)	16	(16.3)	NS

Absolute numbers are shown, with percentages between brackets.
'NS' means that no significant differences were observed between the populations.
[a] Only calculated for group A, percentages are proportions of patients in group A.

In hospitalized patients, resistance rates to tetracycline, oxacillin, gentamicin, erythromycin, chloramphenicol, and trimethoprim-sulfamethoxazole (n=98) were 24 (24.5%), 2 (2.0%), 1 (1.0%), 5 (5.1%), 9 (9.2%), and 7 (7.1%), respectively. Single resistance to tetracycline, erythromycin, chloramphenicol, and trimethoprim-sulfamethoxazole were 16 (16.3%), 1 (1.0%), 2 (2.0%), and 2 (2.0%), respectively.

Antibiotic use

Data on the use of antibiotics in hospitalized patients and community populations are shown in Table 3. In the community (263 individuals), 42 antibiotic courses were prescribed. Seventeen out of 263 (6.5%) people in the community group did not know whether they had used antibiotics recently or not. In the hospital (98 patients), 113 antibiotic courses were prescribed. The most frequently used class of antibiotics in both populations was the penicillins. In hospitalized patients,

Table 2. Resistance patterns observed among *S. aureus* isolates.

Number of isolates (%)	Tetracycline	Oxacillin	Gentamicin	Erythromycin	Chloramphenicol	Trimethoprim-sulfamethoxazole
245 (67.9)	S	S	S	S	S	S
58 (16.1)	R	S	S	S	S	S
12 (3.3)	R	S	S	S	R	S
11 (3.0)	S	S	S	S	R	S
9 (2.5)	R	S	S	S	S	R
5 (1.4)	S	S	S	S	S	R
4 (1.1)	R	S	S	S	R	R
4 (1.1)	S	S	S	R	S	S
3 (0.8)	R	S	S	R	S	S
2 (0.6)	S	S	S	S	R	R
2 (0.6)	R	S	R	S	R	S
2 (0.6)	S	S	S	R	R	R
1 (0.3)	S	S	S	R	S	R
1 (0.3)	S	R	S	R	R	S
1 (0.3)	R	S	R	S	S	S
1 (0.3)	R	R	R	R	S	R

The number of times a given resistance pattern was found is shown in the first column, with the prevalence between brackets. Resistance is represented by an R and susceptibility by an S.

Table 3. Total and single antibiotic use in community and hospital populations.

	Community		Hospital	
	Total use (n)	Single use (%)	Total use (n)	Single use (%)
Tetracycline	3	66	0	0
Penicillins	35	97	65	75
Amphenicols	2	100	3	33
Cephalosporins	0	0	15	47
Carbapenems	0	0	0	0
Sulphonamides	1	100	3	0
Macrolides	0	0	3	0
Aminoglycosides	1	100	10	0
Quinolones	0	0	3	33
Metronidazole	0	0	5	0
Others	0	0	6	0
Total	42	95	113	51

Total use (n) is the number of antibiotic prescriptions; single use (%) is single antibiotic use as percentage of total number of prescriptions.

cephalosporins and aminoglycosides were also regularly consumed. Tetracycline was not used in hospitalized patients, but in the community, 3 tetracycline courses were prescribed.

Determinants of resistance in the community (group A, B, and C)

Analysis of determinants of resistance in the community was performed with resistance to any of the tested antibiotics, any tetracycline resistance (tetracycline resistance with or without other resistance), and multidrug resistance (resistance to two or more antibiotic classes), because more than 10 cases were available for these resistance groups. Admission to a hospital was associated with carriage of *S. aureus* resistant to any of the tested antibiotics (odds ratio [OR] 2.5, 95% confidence interval [95% CI] 1.34.9) and any tetracycline resistance (OR 2.4, 95% CI 1.15.1). Having had no symptoms was associated with less carriage of *S. aureus* with resistance to any of the tested antibiotics (OR 0.5, 95% CI 0.30.9) and any tetracycline resistance (OR 0.5, 95% CI 0.30.9). Crowding (OR 4.5, 95% CI 1.4-15.1) and low income (OR 8.9, 95% CI 1.8-43.9) were associated with multidrug resistance. Antibiotic use variables were not associated with any pattern of resistance.

Determinants of resistance in hospitalized patients (group D)

Analysis of determinants of resistance in hospitalized patients was performed with resistance to any of the tested antibiotics, any tetracycline resistance, single tetracycline resistance, and multidrug resistance. The use of penicillins as monotherapy or in combination was associated with carriage of *S. aureus* with resistance to any of the tested antibiotics (OR 3.9, 95% CI 1.411.6) and any tetracycline resistance (OR 3.7, 95% CI 1.112.0). Sulphonamide use was associated with multidrug resistance (OR 13.5, 95% CI 1.1166.6). Single penicillin use was associated with single tetracycline resistance (OR, 3.8, 95% CI, 1.113.1). The consumption of other antibiotics was not associated with resistance to any of the six tested antibiotics. Patients upon discharge in Semarang had a lower risk of carriage of *S. aureus* with any resistance (OR 0.2, 95% CI 0.10.7), any tetracycline resistance (OR 0.1, 95% CI 0.020.47), and single tetracycline resistance (OR 0.2, 95% CI 0.03-0.9) compared with patients in Surabaya. Discharge from the Department of Obstetrics and Gynecology (OR 5.1, 95% CI 1.1-23.2), rather than from Internal Medicine (reference category), was associated with carriage of *S. aureus* with any tetracycline resistance. Demographic, socioeconomic, healthcare- and disease- related variables were not associated with multidrug resistance.

DISCUSSION

The present analysis, including 361 carriers of *S. aureus*, addresses the determinants of carriage of resistant *S. aureus* strains in individuals in hospitals and in the community in Indonesia. For both clinicians and antibiotic policy makers, it is important to identify populations at risk for infections with resistant microorganisms and to

better understand the epidemiology of antibiotic resistance. It may also aid in the design of more focused studies on preventing the emergence and spread of such microorganisms. Although many factors contribute to the emergence of resistance microorganisms, the use of antimicrobials is considered the most important determinant of resistance (11, 14, 19). The use of antibiotics in hospitalized patients was strikingly high: 84% of the discharged patients were given antibiotics during their hospital stay, mainly aminopenicillins (54%) and cephalosporins (17%). Only 21% of prescriptions were considered definitely appropriate, 15% were inappropriate regarding choice, dosage or duration, and 42% of prescriptions, many for surgical prophylaxis and fever without diagnosis of infection, were deemed unnecessary (6). In this hospital population, the use of penicillins (amoxicillin or ampicillin) was associated with resistance to any of the tested antibiotics and resistance to tetracycline in combination with other antibiotics. It is difficult to say whether this observation reflects the exposure to antibiotics in hospitalized patients, or an association of penicillin use/exposure with a diversity of resistance patterns including tetracycline resistance. Genes encoding for penicillin and tetracycline resistance may co-exist in *S. aureus* and spread in special epidemiologic populations (8). A survey of resistance plasmids circulating in these populations is needed to better understand this process.

In the community, no associations between antibiotic use and certain resistance patterns were observed. However, patients upon admission to hospital were more often colonized with *S. aureus* resistant to any antibiotic and resistant to tetracycline. Indeed, of all patients upon admission to a hospital (n=998), more than one fifth had consumed antibiotics in the four weeks preceding admission. Patients visiting a PHC or (healthy) family members of admitted patients less frequently reported antibiotic use (5). It is likely that antibiotics are mainly taken by patients with health complaints. Indeed, individuals with no symptoms were less often colonized with resistant *S. aureus*. Less individuals from Semarang carried resistant *S. aureus*, than from Surabaya, but more carried resistant *E. coli*, although antibiotic consumption patterns in the two cities were similar (3, 13).

The present analysis has certain limitations. Firstly, the rather small proportion of individuals carrying nasal *S. aureus* and the low number of resistant *S. aureus* limited the statistical power to detect associations in this population. The nasal carriage rate of *S. aureus* in this study was lower than in other developing and western countries (7). Although we screened over 1,200 suspected colonies many of those strains were identified as belonging to other *Staphylococcus* spp. Despite the relatively small sample size, however, logistic regression identified some interesting determinants of resistance. Secondly, antibiotic use in individuals in the community with carriage of resistant *S. aureus* was relatively low, and we may therefore have missed the role of antibiotic use as a determinant of carriage of resistant *S. aureus*. Thirdly, our

'community' population may not be fully representative of the general community population in Java, since the majority of participants in our study had been in contact with healthcare institutions to a varying extent. However, we have shown that the more frequently individuals are in contact with healthcare institutions, the more likely they are to carry resistant *S. aureus*. Fourthly, these data were collected in 2001-2002, and colonization and resistance rates, as well as antibiotic consumption patterns may have changed in the population since that time. However these are as yet the only complete data available from Indonesia. Finally, isolates associated with clinical *S. aureus* infection were not included in our analysis, but since *S. aureus* carriage is the strongest risk factor for subsequent infection it is a key to understand carriage (22).

In conclusion, we have shown that the use of penicillins is a determinant for carriage of resistant *S. aureus* in two hospitals in Java. Factors reflecting increased contact with healthcare institutions play a role as well in Indonesia. Healthcare providers should be made aware of these findings so that they can develop policies including improving diagnosis, treatment and drug delivery process to limit the use of antibiotics in these situations where they are really needed.

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Table S1. Model of selection of candidate variables for final logistic regression model and model for analysis of antibiotic use.

		Interim analyses (selection of candidate variable)							Antibiotic use	
Group	Model	All candidate variables	excluded	Demographic variables	Socio economic adults¶	Socio economic all ages¶	Healthcare & diseases related variables	Significant variables	Any antibiotic use	Single antibiotic use
Community (group A, B, and C)	Any resistance versus completely susceptible (n=263)	Origin Sex Age Ethnicity Provenance Health insurance Income Crowding **Group** Fever symptoms Diarrhoeal symptoms Respiratory symptoms Other symptoms **No symptoms** Antibiotic use yes/no	Infection diagnosis on admission*	Origin Sex Age Ethnicity Provenance	Health insurance Income **Crowding** Education Employment	Health insurance Income Crowding	**Group** Fever symptoms Diarrhoeal symptoms Respiratory symptoms Other symptoms No symptoms	Group No symptoms Nagelkerke R2: 0.077	-	-
Group A, B, and C	Any tetracycline resistance (n=245)	Origin Sex Age Ethnicity Provenance Health insurance Income Crowding **Group** Fever symptoms Diarrhoeal symptoms Respiratory symptoms Other symptoms No symptoms Antibiotic use yes/no	Infection diagnosis on admission*	Origin Sex Age Ethnicity Provenance	Health insurance **Income** **Crowding** Education Employment	Health insurance Income **Crowding**	**Group** Fever symptoms Diarrhoeal symptoms Respiratory symptoms Other symptoms **No symptoms**	Group No symptoms Nagelkerke R2: 0.088	-	-

		Interim analyses (selection of candidate variable)							Antibiotic use	
Group	Model	All candidate variables	excluded	Demographic variables	Socio economic adults¶	Socio economic all ages¶	Healthcare & diseases related variables	Significant variables	Any antibiotic use	Single antibiotic use
Group A, B, and C	Multidrug resistance (n=263)	Origin Sex Age Ethnicity Provenance Health insurance **Income** **Crowding** Group Fever symptoms Diarrhoeal symptoms **Respiratory symptoms** Other symptoms **No symptoms** Antibiotic use yes/no	Infection diagnosis on admission*	Origin Sex Age **Ethnicity** Provenance	Health insurance **Income** **Crowding** Education **Employment**	Health insurance **Income** **Crowding**	**Group** Fever symptoms Diarrhoeal symptoms Respiratory symptoms **Other symptoms** No symptoms	Crowding Income Nagelkerke R2: 0.384	-	-
Hospital (group D)	Any resistance versus completely susceptible (n=98)	**Origin** Sex Age Ethnicity Provenance Health insurance Income Crowding Group Department Nursing class Length of stay Antibiotic use yes/no	Infection diagnosis on discharge*	**Origin** Sex Age Ethnicity Provenance	Health insurance Income Crowding Education Employment	Health insurance Income Crowding	Department Nursing class Length of stay	Origin Nagelkerke R2: 0.113	Penicillins	-
Group D	Any tetracycline resistance (n=90)	Origin Sex Age Ethnicity Provenance **Health insurance** Income Crowding Group **Department** Nursing class Length of stay Antibiotic use yes/no	Infection diagnosis on discharge*	**Origin** Sex Age Ethnicity Provenance	Health insurance Income Crowding Education Employment	Health insurance Income Crowding	Department Nursing class Length of stay	Origin Nursing ward (2) Nagelkerke R2: 0.327	Penicillins	-

		Interim analyses (selection of candidate variable)							Antibiotic use	
Group	Model	All candidate variables	excluded	Demographic variables	Socio economic adults¶	Socio economic all ages¶	Healthcare & diseases related variables	Significant variables	Any antibiotic use	Single antibiotic use
Group D	Single tetracyclineresistance (n=82)	Origin Sex Age Ethnicity Provenance **Health insurance** Income Crowding Group Department Nursing class Length of stay Antibiotic use yes/no	Infection diagnosis on discharge*	**Origin** Sex Age Ethnicity Provenance	Health insurance Income Crowding Education Employment	**Health insurance** Income Crowding	Department Nursing class Length of stay	Origin Nagelkerke R2: 0.175	-	Penicillins
Group D	Multidrug resistance (n=98)	Origin Sex Age Ethnicity Provenance Health insurance Income Crowding Group Department Nursing class Length of stay Antibiotic use yes/no	Infection diagnosis on discharge*	Origin Sex Age Ethnicity Provenance	Health insurance Income Crowding Education Employment	Health insurance Income Crowding	Department Nursing class Length of stay	-	Sulphonamides	-

All candidate variables that were significant in one or more of the interim analyses are printed in bold.

Candidate variables that were excluded from the analyses due to very small dispersion (logistic regression could not be performed because of sparse data) are indicated by an asterisk.

¶ Interim analysis of socioeconomic variables was carried out separately for all ages and for adults, since the variables education and employment could not be analyzed for children.

Table S2. Subculture and identification of putative *S. aureus* isolates.

No	Organisms	Surabaya				Semarang				Total
		A	B	C	D	A	B	C	D	
1	*S. aureus*	50	48	61	64	34	34	37	34	362
2	Coagulase-negative staphylococci, no further determination	13	8	21	12	86	60	104	122	426
3	*S. sciuri*	0	0	0	1	12	4	8	30	55
4	*S. lentus*	0	0	0	0	0	0	3	0	3
5	*S. xylosus*	0	0	0	0	0	1	3	2	6
6	*S. haemolyticus*	0	0	0	1	1	0	2	2	6
7	*S. kloosii*	0	0	0	0	0	1	1	0	2
8	*S. saprophyticus*	0	0	0	0	0	0	2	0	2
9	*S. simulans*	0	0	0	0	1	0	1	0	2
10	*S. hyicus*	1	1	0	1	0	0	0	1	4
11	Unidentified	3	0	3	0	4	2	2	6	20
12	Gram negative rod	1	0	0	2	5	8	7	12	35
13	No growth	67	49	36	103	18	21	17	24	335
	Total	**135**	**106**	**121**	**184**	**161**	**131**	**187**	**233**	**1,258**

Table S3. Distribution of variables and resistance patterns in the community (group A, B, and C)

	Any resistance*			Any tetracycline resistance**			Multidrug resistance***		
	(n=263, n resistant=84)			*(n=245, n resistant=66)*			*(n=263, n resistant=27)*		
Variables	R	S	*P* value	R	S	*P* value	R	S	*P* value
Origin (Surabaya)	54	104	0.340	45	104	0.152	20	138	0.117
Sex (Female)	63	125	0.387	49	125	0.500	22	166	0.224
Age (over 16)	66	141	0.971	53	141	0.793	21	186	0.901
Ethnicity (Javanese)	76	174	0.030	60	174	0.074	22	228	0.006
Provenance (urban)	57	123	0.889	44	123	0.760	17	163	0.518
Health insurance (yes)	30	47	0.116	23	147	0.187	10	67	0.350
Income (below poverty line)	55	99	0.119	42	99	0.242	21	133	0.032
Crowding (8 or more per household)	16	20	0.083	14	20	0.044	8	28	0.018
Group: A (admission)	38	46	0.005	29	46	0.020	15	69	0.010
B (family members)	23	59		18	59		8	74	
Fever symptoms	3	6	1.000	2	6	1.000	1	8	1.000
Diarrhoeal symptoms	4	10	1.000	3	10	1.000	1	13	1.000
Respiratory symptoms	23	53	0.710	19	53	0.900	8	68	1.000
Others symptoms	26	79	0.042	21	79	0.082	4	101	0.005
No symptoms	50	131	0.026	39	131	0.034	12	169	0.004
Any antibiotic use in past month	13	30	0.679	9	30	0.457	3	40	0.586
Any tetracycline use	1	2	1.000	1	2	1.000	0	3	1.000
Any penicillin use	8	27	0.216	6	27	0.223	2	33	0.549

Any amphenicol use	2	0	0.101	1	0	0.269	0	2	1.000
Any sulphonamide use	0	1	0.681	0	1	1.000	0	1	1.000
Any macrolide use	-			-			-		
Any aminoglycoside use	1	0	0.319	-			1	0	0.103
Any quinolone use	-			-			-		
Any metronidazole use	-			-			-		
Single tetracycline use	1	1	0.538	1	1	0.467	0	2	1.000
Single penicillin use	8	26	0.260	6	26	0.263	2	32	0.547
Single amphenicol use	2	0	0.107	1	0	0.269	0	2	1.000
Single sulphonamide use	0	1	1.000	0	1	1.000	0	1	1.000
Single macrolide use	-			-					
Single aminoglycoside use	1	0	0.319	-			1	0	0.103
Single quinolone use	-			-			-		
Single metronidazole use	-			-			-		
No education	4	10	1.000	4	10	1.000	1	13	1.000
only calculated for adults †	(*n*=204, *n* resistant=61)			(*n*=191, *n* resistant=48)			(*n*=204, *n* resistant=20)		
No fixed employment	10	11	0.985	7	11	0.697	6	15	0.513
only calculated for group A ††	(*n*=59, *n* resistant=28)			(*n*=54, *n* resistant=23)			(*n*=59, *n* resistant=13)		
Dept: Surgery	13	12	0.319	10	12	0.191	8	14	0.323
Obstetrics & Gynecology	17	17		15	17		9	25	
Pediatrics	6	9		2	9		1	14	
Nursing class III	28	37	0.462	20	37	0.257	1	54	0.736
Admission diagnosis infection	4	7	0.747	2	7	0.468	1	10	0.680

In this table, the numbers of resistant and susceptible isolates are shown for each variable (e.g. in the first columns the number of resistant and susceptible isolates from Surabaya are shown, respectively). From this table, cross-tabulations can be constructed for each variable.
Corresponding *P* values result from χ^2 testing for each combination of variable and resistance pattern.
Resistance patterns: * resistance to any of the tested antibiotics (tetracycline, oxacillin, gentamicin, erythromycin, chloramphenicol, and/or trimethoprim-sulfamethoxazole), ** resistance to tetracycline with or without other resistance and *** resistance to two or more antibiotic classes.
† Different denominators (204 for resistance to any of the tested antibiotics and multidrug resistance and 191 for any tetracycline resistance), because this population characteristic could only be analyzed for the adult population.
†† Different denominators, because these population characteristics could only be analyzed in subjects upon admission to hospital.

Table S4. Distribution of variables and resistance patterns in the hospital (group D).

Variables	Any resistance*			Any tetracycline Resistance**			Single tetracycline resistance***			Multidrug resistance ****		
	(n=98, n resistant=32)			*(n=90, n resistant=24)*			*(n=82, n resistant=16)*			*(n=98, n resistant=11)*		
	R	S	*P* value	R	S	*P* value	R	S	*P* value	R	S	*P* value
Origin (Surabaya)	27	37	0.006	22	37	0.002	14	37	0.020	10	54	0.058
Sex (Female)	24	41	0.206	19	41	0.129	13	41	0.148	7	58	0.841
Age (over 16)	28	45	0.040	3	21	0.067	15	45	0.057	8	65	0.887
Ethnicity (Javanese)	29	60	0.000	21	60	-	13	60	0.368	11	78	0.592
Provenance (urban)	21	45	0.800	15	45	0.613	12	45	0.765	6	60	0.337
Health insurance (yes)	11	17	0.376	10	17	0.145	8	17	0.074	3	25	0.000
Income (below poverty line)	20	36	0.465	15	36	0.507	12	36	0.136	5	51	0.406
Crowding (8 or more per household)	3	8	0.686	1	8	0.266	0	8	0.344	1	10	0.812
Dept: Surgery	4	15	0.080	2	15		0	15	0.014	3	16	0.822
Obstetrics & Gynecology	21	25		17	25		13	25		5	41	
Pediatrics	3	13		2	13		1	13		2	14	
Room class in hospital: class III	22	51	0.453	15	51		9	51		8	65	
Length of stay 9 days or more	14	31	0.764	9	31	0.424	2	31	0.012	8	37	0.058
Discharge diagnosis infection	5	11	0.896	4	11	1.000	4	11	0.477	1	15	0.491
Any antibiotic use during hospitalization	28	50	0.176	21	50	0.227	14	50	0.502	10	68	0.323
Any tetracycline use	-			-			-			-		
Any penicillin use	27	38	0.008	20	38	0.024	13	38	0.080	10	55	0.061
Any amphenicol use	1	2	0.980	1	2	0.610	0	2	1.000	1	2	0.218
Any cephalosporin use	4	11	0.591	2	11	0.265	1	11	0.444	3	12	0.242
Any carbapenem use	0	0		-			-			-		
Any sulphonamide use	2	1	0.202	0	1	1.000	0	1	1.000	2	1	0.002
Any macrolide use	0	3	0.221	0	3	0.562	0	3	1.000	0	3	0.532
Any aminoglycoside use	5	5	0.217	2	5	1.000	1	5	1.000	3	7	0.047
Any quinolone use	1	2	0.980	1	2	1.000	0	2	1.000	1	2	0.218
Any metronidazole use	2	3	0.719	1	3	1.000	0	3	1.000	2	3	0.095
Any other antibiotic use	2	4	0.971	1	4	1.000	0	4	0.581	2	4	0.134
Single tetracycline use				-			-			0	0	-
Single penicillin use				16	29	0.057	12	29	0.026	5	44	0.749
Single amphenicol use	0	1	0.484	0	1	1.000	0	1	1.000	0	1	1.000
Single cephalosporin use	1	6	0.282	1	6	0.670	1	6	1.000	0	7	1.000
Single sulphonamide use	-			-			-			-		
Single macrolide use	-			-			-			-		
Single aminoglycoside use	-			-			-			-		
Single quinolone use	0	1	1.000	0	1	1.000	0	1	1.000	0	1	1.000

only calculated for adults †	(*n*=73, *n resistant*=28)			(*n*=66, *n resistant*=21)			(*n*=60, *n resistant*=15)			(*n*=73, *n resistant* =8)		
No education	1	3	1.000	1	3	1.000	0	3	0.561	1	3	0.350
only calculated for adults ††	(*n*=70, *n resistant*=27)			(*n*=63, *n resistant*=20)			(*n*=58, *n resistant*=15)			(*n*= 70, *n resistant* =7)		
No fixed employment	7	12	0.810	6	12	0.908	6	12	0.521	1	18	0.664
only calculated for adults †††	(*n*= 69, *n resistant*=27)			(*n*= 62, *n resistant*=20)			(*n*= 57, *n resistant*=15)			(*n*=69, *n resistant* =7)		
Dept: Surgery	4	15	0.080	2	15	0.048	0	15	0.014	3	16	0.822
Obstetrics & Gynecology	21	25		17	25		13	25		5	41	
Pediatrics	3	13		2	13		1	13		2	14	

In this table, the numbers of resistant and susceptible isolates are shown for each variable (e.g. in the first columns the number of resistant and susceptible isolates from Surabaya are shown, respectively). From this table, cross-tabulations can be constructed for each variable.

Corresponding *P* values result from χ^2 testing for each combination of variable and resistance pattern.

Resistance patterns: * resistance to any of the tested antibiotics (tetracycline, oxacillin, gentamicin, erythromycin, chloramphenicol, and/or trimethoprim-sulfamethoxazole), ** any tetracycline resistance: resistance to tetracycline with or without other resistance, resistance to tetracycline only and *** multidrug resistance: resistance to two or more antibiotic classes.

† Different denominators (73 for any resistance and multidrug resistance, 66 for any tetracycline resistance and 60 for resistance to tetracycline only), because this population characteristic could only be analyzed for the adult population.

†† Different denominators (70 for any resistance and multidrug resistance, 63 for any tetracycline resistance and 58 for resistance to tetracycline only), because this population characteristic could only be analyzed for the adult population.

††† Different denominators, because for 29 subjects, these date were missing.

Chapter 7

Unusually high prevalence of Panton-Valentine leukocidin genes among methicillin-sensitive *Staphylococcus aureus* carried in the Indonesian population

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ABSTRACT

Few data on the molecular characteristics and epidemiology of *Staphylococcus aureus* from Indonesia are available. The purpose of the present study was to define *S. aureus* reservoirs in both the Indonesian community and the hospital using a collection of 329 nasal carriage isolates obtained during a survey of 3,995 healthy individuals and patients from Java, Indonesia. Only one strain (0.3%) was identified as methicillin-resistant *S. aureus* (MRSA) by *mecA* gene PCR. The Panton-Valentine leukocidin (PVL) genes were detected in 35 methicillin-sensitive *S. aureus* (MSSA) strains (10.6%). Molecular typing by pulsed-field gel electrophoresis of the 329 isolates showed extensive genetic diversity among both PVL-positive and PVL-negative strains. In Surabaya, Indonesia, however, a cluster was identified that was strongly associated with the presence of the PVL locus ($P<0.0001$). As determined by high-throughput amplified fragment length polymorphism (AFLP), PVL-positive strains occurred throughout all major AFLP clusters (I-IV). Multilocus sequence typing of a subset of isolates showed that most PVL-positive strains belonged to sequence type (ST) 188, while most PVL-negative isolates belonged to ST45. The high prevalence of PVL-positive *S. aureus* strains in certain regions of Indonesia is of concern since these strains may cause severe infections in the community and in hospitals.

INTRODUCTION

Humans are a natural reservoir of *Staphylococcus aureus*, with the moist squamous epithelium of the anterior nares acting as the primary ecological niche (12, 33). Nasal carriage of *S. aureus* has been identified as a major risk factor for the development of infections, including *S. aureus* bacteremia (13, 31).

In the past four decades, the incidences of both community-acquired and hospital-acquired *S. aureus* infections have increased, while antibiotic treatment options are increasingly hampered by the spread of methicillin-resistant *S. aureus* (MRSA) strains and, more recently, *S. aureus* strains resistant to other classes of antibiotics (26, 27). The prevalence of MRSA in some Asian countries, such as Taiwan and China, is among the highest in the world (2). Community-acquired MRSA (CA-MRSA) infections also appear to be an emerging phenomenon in some Asian countries (6, 10, 11). The majority of these CA-MRSA isolates carry the Panton-Valentine leukocidin (PVL), a virulence factor that is strongly associated with skin infections and severe necrotizing pneumonia (8, 9, 30).

Several studies have assessed local population structures of *S. aureus* in order to investigate clonality and associated virulence. In a previous analysis of over 1,000 *S. aureus* strains from The Netherlands, by using amplified fragment length polymorphism (AFLP) and multilocus sequence typing (MLST), evidence was provided showing that essentially any *S. aureus* genotype carried by humans can transform into a life-threatening human pathogen, but that certain clones may be more virulent than others (20). A smaller study of 74 MRSA strains of unknown clinical or nonclinical origin from 12 Asian countries revealed two major genotypes with a distinct geographic distribution (14). Most of the Korean and Japanese isolates belonged to clonal complex (CC) 5, while most MRSA strains from other Asian countries, including seven strains from Indonesia, belonged to sequence type (ST) 239, a distinct lineage within CC8. This finding was recently corroborated by an analysis of 615 MRSA isolates from 11 Asian countries by Chongtrakool et al. (7). They found the same MLST profile (ST239) for three representative clinical isolates from Jakarta, Indonesia. However, further data on the genotypic characteristics of methicillin-susceptible *S. aureus* (MSSA) and MRSA from Indonesia are not available to our knowledge.

Recently, a population-based survey of 4,000 people in two cities on the island of Java (Surabaya and Semarang) was conducted by the "Antimicrobial Resistance in Indonesia, Prevalence and Prevention" (AMRIN) study group in order to quantify human carriage of resistant microorganisms (17). The purpose of the present study was to define *S. aureus* reservoirs in both the Indonesian community and the hospital using *S. aureus* strains obtained in the AMRIN study.

MATERIALS AND METHODS

Bacterial isolates

The AMRIN study is a population-based study among four groups of individuals in two cities, Semarang and Surabaya, aimed at investigating the level of carriage of resistant microorganisms. These groups involved patients on the day of admission to the hospital (group 1), patients on the day of discharge after five or more days of hospitalization (group 2), persons visiting a primary health center (group 3), and healthy relatives or household members of the group 1 patients (group 4). Nasal swabs were taken from patients or healthy individuals in the Internal Medicine, Surgery, Gynecology and Obstetrics, and Pediatrics departments (group 1, 2, and 4). The specimens were collected from July to October 2001 in Surabaya and from January to May 2002 in Semarang. The Medical Ethics Committees of both hospitals approved of the study protocol.

Cultures of the anterior nares were obtained with sterile cotton swabs from 3,995 persons after they gave informed consent. Within 24 hours, these swabs were inoculated on phenol red mannitol agar (Becton Dickinson, Heidelberg, Germany). Colonies suspected of being *S. aureus* were stored in trypticase soy agar (TSA). This collection, comprising more than 1,200 putative *S. aureus* strains, was subsequently speciated with an agglutination test (Slidex Staph Plus, bioMérieux, Marcy l' Etoile, France) and the Vitek® 2 system (bioMérieux, Inc., Hazelwood, MO, USA). In case of doubt, an *S. aureus*-specific DNA hybridization test (AccuProbe, Gen-Probe Inc., San Diego, CA, USA) was performed. Susceptibility testing was performed in Indonesia, and the results have been published elsewhere (17). In the present analysis, we included the first 329 confirmed *S. aureus* isolates (Table 1).

Pulsed-field gel electrophoresis (PFGE)

PFGE of *Sma*I digests of chromosomal DNA from all 329 strains was performed as described previously (15). Relatedness among the PFGE profiles was evaluated with Bionumerics software (version 3.0; Applied Maths, Ghent, Belgium). A dendrogram was produced using the Dice coefficient and an unweighted-pair group method using arithmetic averages (UPGMA). Band tolerance was set at 2.0%.

DNA isolation and detection of *mecA* and *pvl* genes

Chromosomal DNA was extracted with the MagNA Pure LC DNA Isolation Kit III (Bacteria, Fungi) using the MagNA Pure LC Instrument (Roche Diagnostics, Almere, The Netherlands) (20). The DNA concentration was assessed spectrophotometrically, and samples were stored at -20°C. The presence of the *mecA* and *pvl* genes was determined by PCR (18, 24). *mecA*-positive strains and isolates containing the *pvl*

Table 1. Origin of the 329 *S. aureus* strains included in the study.

Population group		No. of isolates per city (%)	
	Department	Semarang	Surabaya
Admission		29 (23.2)	45 (22.1)
	Internal Medicine	4	4
	Surgery	10	13
	Gynecology/ Obstetrics	7	25
	Pediatrics	8	3
Discharge		31 (25.6)	60 (29.4)
	Internal Medicine	3	13
	Surgery	7	10
	Gynecology/ Obstetrics	12	31
	Pediatrics	9	6
Primary health center		33 (26.4)	56 (27.4)
Relatives		32 (24.8)	43 (21.1)
	Internal Medicine	4	6
	Surgery	11	12
	Gynecology/ Obstetrics	5	16
	Pediatrics	12	9
Total		125 (100)	204 (100)

genes (whether *mecA*-positive or not) were further subjected to a multiplex PCR to identify staphylococcal cassette chromosome *mec* (SCC*mec*) types I to V (5). Positive and negative control strains were included in each PCR run.

High-throughput AFLP

A selection of 81 isolates was analyzed by high throughput-AFLP. We selected every fourth isolate when going from top to bottom through the PFGE dendrogram. DNA restriction, ligation of AFLP adapters, and amplification of the modified fragments were carried out as described previously (20). Briefly, bacterial DNA was digested with the enzymes *Mbo*I and *Csp6*I (New England Biolabs, Westburg, Leusden, The Netherlands). Ligation was performed by using specific linker oligonucleotide pairs (for *Mbo*I, 5'-CTCGTAGACTGCGTACC-3' and 5'-ATCGGTACGCAGTCTAC-3'; for *Csp6*I, 5'-ACGATGAGTCCTGAC-3' and 5'-TAGTCAGGACTCAT-3'). Subsequently, a nonselective preamplification was performed using the *Mbo*I primer (5'-GTAGACT-GCGTACCGATC-3') and *Csp6*I primer (5'-ACGATGAGTCCTGACTAC-3'). In the final amplification, a ^{33}P-labeled *Mbo*I primer containing one selective nucleotide (either +C or +G) and a *Csp6*I primer containing two selective nucleotides (+TA) were used. The amplified material was analyzed by polyacrylamide gel electrophoresis and autoradiography.

AFLP database

We compared the genetic structure of the 81 Indonesian *S. aureus* isolates with the (previously determined) natural population structure of *S. aureus* carriage isolates from healthy individuals from the Rotterdam area (The Netherlands) (20). The AFLP database comprises high throughput-AFLP patterns from 829 nonclinical *S. aureus* isolates from the Dutch study (20).

AFLP data analysis

For two-dimensional clustering of the AFLP genotype patterns in the AFLP database, an agglomerative (successive) hierarchical procedure was performed using the UPGMA distance algorithm (20). The Tanimoto method was used to calculate the similarity matrix (Spotfire DecisionSite 7.2, Spotfire, Göteborg, Sweden). Principal component analysis (PCA) was used to express the data three-dimensionally after mathematical data reduction. In this way similarities and differences are highlighted in components that reveal optimal variability. PCA analysis was performed using Spotfire DecisionSite 7.2 software.

MLST

MLST was carried out for 36 *S. aureus* strains from the AFLP set using DNA arrays (Affymetrix, Santa Clara, California, USA; bioMérieux, Marcy l'Etoile, France) (28). The selected isolates were equally distributed across the PFGE dendrogram by selecting alternately one out of two and one out of three strains that had been analyzed by AFLP, going from top to bottom through the PFGE dendrogram.

Statistical analysis

Data were analyzed using statistical software packages SPSS Version 11.0 (SPSS, Chicago, IL, USA) and EpiInfo version 5.00 (Centers for Disease Control and Prevention, Atlanta, GA, USA). Chi-square or Fisher's exact tests (two-tailed) were used when appropriate for comparisons of proportions. *P* values less than 0.05 were considered significant.

RESULTS

PFGE

Molecular typing by PFGE revealed extensive strain heterogeneity (Figure 1). At a similarity value of circa 50%, however, three PFGE clusters, designated A to C, could be distinguished (Figure 1, Table 2). Strains from Semarang and Surabaya were equally distributed among clusters A and B. Cluster C consisted exclusively of strains

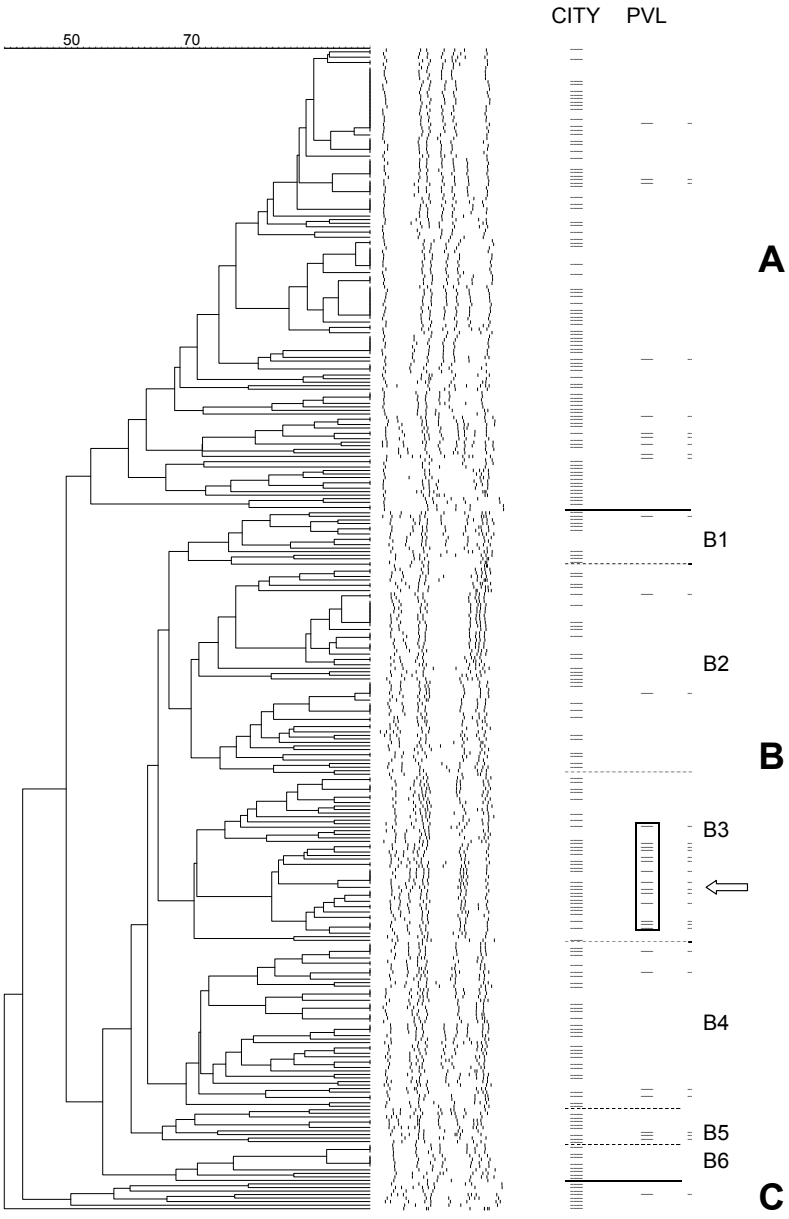

Figure 1. Dendrogram based on PFGE *Sma*I restriction pattern analysis of 329 nares-colonizing *S. aureus* isolates. Similarity analysis was performed with the Dice coefficient and clustering by the UPGMA method. The scale on the top shows percentages of similarity. Further information about the strains is shown on the right side of the figure in two columns. In the first column (CITY), isolates from Surabaya are indicated by a dash, whereas isolates from Semarang are left blank. In the second column (PVL), only PVL-positive strains are marked by a dash. The single MRSA isolate is indicated by an arrow. Clusters are designated A to C, and subclusters within cluster B are designated B1 to B6. The rectangle highlights the 14 PVL-positive strains in subcluster B3.

Table 2. Molecular characteristics of 329 Indonesian *S. aureus* isolates.

Characteristic	Total no. of isolates (%)	No. of isolates per city (%)		*P* value
		Semarang n=125	Surabaya n=204	
PFGE[a]				
cluster A	131 (39.8)	46 (36.8)	85 (41.7)	0.448
cluster B	190 (57.8)	79 (63.2)	111 (54.4)	0.147
cluster C	8 (2.4)	0	8 (3.9)	0.026
mecA				
positive	1 (0.3)	0	1 (0.5)	1.000
negative	328 (99.7)	125 (100)	203 (99.5)	
PVL[b]				
positive	35 (10.6)	9 (7.2)	26 (12.7)	0.141
negative	294 (89.4)	116 (92.8)	178 (87.3)	

[a] PFGE, pulsed-field gel electrophoresis.
[b] PVL, Panton-Valentine leukocidin.

from Surabaya (n=8, P=0.026) from patients visiting a primary health center (P<0.001). However, the observed profiles within this cluster were variable. Therefore, it would be incorrect to define this cluster as a single clone. Both clusters A and B contained isolates from all four population groups and all four hospital departments, with similar distributions (data not shown). As shown in Figure 1, cluster B could be further subdivided into six subclusters, B1 to B6, on the basis of segregated branches. Subcluster B3 was the most homogeneous, with a similarity level of >71%. Strains from Semarang were more frequently found in subcluster B2 (32/125 [25.6%]), compared with isolates from Surabaya (27/204 [13.2%], P=0.007). No other significant geographical differences in the distributions of the isolates were found.

mecA- and PVL-positive strains

Only 1/329 strains harbored the *mecA* gene (0.3%). This MRSA strain was isolated from the nose of a 41-year-old male patient upon discharge after 45 days of hospitalization in the Surgery department in Surabaya. The discharge diagnosis was a malignancy. In the PFGE analysis, the strain clustered in B3. The SCC*mec* of this isolate was identified as type V.

The *pvl* genes were detected in 35 of 329 isolates (10.6%). The proportion of PVL-positive isolates from Surabaya was 12.6%, whereas 7.2% of the isolates from Semarang carried PVL (P=0.141) (Table 2). The PVL-positive strains were evenly distributed among the four groups of patients and healthy persons (7, 9, 9, and 10 carriers in groups 1, 2, 3, and 4, respectively) and among individuals from the four departments (data not shown). In the PFGE analysis, the *pvl* genes were found in isolates from all three clusters. A cluster of PVL-positive strains could be identified within

cluster B (Figure 1). Strains from PFGE subcluster B3 were significantly enriched for the presence of PVL in comparison with the other PFGE clusters (14/47 [29.8%] cluster B3 isolates versus 21/282 [7.4%] non-B3 isolates, $P<0.0001$). Twelve of these 14 PVL-positive PFGE subcluster B3 strains were isolated in Surabaya, and four of these were from patients who were discharged from the Department of Gynecology and Obstetrics. The single MRSA isolate did not harbor the *pvl* genes. In order to determine whether SCC*mec* elements were present in these 35 *mecA*-negative PVL-positive isolates, a multiplex PCR for SCC*mec* types I to V was carried out. One strain (1/35, 2.9%) was positive for SCC*mec* type I (IS*1272*). Other SCC*mec* types were not found. The SCC*mec*-positive isolate was cultured from a healthy relative accompanying a patient that was admitted to the Gynecology and Obstetrics department in Surabaya. In the PFGE analysis, the isolate clustered in B3.

AFLP

The AFLP patterns obtained for the 81 Indonesian strains, including 12 PVL-positive strains, were compared with those of *S. aureus* carriage isolates obtained from healthy individuals in The Netherlands (Figure 2 and Figure 3). Essentially, the Indonesian strains clustered within the previously defined AFLP clusters (I to IV) (Table 3) (20). However, in comparison with the representation of the Dutch isolates in AFLP cluster II (similar to CC30), the Indonesian strains were significantly underrepresented in this cluster (216/829 versus 1/81, $P<0.0001$). The single strain in cluster II was isolated from a patient on the day of admission at the department of Gynecology and Obstetrics in Semarang. Vice versa, the Indonesian strains were significantly overrepresented in cluster III (similar to CC45) (27/81 versus 176/829, $P=0.018$).

The distributions of the isolates from Semarang and Surabaya across the major AFLP clusters was similar (Table 3). Strains from all four population groups and from the three nonpediatric departments were found in major clusters I, III, and IV, without any significant differences. AFLP cluster IV did not contain strains from the pediatrics department. However, this was not statistically significant (data not shown). All major clusters contained PVL-positive strains. Isolates from minor cluster IVb were significantly enriched for the presence of PVL in comparison with the isolates from the other AFLP clusters (3/5 versus 9/76, $P=0.022$).

MLST

We identified nine different STs among 30 strains and three new profiles for which no ST has been defined yet (Table 4). Three other isolates could not be typed by MLST, since no PCR product could be obtained repeatedly for the *aroE* gene. The strains that were classified in AFLP cluster I revealed seven different known STs and the three unknown STs. In contrast, AFLP clusters III and IVb were more homogeneous. These

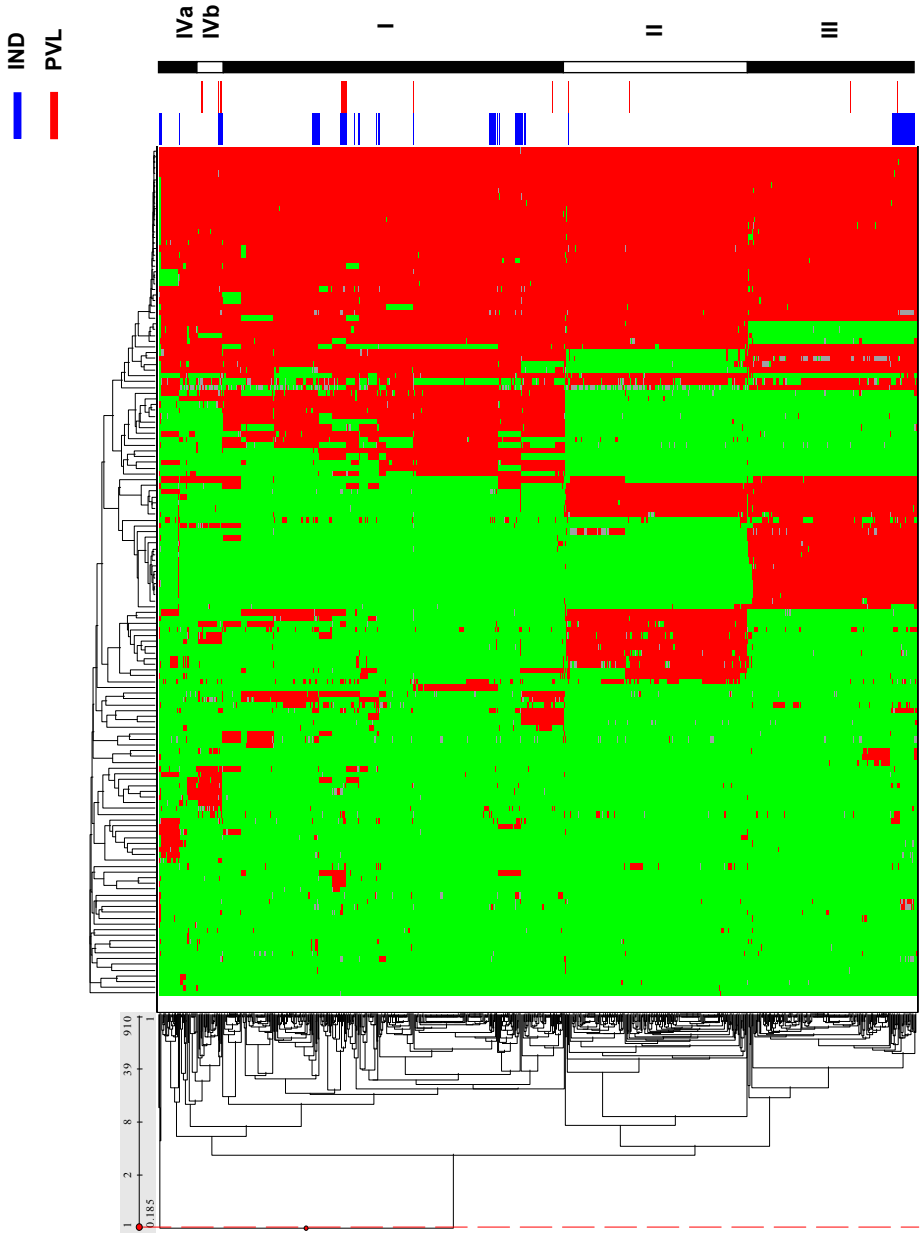

Figure 2. Two-dimensional clustering of 81 carriage strains from Indonesia and 829 carriage strains from The Netherlands (20). The dendrogram on the *y* axis represents the phylogenetic clustering of the 910 strains. The dendrogram on the *x* axis shows the clustering of the AFLP markers. AFLP marker fragments are shown in red and green; red represents marker presence and green indicates absence. The blue horizontal bars at the right side of the figure indicate the position of the Indonesian isolates; the small red bars represent PVL-positive strains from both Indonesia and The Netherlands. The black and white bar at the far right of the figure represents the three main clusters (I, II, and III) and two minor clusters (IVa and IVb), as defined before (20).

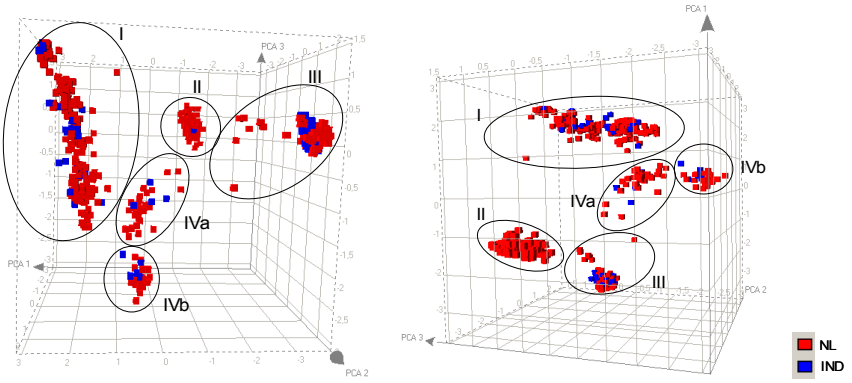

Figure 3. Principal component analysis of 81 carriage strains from Indonesia and 829 carriage strains from the Netherlands (20). Every cube represents one strain from either Indonesia (blue) or the Netherlands (red). The three main clusters (I, II, and III) and two minor clusters (IVa and IVb) are indicated by the five circles. Two different angle views are shown.

Table 3. Distribution of the Indonesian *S. aureus* collection of 81 strains and its subcollections in the five phylogenetic AFLP clusters and the distribution of the Dutch carriage strains in de five clusters for comparison.

	No. of strains (%) in AFLP[a] cluster				
Collection (no. of strains)	I	II	III	IVa	IVb
Dutch carriage strains (829)[b]	367 (44)	216 (26)[c]	176 (21)[d]	46 (6)	24 (3)
Indonesia (81)	44 (54)	1 (1)[c]	27 (33)[d]	4 (5)	5 (6)
Semarang (33)	20 (61)	1 (3)	11 (33)	1 (3)	0
Surabaya (48)	24 (50)	0	16 (33)	3 (6)	5 (10)
Admission (19)	7 (37)	1 (5)	8 (42)	1 (5)	2 (11)
Discharge (25)	17 (68)	0	5 (20)	2 (8)	1 (4)
Primary health center (18)	10 (56)	0	7 (39)	1 (6)	0
Relatives (19)	10 (53)	0	7 (37)	0	2 (11)
PVL[e] positive (12)	7 (58)	1 (8)	1 (8)	0	3 (25)[f]
PVL negative (69)	37 (54)	0	26 (38)	4 (6)	2 (3)
PFGE[g] cluster A (32)	2 (6)	1 (3)	23 (72)[h]	2 (6)	4 (13)
PFGE cluster B (47)	40 (85)[i]	0	4 (9)	2 (4)	1 (2)
PFGE cluster C (2)	2 (100)	0	0	0	0

[a] AFLP, amplified fragment length polymorphism.
[b] From reference (20).
[c] Underrepresentation of Indonesian strains in cluster II compared with the Dutch strains ($P<0.001$).
[d] Overrepresentation of Indonesian strains in cluster III compared with the Dutch strains ($P=0.018$).
[e] PVL, Panton-Valentine leukocidin.
[f] Cluster IVb was significantly enriched with PVL in comparison with the other AFLP clusters ($P=0.022$).
[g] PFGE, pulsed-field gel electrophoresis.
[h] Proportionately more PFGE cluster A strains compared with the other PFGE cluster strains ($P<0.001$).
[i] Proportionately more PFGE cluster B strains compared with the other PFGE cluster strains ($P<0.001$).

Table 4. Sequence types of 36 *S. aureus* strains assigned by MLST.

	MLST[a] sequence type (ST) (no. of strains)	
AFLP[b] cluster	Semarang	Surabaya
I	ST5 (1), ST8 (1), ST9 (1), ST15 (2), unknown ST (2)[c]	ST1 (3), ST5 (1), ST15 (1), ST20 (1), ST188 (4), unknown ST (1)[c]
II	-	-
III	ST45 (6)	ST45 (6)
IVa	ND[d] (1)	ND[d] (2)
IVb	-	ST121 (3)

[a] MLST, multilocus sequence typing.
[b] AFLP, amplified fragment length polymorphism.
[c] Three different profiles were observed.
[d] ND, not determinable, because no PCR product could be obtained for one of the housekeeping genes.

results were in agreement with those published before (20, 29). Cluster IVa harbored the three non-typeable strains. The six PVL-positive strains that were analyzed by MLST revealed three different STs: ST188/CC1 (n=3), ST121/CC121 (n=2), and ST45/CC45 (n=1). The three isolates with ST188 were all from Surabaya and clustered in subcluster B3 in the PFGE analysis. Using the AFLP database (22), the STs of six other PVL-positive isolates could be deduced: ST188/CC1 (n=3, including the SCC*mec* type I-positive strain), ST121/CC121 (n=1), ST25/CC25 (n=1), ST30/CC30 (n=1).

DISCUSSION

Nasal carriage of *S. aureus* plays a key role in the epidemiology and pathogenesis of *S. aureus* infections (12, 33). To define reservoirs of this pathogen in both the hospital and the community, it is necessary to study the molecular epidemiology of carriage isolates of patients and healthy individuals. This is the first report of such an analysis of a well-defined population-based collection of *S. aureus* isolates from Indonesia.

Interestingly, we found a low prevalence of MRSA (0.3%), but a high prevalence of PVL-positive MSSA (10.6%) in this collection of Indonesian carriage strains. Molecular typing by PFGE showed extensive genetic diversity among both PVL-positive and PVL-negative strains. In Surabaya, however, we identified a cluster that was strongly associated with the presence of the PVL locus. The high prevalence of PVL-positive commensal *S. aureus* is in contrast with the low prevalences in carriage isolates previously reported: 0.6% by Melles et al. (The Netherlands), 0% by Prevost et al. (France), and 1.4% by Von Eiff et al. (Germany) (21, 25, 32). In populations with high MRSA carriage rates, more PVL-positive isolates can be found. Kuehnert et al. reported the presence of PVL in 1.0% of 297 American MSSA nasal isolates but in 8.0% of 75 MRSA strains (16). In a recent study from Taiwan, 18 (6.0%) of 300 colonizing isolates from children

carried the *pvl* genes, 15 of which were MRSA (19). Evidence was provided that linked these PVL-positive carriage strains from the community to CA-MRSA-infecting strains. The PVL locus seems to represent a genetic marker of CA-MRSA strains worldwide (30). In Indonesia, however, PVL is apparently not associated with MRSA. Although small numbers of PVL-positive MSSA strains have been reported from other countries, this is, to the best of our knowledge, a rare phenomenon (1, 16, 21). In only one study, from the Cape Verde islands, was a similar finding reported (3). The *pvl* genes were detected in 34.9% of 63 nosocomial MSSA strains isolated from nasal and wound swabs from patients and health care workers. Eighteen of the 22 PVL-positive strains were from nasal samples, but since it is unknown whether these carriers suffered from skin infections, a possible association with active disease cannot be ruled out. In our study, none of the patients from groups 1 and 2 that carried a PVL-positive isolate had a diagnosis of a skin infection at the moment of inclusion in the study.

It has been suggested by Vandenesch et al. that first, intercontinental exchange of MSSA or MRSA had occurred, which was then possibly followed by the introduction of a *mecA* gene harboring SCC*mec* in MSSA and the *pvl* genes in MSSA or MRSA (30). Since the two loci (PVL and SCC*mec*) are widely separated on the *S. aureus* chromosome, co-acquisition on a single mobile genetic element is unlikely (4). In the present study, we found one *mecA* gene-positive strain that was PVL negative and one *mecA* gene-negative SCC*mec* type 1-positive strain that was PVL positive. Therefore, we have shown that the SCC*mec* has only rarely been introduced in the Indonesian carriage *S. aureus*, but the *pvl* genes, on the other hand, have been integrated in distinct phylogenetic subpopulations, as demonstrated by PFGE and AFLP. Overall, in contrast with the Dutch situation, the Indonesian strains were virtually absent from AFLP cluster II (similar to CC30), but PVL-positive strains occurred throughout all major AFLP clusters (I to IV). AFLP cluster IVb (similar to ST121) was significantly enriched with PVL compared with the other clusters. MLST of a subset of isolates showed that PVL-positive strains mainly belonged to ST188/CC1 and ST121/CC121, while most PVL-negative isolates belonged to ST45/CC45. PVL-positive MSSA of ST188 has not been described before, but ST121 has been detected among MSSA isolates carrying PVL in other countries, such as Portugal, Cape Verde, The Netherlands, and Germany (1, 3, 21, 23). CC239, to which previous MRSA strains from Jakarta reportedly belong, was not found, indicating that geographical differences may exist within Indonesia (7, 14). Three PVL-negative strains from AFLP cluster IVa were not typeable by MLST because no PCR product could be obtained for the *aroE* gene, which encodes shikimate dehydrogenase. We assume that this is caused by a primer binding site mutation, but this needs further research. The PVL-positive MSSA strains seem to be successful commensals in Indonesia. Whether they are also successful pathogens needs to be corroborated by analyzing clinical isolates. Although

the *mecA* gene may be transmitted to any MSSA strain, it is of concern that the MRSA isolate clustered in PFGE subcluster B3 together with the successful PVL-positive strains, because this may be an early warning for CA-MRSA emergence in Indonesia. Since this single MRSA strain in our study was isolated from a patient at the moment of discharge after a hospital stay of more than five days and the strain did not contain the *pvl* genes, we assume that the strain was nosocomially acquired. The SCC*mec* type of the isolate was identified as type V. The strain was resistant to erythromycin and chloramphenicol, but sensitive to trimethoprim-sulfamethoxazole, tetracycline, and gentamicin (data not shown).

The low prevalence of MRSA in this collection of Indonesian carriage strains is comparable to the prevalence in the Dutch population (0.1% of 2,332 carriage strains isolated from 9,859 individuals) (34). The low prevalence in The Netherlands is ascribed to restrictive antibiotic use and a national search-and-destroy policy when dealing with MRSA. Since there is no search-and-destroy policy in Indonesia, the low MRSA prevalence in Java may be due to limited antibiotic consumption. On the other hand, a scenario of a low carriage rate of MRSA in the community and a high prevalence of invasive MRSA in the hospital, such as in Portugal, remains a possibility (1). This hypothesis, however, needs to be explored further.

In summary, our data provide a unique insight into the molecular characteristics and population structure of *S. aureus* carried by healthy individuals and patients from Java, Indonesia. There is good news with respect to the low prevalence of MRSA. The picture is less bright with respect to the presence of PVL. Nasal isolates that harbor the *pvl* genes may serve as an endogenous reservoir for infections or may be spread to other individuals. Further research is needed and continued surveillance is warranted, as the epidemiology of *S. aureus* is constantly changing.

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Chapter 8

Nasal carriage of methicillin-resistant and methicillin-sensitive strains of *Staphylococcus sciuri* in the Indonesian population: epidemiology and risk factors

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ABSTRACT

Fifty-five strains of *Staphylococcus sciuri* were unexpectedly cultured from the nares of healthy persons and patients from Java, Indonesia, during a population-based survey on nasal *Staphylococcus aureus* carriage. Fifty-one *S. sciuri* isolates were further characterized and risk factors for carriage were determined using logistic regression analysis. Pulsed-field gel electrophoresis (PFGE) revealed a high degree of genomic diversity. The *S. aureus mecA* gene was detected by PCR in 22 isolates (43.1%), whereas the *S. sciuri mecA* was found in 33 isolates (64.7%). The staphylococcal chromosomal cassette *mec* (SCC*mec*) regions of *S. aureus mecA*-positive isolates were not typeable by the current classification scheme as defined for *S. aureus*, but most of the strains contained elements of the classical SCC*mec* types II and/or III. Multivariate analysis showed that patients at the time of discharge from the hospital were at highest risk of *S. sciuri* carriage (OR 12.49, 95% CI 4.30-36.35). Although antibiotic consumption was not a risk factor for overall *S. sciuri* carriage, it was associated with carriage of an *S. sciuri* with the *S. aureus mecA* gene (P=0.002). *S. aureus mecA*-positive *S. sciuri* strains were more resistant to non-β-lactam antibiotics compared to *S. aureus mecA*-negative *S. sciuri* strains. In Indonesia, hospitalized patients with recent antibiotic use are thus at risk of becoming colonized with a multiresistant *S. sciuri*. The clinical implications are currently unknown and need to be investigated further.

INTRODUCTION

The genus *Staphylococcus* comprises more than 40 species and subspecies, about one-half of which are indigeneous to humans (3). *Staphylococcus sciuri* has traditionally been described as a commensal species of a variety of domestic and wild animals. In goats and cows, this oxidase-positive and novobiocin-resistant *Staphylococcus* species has been reported as the causative agent of mastitis, and in other animals, it has been associated with wound and skin infections (1, 8, 32, 34). In humans, it has been described as a cause of endocarditis, peritonitis, endophthalmitis, and (nosocomial) wound infections (5, 17, 35, 36, 40). Colonization of humans has been reported less frequently. *S. sciuri* has been isolated from the anterior nares, axillae, nasopharynges, and urogenital tract of healthy persons and hospitalized patients (2, 12, 39, 44). Although infection and colonization of humans with *S. sciuri* seems to be rare, the bacterium has recently gained interest after it was discovered that *S. sciuri* strains ubiquitously carry a genetic element (*S. sciuri mecA*) that is closely related to the *mecA* gene found in methicillin-resistant *S. aureus* strains (MRSA) (11, 43). This finding led to the proposal that the *S. sciuri mecA* might be the evolutionary origin of the *mecA* element carried by MRSA. The biochemical properties of both *mecA* encoded proteins were recently found to be similar, which further supports its putative evolution (15). In *S. sciuri*, however, the *mecA* gene exists as a silent gene of unknown function, since it does not confer resistance to methicillin. Some *S. sciuri* strains also carry a second copy of the gene, identical to *S. aureus mecA*. Only isolates with both *mecA* genes are phenotypically methicillin-resistant (12).

The 'Antimicrobial Resistance in Indonesia: Prevalence and Prevention' (AMRIN) study group investigated antimicrobial resistance rates among potential pathogens in the commensal microflora from patients and healthy persons on the island of Java, Indonesia (24). Nasal *Staphylococcus aureus* was among the studied microorganisms, since carriage of this bacterium in the nose is a risk factor for subsequent infection (41). The survey was carried out by culturing nasal swabs on phenol red mannitol agar (PHMA) on which *S. aureus* produces yellow colonies due to its ability to ferment mannitol. Interestingly, we found 55 mannitol-fermenting bacteria (MFB) that, on further identification, appeared to be *S. sciuri*. In the present work, we defined a number of phenotypic and molecular characteristics of these isolates. Furthermore, we investigated whether demographic and socioeconomic variables could be identified as risk factors for nasal carriage of *S. sciuri*.

MATERIALS AND METHODS

Bacterial isolates

The AMRIN study was carried out in two governmental teaching hospitals (Dr. Soetomo Hospital in Surabaya, Dr. Kariadi Hospital in Semarang) and three primary health centers (two in Surabaya and one in Semarang). A total of 3,995 individuals were screened for nasal *S. aureus* carriage. These individuals were patients sampled within 24 hours after hospital admission (admission group, n=998), patients sampled at discharge after a minimum of five days of hospitalization (discharge group, n=999), and patients sampled when visiting a primary health center (PHC group, n=1,000). In addition, healthy relatives or household members of admission group patients were sampled (relatives group, n=998). Included were patients from the Internal Medicine, Surgery, Gynecology and Obstetrics, and Pediatrics departments (admission and discharge group). The specimens were collected from July to October 2001 in Surabaya and from January to May 2002 in Semarang. Demographic and socioeconomic variables were recorded for each individual as described elsewhere (14).

Nasal swabs were inoculated on PHMA (Becton Dickinson, Heidelberg, Germany). Suspect *S. aureus* colonies were stored in trypticase soy agar (TSA). This collection, comprising more than 1,200 putative *S. aureus* strains, was subsequently speciated with an agglutination test (Slidex Staph Plus, bioMérieux, Marcy l' Etoile, France), and the Vitek® 2 system (bioMérieux). During the first phase of the study both Slidex Staph Plus-negative and -positive isolates were speciated using the Vitek® 2 system. Later this was only performed for Slidex Staph Plus-positive isolates.

Phenotypic and genetic confirmation of the species

Additional phenotypic tests for the identification of *S. sciuri* included an oxidase test (BBL™ DrySlide™ Oxidase, Becton Dickinson) and novobiocin susceptibility, which was determined on Mueller-Hinton agar with a disk containing 5 µg of novobiocin (Oxoid, Basingstoke, UK). Zone diameters of ≤16 mm were regarded as resistant (37). For confirmation purposes, sequence analysis of the 16S ribosomal RNA gene was carried out for 13 randomly chosen isolates. Chromosomal DNA was extracted with the MagNA Pure LC DNA Isolation Kit III (Bacteria, Fungi) using the MagNA Pure LC Instrument (Roche Diagnostics, Almere, The Netherlands) as described elsewhere (28). The DNA concentration was assessed spectrophotometrically. PCR amplification was performed using the primers EUB-L (5'-CTTTACGCCCA(AG)T(AG)A(AT)TCCG-3') and EUB-R (5'-AGAGTTTGATC(AC)TGG(CT)TCAG-3'). Amplification conditions were: initial denaturation at 94°C for 14 min; 40 cycles of 94°C for 1 min, 55°C for 1 min and 72°C for 1 min. The PCR fragments thus generated were sequenced by Service XS (Leiden, The Netherlands). Analysis and comparisons of nucleotide

sequence data were carried out using MegAlign software (DNAStar Inc., Madison, USA) and programs available at the National Centre for Biotechnology Information website (http://www.ncbi.nlm.nih.gov).

Antimicrobial susceptibility

The Clinical and Laboratory Standards Institute (CLSI) recommends disk diffusion using cefoxitin for screening for oxacillin or methicillin resistance in staphylococci (10). Cefoxitin susceptibility was performed by disk diffusion according to the criteria set by the CLSI (10). Resistance to cefoxitin (30 µg disk) was defined as a zone diameter of ≤24 mm. Antimicrobial susceptibility of the following antibiotics was determined using the Vitek® 2 system (card AST-P549): vancomycin, gentamicin, norfloxacin, erythromycin, trimethoprim-sulfamethoxazole, tetracycline, fusidic acid, and rifampicin. Results were interpreted according to the CLSI criteria (10).

Pulsed-field gel electrophoresis (PFGE)

Molecular typing of the isolates was performed by PFGE as described previously for *S. aureus* (22). Relatedness among the PFGE profiles was evaluated with Bionumerics software (version 3.0; Applied Maths, Ghent, Belgium). A dendrogram was produced using the Dice coefficient and an unweighted pair group method using arithmetic averages (UPGMA). Band tolerance was set at 2.0%.

Detection of the *S. aureus mecA*, *S. sciuri mecA*, and Panton-Valentine leukocidin (*pvl*) genes

The presence of the *S. aureus mecA* and *pvl* genes was determined by PCR as described before (26, 30). Detection of the *S. sciuri mecA* gene was performed by PCR as described by Couto et al. (12). A PCR of the 16S ribosomal RNA gene was carried out for all *S. sciuri mecA* negative isolates as an internal amplification control. Positive and negative control strains were included in each PCR run.

Analysis of the "staphylococcal chromosomal cassette *mec* (SCC*mec*) regions"

The presence of areas homologous to regions of the *S. aureus* SCC*mec* types I to VI was examined in a randomly chosen subset of *S. aureus mecA*-positive and *S. aureus mecA*-negative *S. sciuri* isolates. The *S. aureus mecA*-positive isolates were analyzed using the primer sets for detection of loci A to H as described by Oliveira and de Lencastre and for detection of the cassette chromosome recombinases (*ccr*) genes (18-20, 25, 31). The *S. aureus mecA*-negative *S. sciuri* isolates were subjected exclusively to the PCR method of Oliveira and de Lencastre (31). Instead of the usual multiplex assays, they were performed in single reactions. Positive and negative control strains were included in each PCR run.

Statistical analysis

A statistical analysis was performed to investigate whether demographic and socio-economic variables and antibiotic use could be identified as determinants for the carriage of *S. sciuri*. Data were collected as described elsewhere (14). Cases were nasal carriers of *S. sciuri*. Control subjects were all individuals included in the AMRIN study whose nasal culture did not reveal growth of any MFB and individuals whose culture revealed *S. aureus* or a coagulase-negative staphylococcus that was identified as a non-*S. sciuri*. Differences in potential determinants of *S. sciuri* carriage were tested univariately between cases and controls using Pearsons' χ^2 test or, if necessary, Fisher's exact test or Student's *t* test (for continuous variables). Variables tested were sex, age (≤16 yrs, 17-50 yrs, or ≥51 yrs), study group (admission, discharge, PHC, or relatives group), ethnicity, provenance (urban versus rural), health insurance (yes or no), income level (below or above poverty line) (7), employment (job with animal contact or no job/other job), and persons per household (1-8 versus ≥8). For patients of the discharge group separately, the department, duration of hospitalization (in days), and antibiotic consumption during hospitalization (yes or no) were tested. For the other groups, antibiotic consumption during the preceding month as reported by the participant was tested (yes, no, or unknown). All variables with a $P<0.2$ were included in a multivariate logistic regression model. Backward selection based on the likelihood-ratio test was used to identify significant variables. $P<0.05$ was considered to be statistically significant. SPSS version 15.0 for Windows (SPSS Inc., Chicago, IL, USA) was used for the analysis.

RESULTS

Identification of *S. sciuri* strains

Within the scope of the AMRIN project, MFB were found in the cultures of 546 out of 1,995 individuals from Surabaya and 712 out of 2,000 participants from Semarang. Due to storage problems, 255 isolates from Surabaya and 80 isolates from Semarang could not be re-cultured for further testing. Of the remaining isolates, 362 isolates were identified as *S. aureus* and 55 as *S. sciuri* by the Vitek® 2 system. Four *S. sciuri* isolates were lost during storage. Thus, 51 isolates were available for phenotypic and genotypic analyses. The majority of these *S. sciuri* isolates was from Semarang (n=50).

The 51 isolates identified as *S. sciuri* by the Vitek® 2 system were oxidase positive and resistant to novobiocin. Twenty-five strains (49.0%) gave a positive Slidex Staph Plus agglutination test. As a check for the phenotypic identification, the 16S ribosomal RNA gene from 13 randomly chosen isolates was sequenced and all appeared specific for *S. sciuri*. Eleven isolates belonged to subspecies *sciuri* and two to subspecies

rodentium. Additional information on the Slidex Staph Plus agglutination-negative and -positive *S. sciuri* isolates is shown in Table S1.

PFGE

Molecular typing revealed a high degree of genomic diversity among the *S. sciuri* isolates (Figure 1). Two pairs of strains showed indiscriminate genotypes without an obvious epidemiological link in time or location.

Antimicrobial susceptibility and detection of *mecA* genes

The results of the PCR for *S. aureus* and *S. sciuri mecA* genes, cefoxitin disk diffusion susceptibility results, and antimicrobial susceptibility results from Vitek® 2 system are shown in Table 1. The *S. aureus mecA* gene was detected in 22 isolates (43.1%), whereas the *S. sciuri mecA* gene was found in 33 isolates (64.7%). The presence of both the *S. aureus mecA* and the *S. sciuri mecA* was demonstrated in 13 strains (25.5%), the *S. aureus mecA*, but not the *S. sciuri mecA* was found in 9 strains (17.6%), the *S. sciuri mecA* gene, but not the *S. aureus mecA* was detected in 20 strains (39.2%), and 9 strains were negative for both *mecA* genes (17.6%). The 18 *S. sciuri mecA*-negative isolates revealed a clear product in the PCR of the 16S ribosomal RNA gene, thereby ruling out a false-negative *S. sciuri mecA* PCR.

Phenotypic resistance to cefoxitin was observed for 19 *S. aureus mecA*-positive strains (Table 1). Three additional isolates were *S. aureus mecA* positive, but had zone diameters of ≥25 mm for cefoxitin. The 29 *S. aureus mecA*-negative strains were susceptible to cefoxitin.

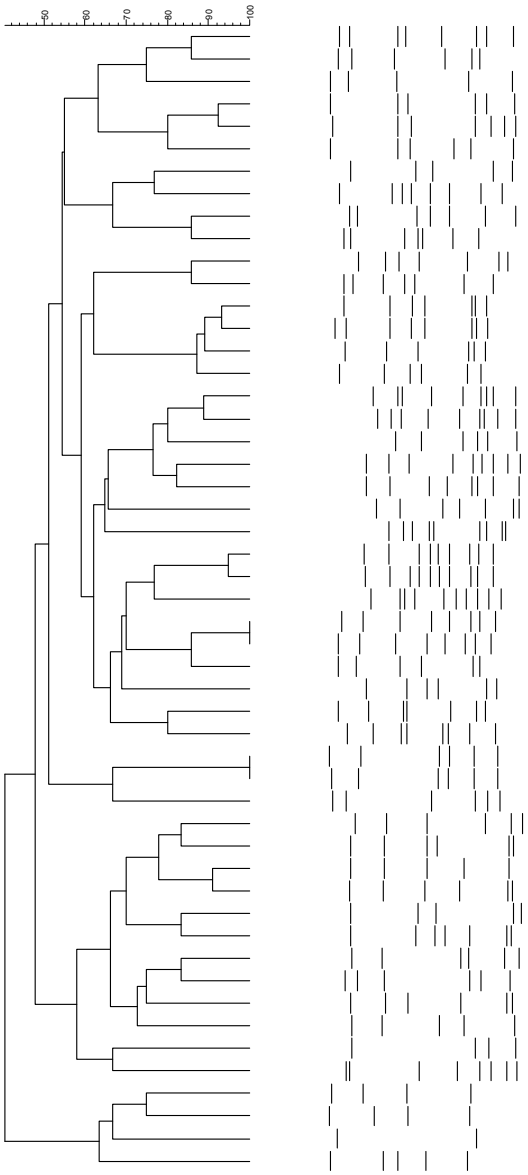
The *S. aureus mecA*-positive strains were not evenly distributed across the PFGE dendrogram but were found to cluster in the lower part of the dendrogram (rectangle,

Table 1. Correlation between presence of *S. aureus mecA* and *S. sciuri mecA* genes, cefoxitin disk diffusion susceptibility, and antimicrobial susceptibility of 51 isolates of *S. sciuri*.

PCR result for		No. of isolates with cefoxitin zone diameter		% resistant to[a]:							
S. aureus mecA	*S. sciuri mecA*	≤24 mm	≥25 mm	ERY	FUS	GEN	NOR	RIF	SXT	TET	VAN
negative	negative	0	9[b]	0	55.6	0	0	0	0	11.1	0
negative	positive	0	20	0	60.0	0	0	0	0	5.0	0
positive	negative	7	2[b]	33.3	44.4	55.5	66.7	11.1	22.2	77.8	0
positive	positive	12	1	23.1	69.2	38.5	15.4	15.4	7.7	46.2	0
Total		19	32	11.8	58.8	19.6	15.7	5.9	5.9	29.4	0

[a] Rates of resistance include resistant as well as intermediate susceptible isolates as tested by Vitek® 2. Abbreviations: ERY, erythromycin; FUS, fusidic acid; GEN, gentamicin; NOR, norfloxacin; RIF, rifampicin; SXT, trimethoprim-sulfamethoxazole; TET, tetracycline; VAN, vancomycin.

[b] These included two *S. sciuri* subsp. *sciuri*.



SG	Dpt	SA	Slidex	City	SS	16S
1	3	neg	pos	Sem	pos	subsp. sciuri
3		neg	pos	Sem	neg	-
1	1	neg	pos	Sem	neg	subsp. sciuri
4		neg	pos	sem	pos	-
2	1	pos	pos	Sem	pos	-
2	3	neg	pos	Sem	pos	-
2	3	pos	neg	Sem	pos	-
3		neg	neg	Sem	pos	subsp. sciuri
2	1	neg	pos	Sem	neg	-
4		neg	neg	Sem	neg	-
3		neg	pos	Sem	pos	-
4		pos	pos	Sem	neg	-
1	1	neg	neg	Sem	pos	-
2	2	neg	pos	Sem	pos	-
2	2	neg	neg	Sem	pos	subsp. sciuri
2	2	neg	pos	Sby	pos	-
2	3	neg	neg	Sem	pos	-
3		neg	neg	Sem	pos	-
1	4	pos	neg	Sem	pos	-
1	1	neg	pos	Sem	neg	subsp. sciuri
3		neg	pos	Sem	pos	-
1	2	neg	neg	Sem	pos	-
3		neg	neg	Sem	pos	-
2	3	pos	neg	Sem	neg	-
2	3	pos	neg	Sem	neg	subsp. sciuri
2	1	neg	neg	Sem	neg	-
2	2	pos	neg	Sem	neg	-
2	3	pos	neg	Sem	neg	subsp. sciuri
2	4	neg	neg	Sem	neg	-
1	2	neg	neg	Sem	pos	subsp. sciuri
3		neg	neg	Sem	pos	-
2	1	pos	neg	Sem	pos	-
1	2	pos	neg	Sem	pos	subsp. rodentium
2	4	pos	pos	Sem	pos	-
3		neg	neg	Sem	neg	-
2	3	neg	pos	Sem	pos	-
2	2	pos	pos	Sem	pos	-
1	3	pos	pos	Sem	pos	subsp. sciuri
2	2	pos	pos	Sem	neg	-
2	2	pos	pos	Sem	pos	-
1	3	pos	pos	Sem	pos	-
2	2	neg	pos	Sem	neg	-
2	3	pos	pos	Sem	pos	subsp. sciuri
2	2	pos	pos	Sem	neg	-
2	3	neg	neg	Sem	pos	-
2	3	pos	neg	Sem	pos	-
1	2	neg	neg	Sem	neg	subsp. rodentium
2	1	pos	neg	Sem	neg	-
2	1	pos	pos	Sem	neg	-
1	1	neg	neg	Sem	pos	subsp. sciuri
2	3	pos	pos	Sem	pos	-

Figure 1. Dendrogram based on PFGE *Sma*I restriction pattern analysis of 51 nares-colonizing *S. sciuri* isolates. Similarity analysis was performed with Dice's coefficient and clustering by the UPGMA method. The scale on the top shows percentages of similarity. Further information is shown on the right side of the figure: study group (SG; 1, admission group; 2, discharge group; 3, PHC group; 4, relatives group), department (dpt; 1, Internal Medicine; 2, Surgery; 3, Gynecology and Obstetrics; 4, Pediatrics), presence (pos) or absence (neg) of the *S. aureus mecA* gene (SA), positive (pos) or negative (neg) Slidex Staph Plus agglutination test (Slidex), city (Sem, Semarang; Sby, Surabaya), presence (pos) or absence (neg) of the *S. sciuri mecA* gene (SS), and the result of the 16S ribosomal RNA gene sequencing (16S; –, not performed). The rectangle highlights a clustering of *S. aureus mecA*-positive *S. sciuri* strains.

Figure 1). However, this may be a coincidental observation, since the genetic element as such was found in many different clones of *S. sciuri*.

There was no significant difference in the presence of the *mecA* genes when comparing the Slidex Staph Plus-negative and -positive isolates (Table S1).

Antimicrobial susceptibility to other antibiotics is shown in Table 1. Overall, resistance rates of *S. aureus mecA*-positive strains were higher than those of *S. aureus mecA*-negative strains. Resistance to fusidic acid was prevalent in both *S. aureus mecA*-negative and -positive strains. All strains were susceptible to vancomycin.

Analysis of the "SCC*mec* region"

Eighteen *S. aureus mecA*-positive isolates, including 8 *S. sciuri mecA*-negative isolates, were analyzed in more detail (Table 2). Using PCRs for several parts of the known SCC*mec* types, none of the strains was typeable by the current classification system. Therefore, PCR profiles, designated a to g, are shown in Table 2. Profile a was the most prevalent (n=6) with loci C (*mecI* gene), D (*dcs* gene), and G (left junction between IS*431* and pUB110), and *ccr3*. The presence of loci C, D, and G is consistent with SCC*mec* type II, but *ccr3* is part of SCC*mec* type III. *ccrC* and locus E were absent in these strains, suggesting the absence of the SCC*mercury* element (9). Locus C was found in 12 strains, locus D in 8, locus G in 6, and *ccr3* in 10 strains. PCRs for other regions were negative.

Ten *S. aureus mecA*-negative isolates, including 3 *S. sciuri mecA*-negative isolates, were also analyzed. None of the targets of the PCR method of Oliveira and de Lencastre could be demonstrated, indicating that these regions were not part of the *S. sciuri* genome (31).

Table 2. SCC*mec* typing of 18 *S. aureus mecA*-positive *S. sciuri* strains

Profile	No. of isolates	No. of isolates with fox[a] zone ≤24 mm	Locus from Oliveira et al.[b]								ccr locus					No. of isolates with positive *S. sciuri mecA* PCR
			A	B	C	D	E	F	G	H	*ccr1*	*ccr2*	*ccr3*	*ccr4*	*ccrC*	
a	6	3	–	–	+	+	–	–	+	–	–	–	+	–	–	3
b	4	4	–	–	+	–	–	–	–	–	–	–	–	–	–	2
c	3	3	–	–	–	–	–	–	–	–	–	–	–	–	–	1
d	2	2	–	–	+	–	–	–	–	–	–	–	+	–	–	2
e	1	1	–	–	–	+	–	–	–	–	–	–	–	–	–	1
f	1	1	–	–	–	+	–	–	–	–	–	–	+	–	–	0
g	1	1	–	–	–	–	–	–	–	–	–	–	+	–	–	1

[a] fox, cefoxitin.

[b] Loci described by Oliveira et al. (31). A, downstream of *pls* gene; B, *kdp* operon; C, *mecI* gene; D, *dcs* gene; E, region between pI258 and Tn554; F, region between Tn554 and *orfX*; G, left junction between IS431 and pUB110; H, left junction between IS431 and pT181. +, locus present; –, locus not present.

Detection of the *pvl* genes

None of the isolates carried the *pvl* genes.

Determinants of *S. sciuri* carriage

As all but one of the *S. sciuri* isolates were from Semarang, we performed univariate and multivariate logistic regression analyses with 54 carriers of *S. sciuri* and 1,481 non-*S. sciuri* carriers, all from Semarang (Table 3). The four persons whose *S. sciuri* was lost during storage were included. Multivariate analysis showed that patients cultured at the day of discharge after hospitalization for at least five days were at highest risk of *S. sciuri* carriage (odds ratio [OR] 12.49, 95% confidence interval [CI] 4.30-36.35). Admission to hospital was also independently associated with carriage of *S. sciuri* (OR 3.94, 95% 1.25-12.45). Children were less likely to carry *S. sciuri*, as demonstrated in the overall analysis (OR 0.39, 95% CI 0.18-0.83) and in the analysis that included the discharge group only (OR 0.21, 95% CI 0.06-0.73). The use of antibiotics during the preceding month or, in case of discharge group patients, during hospitalization, did not influence the carrier state. The use of antibiotics, however, was associated with the carriage of an *S. aureus mecA*-positive *S. sciuri* (Fisher's exact, *P*=0.002). Among

Table 3. Risk factors for nasal carriage of *S. sciuri*.

Variable	Univariate analysis			Multivariate analysis	
	Frequency (%)		*P* value	OR (95% CI)	*P* value
	Carriage of *S. sciuri* (n=54)	No carriage of *S. sciuri* (n=1,481)			
Sex			0.457		
Female	35 (65)	885 (60)			
Male	19 (35)	596 (40)			
Age			0.070		0.020
≤16 yrs	9 (17)	402 (27)		0.39 (0.18 – 0.83)	
17 – 50 yrs	33 (61)	888 (60)		1	
>50 yrs	12 (22)	191 (13)		1.31 (0.65 – 2.63)	
Study group			< 0.001		< 0.001
Admission	12 (22)	383 (26)		3.94 (1.25 – 12.45)	
Discharge	30 (56)	316 (21)		12.49 (4.30 – 36.35)	
PHC[a]	8 (15)	368 (25)		3.00 (0.89 – 10.14)	
Relatives	4 (7)	414 (28)		1	
Ethnicity			1.000		
Madurese		4 (0)			
Javanese	54 (100)	1,474 (100)			
Chinese		1 (0)			
Other		2 (0)			

Provenance			0.934		
Urban	27 (50)	732 (49)			
Rural	27 (50)	749 (51)			
Health insurance			0.945		
Yes	18 (33)	487 (33)			
No	36 (67)	994 (67)			
Income level			0.178		0.443
Below poverty line	25 (46)	551 (37)			
Above poverty line	29 (54)	930 (63)			
Employment			0.319		
Animal contact	2 (4)	109 (7)			
No job / other job	52 (96)	1,372 (93)			
Persons per household			0.285		
1 – 8	49 (91)	1,396 (94)			
>8	5 (9)	85 (6)			

Variable (admission, PHC, and relatives group only)	Univariate analysis			Multivariate analysis	
	Frequency		*P* value	OR (95% CI)	*P* value
	Carriage of *S. sciuri* (n=24)	No carriage of *S. sciuri* (n=1,165)			
Self-reported antibiotic use			0.276		
Yes	6 (25)	201 (17)			
No	14 (58)	851 (73)			
Unknown	4 (17)	113 (10)			

Variable (discharge group only)	Univariate analysis			Multivariate analysis	
	Frequency or mean ± SD		*P* value	OR (95% CI)	*P* value
	Carriage of *S. sciuri* (n=30)	No carriage of *S. sciuri* (n=316)			
Department			0.041		0.344
Internal medicine	8 (27)	76 (24)			
Surgery	8 (27)	73 (23)			
Gyn/Obst[a]	12 (40)	66 (21)			
Pediatrics	2 (7)	101 (32)			
Age			0.005		0.005
≤16 yrs	3 (10)	124 (39)		0.21 (0.06 – 0.73)	
17 – 50 yrs	17 (57)	147 (47)		1	
>50 yrs	10 (33)	45 (14)		1.92 (0.82 – 4.49)	
Length of hospital stay	10.2 ± 6.4	11.3 ± 8.4	0.498		
Antibiotic use during hospitalization			0.307		
Yes	27 (90)	261 (83)			
No	3 (10)	55 (17)			

[a] PHC, Primary Health Center; Gyn/Obst, Gynecology/Obstetrics

the 22 carriers of an *S. aureus mecA*-positive strain, 19 had used antibiotics and 2 had not (1 person did not know). Among the 29 carriers of an *S. aureus mecA*-negative strain, 12 had used an antibiotic and 14 had not (3 persons did not know). This association was independent of the study group (data not shown).

Since we could not rule out nasal *S. sciuri* carriage in nasal *S. aureus* carriers, univariate and multivariate logistic regression analyses were repeated using individuals whose nasal culture did not reveal growth of any MFB as control subjects. The results described above were confirmed (data not shown).

DISCUSSION

We here show that *S. sciuri* is a colonizer of the nares of people in Indonesia and that carriage is associated with hospitalization. Although antibiotic consumption was not a risk factor for *S. sciuri* carriage, it was associated with carriage of an *S. sciuri* that harbours the *S. aureus mecA* gene. In addition, the *S. aureus mecA*-positive *S. sciuri* strains were more resistant to non-β-lactam antibiotics than *S. aureus mecA*-negative strains, indicating that *S. aureus mecA*-positive isolates have additional resistance genes linked to or integrated in their SCC*mec* or at other sites of the genome. Hospitalized patients in Semarang with recent antibiotic use are, thus, at risk of becoming colonized with a multiresistant *S. sciuri*. Colonization with (multiresistant) MRSA alike, carriage of *S. sciuri* seems to be associated with health care exposure. Interestingly, *S. sciuri* has shown to be a colonizer of the hospital environment in Serbia, but whether transmission to patients occurred is unknown (13). The polyclonal nature of *S. sciuri* found by PFGE in our study suggests repeated introduction of new clones from the community into the hospital. Possible sources are health care workers and patients, but this needs to be further investigated. Based on previous reports, we hypothesized that factors involving exposure to animals would be associated with nasal carriage of *S. sciuri* (12). Within our AMRIN database we could not study the direct exposure to animals. Instead, we investigated whether certain jobs with animal exposure, such as farmers and gardeners, would be associated with *S. sciuri* carriage, but no association was found.

The *S. sciuri* isolates were an unexpected finding during an *S. aureus* surveillance study. Using the PHMA for the detection of *S. aureus* as MFB, we found a prevalence of nasal carriage of *S. sciuri* in Java, Indonesia, of at least 55/3,995 (1.4%). Both *S. aureus* and *S. sciuri* are mannitol fermenting bacteria, producing yellow colonies on PHMA. *S. sciuri*, like *S. aureus*, may grow as yellow colonies on blood agar, and may give a positive Slidex Staph Plus agglutination and Staphaurex test (Remel), which may lead to false species identification (17, 35, 44). Differences between the

two species are, among others, novobiocin susceptibility and oxidase and coagulase production. By using the PHMA and some additional tests, we found a prevalence of nasal carriage of *S. sciuri* of at least 54/2,000 (2.7%) in Semarang. However, we did not speciate all Slidex Staph Plus agglutination-negative staphylococci found in the survey, and the actual prevalence may, thus, be higher. In Surabaya, a coagulase test was often performed before an isolate was stored as MFB, thereby increasing the number of 'true' *S. aureus* isolates. Interestingly, in a large survey of nasal *S. aureus* carriage in the USA, a similar culture strategy was used, but isolates were only identified using the Staphaurex test (23). *S. sciuri* strains may, thus, have been misclassified as *S. aureus*. In a survey among patients at admission in Germany, in which all *Staphylococcus* spp. were identified using multiple biochemical tests, *S. sciuri* was found in 0.4% of cases (4). In Indonesia, *S. sciuri* in nasal cultures was also frequently observed recently in Malang, Java and Denpasar, Bali, during a survey to estimate the prevalence of MRSA among discharged patients. In this survey, the MRSA CHROMagar (ITK Diagnostics) was used, on which *S. sciuri* produced green colonies, and MRSA pink colonies (unpublished data).

The *S. aureus mecA* gene was detected in as many as 22 of our *S. sciuri* isolates (43.1%), whereas the *S. sciuri mecA* was found in 33 isolates by PCR (64.7%). Although *S. sciuri* is described to always contain an *S. sciuri mecA* gene, we were unable to demonstrate the presence of the gene in 18 strains (35.3%) using a quality controlled well-described PCR (11, 12). This suggests that a genetic event such as deletion of the gene or mutation or deletion at the primer binding site may have occurred in these isolates. The latter possibility seems more likely, since sequence diversity in the *mecA* homologue of *S. sciuri* has been documented before (34). On the other hand, the loss of the putative native *mecA* has been reported before in a strain isolated from a rodent (42). Our findings are supported by the study of Marsou et al, in which they were also unable to detect the *S. sciuri mecA* by PCR in ten out of 30 isolates (27). The *S. aureus mecA* gene was carried by a high percentage of isolates in our collection (43.1%). Similar results were found among isolates from healthy Portuguese carriers (47.8%), and strains from a hospital environment (38.1%) (13). Among clinical human isolates and animal isolates the prevalence was lower, 28.6% and 26.5%, respectively (38). In general, *S. sciuri* strains with both *mecA* genes are oxacillin-resistant, and strains without the *S. aureus mecA* gene are oxacillin-susceptible (12). This was largely corroborated in the present study. We found 3 *S. aureus mecA*-positive strains with cefoxitin disk diffusion zones of ≥25 mm. In these strains the *S. aureus mecA* gene is probably not expressed at significant levels. Such strains have been identified before (21).

Coagulase-negative staphylococci (CoNS) are believed to constitute a reservoir of resistance genes and SCC*mec* elements for *S. aureus*. Most of the *S. sciuri* strains

were isolated from patients at discharge. The presence of such CoNS in a nosocomial setting where a selective antibiotic pressure exists can perhaps provide a source for new MRSA lineages (16). Therefore, we analyzed the SCC*mec* region of 18 *S. aureus mecA*-positive *S. sciuri* isolates, including 15 strains from discharge group patients, and ten *S. aureus mecA*-negative isolates. None of the SCC*mec* regions of the 18 *S. aureus mecA*-positive isolates were typeable by the current classification scheme, but most of the strains contained elements of the classical SCC*mec* type II (loci C, D, and G) and/or III (*ccr3*). Although we did not search for all elements of SCC*mec* in our isolates, we speculate that SCC*mec* in *S. sciuri* is composed of elements of different classical SCC*mec* types, giving rise to mosaic-like structures. This is in agreement with previous reports and has also been demonstrated for *S. epidermidis* (21, 29). Furthermore, MRSA strains with variable elements in their SCC*mec*, possibly originating from CoNS, have been described (33). None of the loci A to H could be detected in our *S. aureus mecA*-negative strains, which is in contrast to the report by Juuti et al. in which they describe the presence of loci G and H in 4 out of 7 *S. sciuri* strains (21). When comparing data of SCC*mec* regions of MRSA strains from Indonesia to those of *S. sciuri*, differences and similarities can be noted. Sixty MRSA strains from Indonesia (University of Indonesia, Jakarta) that were analyzed thoroughly by Chongtrakool et al, carried SCC*mec* type IIIA, including the *ccrC* locus (9). Although parts of the type III cassette were found in our study, *ccrC* was absent in all *S. aureus mecA*-positive strains. The two MRSA strains that were found concurrently in our survey were classified as type III and V by the method of Boye et al. (6). The type III strain was positive for the *ccrC* target and the type V strain for both the *ccrC* and the *mecA*-IS*431* target. Thus, our data do not support the hypothesis of direct genetic exchange of SCC*mec* elements between MRSA and *S. sciuri*, in this specific setting.

We have shown that *S. sciuri* is a colonizer of the nares of people in Indonesia and that carriage is associated with hospitalization. This bacterial species may be misidentified as *S. aureus* when an oxidase test is not performed in addition to a Slidex Staph Plus agglutination or Staphaurex test. Further research is needed to investigate the clinical significance of this *Staphylococcus* species in Indonesia. In the hospital setting *S. sciuri* may serve as a reservoir of the *S. aureus mecA* gene for *S. aureus*. This potential interaction with *S. aureus* should be investigated as well.

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Table S1. Origin, presence of *mecA* genes and 16S ribosomal RNA gene sequence result of Slidex Staph Plus agglutination test-negative and -positive *S. sciuri* isolates.

Characteristic	Slidex Staph Plus agglutination test		*P* value
	negative (n=26)	positive (n=25)	
Study group			NS[a]
Admission	7	5	
Discharge	13	15	
PHC	5	3	
Relatives	1	2	
S. aureus mecA			NS
negative	16	13	
positive	10	12	
S. sciuri mecA			NS
negative	9	9	
positive	17	16	
16S ribosomal RNA gene sequence			NS
S. sciuri subsp. *sciuri*	6	5	
S. sciuri subsp. *rodentium*	2		

[a] NS, not significant (χ^2 test or, if necessary, Fisher's exact)

Part IV

Focus on Enterobacteriaceae

Chapter 9

Determinants of carriage of resistant *Escherichia coli* in the Indonesian population inside and outside hospitals

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ABSTRACT

Objectives: Antibiotic resistance is a worldwide healthcare problem exacerbated by antibiotic use and transmission of resistant bacteria. Not much is known about resistance in commensal flora and about determinants for resistance in Indonesia. This study analyzed recent antibiotic use as well as demographic, socioeconomic, disease-related and healthcare-related determinants of rectal carriage of resistant *Escherichia coli* in the community and in hospitals in Indonesia.

Methods: Carriers of susceptible *E. coli* were compared with carriers of *E. coli* with resistance to any of the tested antibiotics. Logistic regression analysis was performed to determine which variables were associated with carriage of resistant *E. coli*. Individuals in the community with varying levels of contact with healthcare institutions and hospitalized patients were analyzed as separate populations.

Results and conclusion: Of 3,275 individuals (community 2,494, hospital 781), 54% carried resistant *E. coli*. Recent antibiotic use was the most important determinant of resistance in both populations (community: odds ratio (OR) 1.8, 95% confidence interval (95%CI) 1.52.3; hospital: OR 2.5, 95%CI 1.63.9). In the community, hospitalization (OR 2.4, 95%CI 2.03.0), diarrhoeal symptoms (OR 1.9, 95%CI 1.32.7) and age under 16 years (adults: OR 0.4, 95%CI 0.30.5) were associated with carriage of resistant *E. coli*. For hospitalized patients, having no health insurance was associated with less resistance (OR 0.6, 95%CI 0.40.9) and differences were observed between hospitals (Semarang: OR 2.2, 95%CI 1.53.3) and departments (Pediatrics: OR 4.3, 95%CI 1.710.7). Further research is needed to investigate whether transmission is responsible for these differences.

INTRODUCTION

Antibiotic resistance is a worldwide healthcare problem that threatens the progress in healthcare in developing countries (23, 26). Limited published data are available on antibiotic resistance in *Escherichia coli* in the Far East and these primarily concern clinical isolates (2, 6, 10, 12, 13, 15, 16, 21, 25, 27, 28, 31). Resistance data from Indonesia are mostly limited to pathogens of diarrhoeal disease (1, 7, 17, 18, 20, 25, 28, 31). The use of antibiotics is the most important determinant for emergence of resistant microorganisms (4, 8). Little is known about other determinants for carriage of resistant bacteria, such as demographic (11) and socioeconomic (14, 19) factors.

The study group 'Antimicrobial Resistance in Indonesia: Prevalence and Prevention' (AMRIN) investigated rectal carriage of resistant bacteria among inhabitants of the island of Java. Rectal swabs of individuals in the community and the hospital were cultured for the presence of *E. coli*, a commensal intestinal bacterium frequently used as an indicator of antibiotic resistance in populations (5). Antibiotic susceptibility testing of the *E. coli* isolates was conducted for six antibiotics commonly used in Indonesia: ampicillin, ciprofloxacin, cefotaxime, gentamicin, chloramphenicol, and trimethoprim-sulfamethoxazole.

The aim of the present study is to investigate whether recent antibiotic use as well as demographic, socioeconomic, healthcare-related and disease-related variables are risk factors for carriage of resistant *E. coli*. We hypothesized that recent antibiotic use would be associated with carriage of resistant *E. coli,* and that due to transmission of resistant bacteria differences would be found between nursing wards, departments, and hospitals.

MATERIALS AND METHODS

Two government hospitals, the Dr. Soetomo Hospital in Surabaya, East Java, and the Dr. Kariadi Hospital in Semarang, Central Java, Indonesia, as well as three primary health centers (PHC, two in Surabaya and one in Semarang) were selected for this study. The hospital in Surabaya has approximately 60,000 and that in Semarang 26,000 admissions per year. The Medical Ethics Committees of the hospitals approved of the study protocol (ethical clearance No/Panke.KKE/2001 (Surabaya) and 11/EC/FK/RSDK/2001 (Semarang)). Patients upon admission to hospital (group A), healthy family members accompanying them (group B), people visiting a PHC for consultation or vaccination (group C), and patients upon discharge after hospitalization for five days or more (group D) were enrolled after giving informed consent. The aim

was to include 4,000 individuals; 500 individuals per group per city, whereby each department was equally represented.

For the purpose of analysis, individuals who had not been hospitalized (groups A, B, and C) were combined into a community population, whereas patients upon discharge from hospital (group D) formed the hospital population.

Group A patients were included within the first 24 hours of admission. Persons in group B were included on admission of group A patients at a rate of one contact per patient. Patients in group C were included on specific study days twice weekly in Surabaya and once weekly in Semarang. Individuals were excluded from the study if they had been transferred from another hospital, if they were not accompanied by a family member (group A), or if they had been admitted to a hospital during the previous three months (groups A, B, and C).

Demographic and socioeconomic data and, for community patients, data on health complaints and consumption of antibiotics in the month preceding the study were collected by semi-structured interviews, performed by pairs of trained Indonesian and Dutch data collectors (researchers, residents, and medical students). For group A, diagnosis on admission, and for group D, data on antibiotic consumption during hospitalization and diagnosis on discharge were collected from medical records. Subjects for whom susceptibility testing and data on antibiotic consumption were available were included in the analyses.

Variables

Recent antibiotic use was defined in accordance with the nomenclature and subcategory definitions of the WHO ATC Classification code, subgroup antibacterials for systemic use (32). We analyzed any antibiotic use, i.e. whether or not a patient took any antibiotic in the preceding month or during hospitalization; use of an antibiotic from a specific ATC class, combined or not combined with an antibiotic from a different class; and single antibiotic use, i.e. use of an antibiotic from a specific ATC class not combined with an antibiotic from a different class. Combined use was defined as either simultaneous or successive use of antibiotics from different ATC classes.

Origin (Surabaya or Semarang), sex, age (newborn to sixteen years of age versus over sixteen years of age in accordance with the age limit for the Departments of Pediatrics, and children of less than two years old versus people of more than two years of age in accordance with approximate pre- and post weaning periods), ethnicity, and living area (urban or rural) were the selected demographic variables. Health insurance, income (below or above poverty line(3)), education (primary school not completed versus primary school education and higher), employment, and crowding (one through eight versus nine or more individuals sharing a household) were the chosen socioeconomic variables. Group, Department (Internal Medicine, Surgery, Ob-

stetrics and Gynecology or Pediatrics), nursing ward (sub-department), nursing class (I, II or III, with class I being the most expensive class), and length of stay in hospital (five through eight versus nine days or more) were studied as healthcare-related variables. Only the last ward of admission was recorded; transfers were not recorded. For community patients, clinical signs and symptoms in the month preceding the study (fever, diarrhoea, respiratory symptoms, other symptoms or no symptoms) were the disease-related variables and for patients upon admission and discharge whether or not an infection was diagnosed.

Selection of strains and susceptibility testing

Rectal samples were taken with sterile cotton-tipped swabs, which were transported to the laboratory in Amies transport medium (Copan, Brescia, Italy) in closed boxes at ambient temperature. They were cultured within 24 hours on CHROMagar Orientation (Becton Dickinson, Heidelberg, Germany) for the isolation of *E. coli* (9). From each culture, two colonies representing the dominantly growing bacterium were further analyzed. Pink colonies were assumed to be *E. coli* and used for susceptibility testing without additional determination. From the original 3,995 isolates, almost 400 were confirmed by Vitek 2 (bioMérieux, Marcy-l'Etoile, France) (16). Previously published validation of identification of *E. coli* by CHROMagar yielded a positive predictive value of 0.93, which is comparable to our results (9).

Susceptibility testing was performed by the Clinical and Laboratory Standards Institute (CLSI; formerly the NCCLS)-based disk diffusion method on Mueller-Hinton agar using disks containing ampicillin (10 µg), chloramphenicol (30 µg), gentamicin (10 µg), cefotaxime (30 µg), ciprofloxacin (5 µg) and trimethoprim-sulfamethoxazole (1.25/23.75 µg) (22). The performance of the susceptibility testing was monitored twice weekly with the quality control strain *E. coli* ATCC 25922. Isolates that were susceptible or intermediately susceptible according to the CLSI criteria were categorized as susceptible.

For the purpose of analysis, a maximum of one *E. coli* isolate per enrolled individual, namely the first *E. coli* isolate in the study database, was included in the analysis.

Analysis

Individuals carrying resistant strains were compared with individuals carrying bacteria susceptible to all tested antibiotics. Resistance as an outcome variable for each of the different antibiotics was explored in two different ways:

1. Resistance of *E. coli* to any of the tested antibiotics, irrespective of whether this was resistance to the specific antibiotic considered, or whether the resistance to the antibiotic of interest was part of a pattern of resistance to multiple antibiotics, was taken as the outcome (dependent) variable, and possible determinants for this variable identified.

2. Carriage of *E. coli* resistant to the specific antibiotic of interest was taken as the outcome variable, and determinants for this outcome variable identified. This approach was only pursued when at least 100 isolates with the relevant resistance pattern were available.

To identify determinants for any of these outcome variables, logistic regression analysis with backward selection of variables (statistical package SPSS, version 12.0, SPSS Inc., Chicago, Illinois, USA) was used.

In view of the large number of interrelated candidate determinants, some of which were sparse (i.e. most individuals had the same value for this variable), each of the analyses was performed using a two-step procedure. First, candidate variables were selected by performing logistic regression on four partially overlapping sets of co-variables (Table S1):

(a) any antibiotic use, combined with all demographic, socioeconomic, disease-related and healthcare-related determinants,
(b) demographic determinants,
(c) socioeconomic determinants,
(d) disease-related and healthcare-related determinants (without nursing wards).

Then, a 'final' logistic regression analysis was performed with all variables that were significantly associated with antibiotic resistance in any of these four analyses. The variables that were significantly associated with resistance in this final analysis were presumed to be independently associated (in the sense that the association was not caused by confounding) with resistance. This approach of selecting candidate variables was preferred over the usual strategy of picking variables univariately significantly associated with the outcome variable, as in our experience that strategy sometimes misses variables that are only significantly associated with the outcome variable in conjunction with other variables. Use of antibiotics from specific antibiotic classes and single use of specific antibiotic classes were analyzed as separate sets of variables. When logistic regression could not be performed because of sparse data, variables with very small dispersion were excluded from the analyses.

Possible clustering of susceptibility patterns between groups A and B was investigated by comparing whether included pairs of individuals had similar susceptibility patterns and calculating Pearson's correlation coefficient.

RESULTS

Between July and October 2001 in Surabaya and January and May 2002 in Semarang, 3,995 subjects were included. In 3,275 individuals, culture and susceptibility data on *E. coli* and antibiotic use data were complete. In 720 patients, data were not suitable

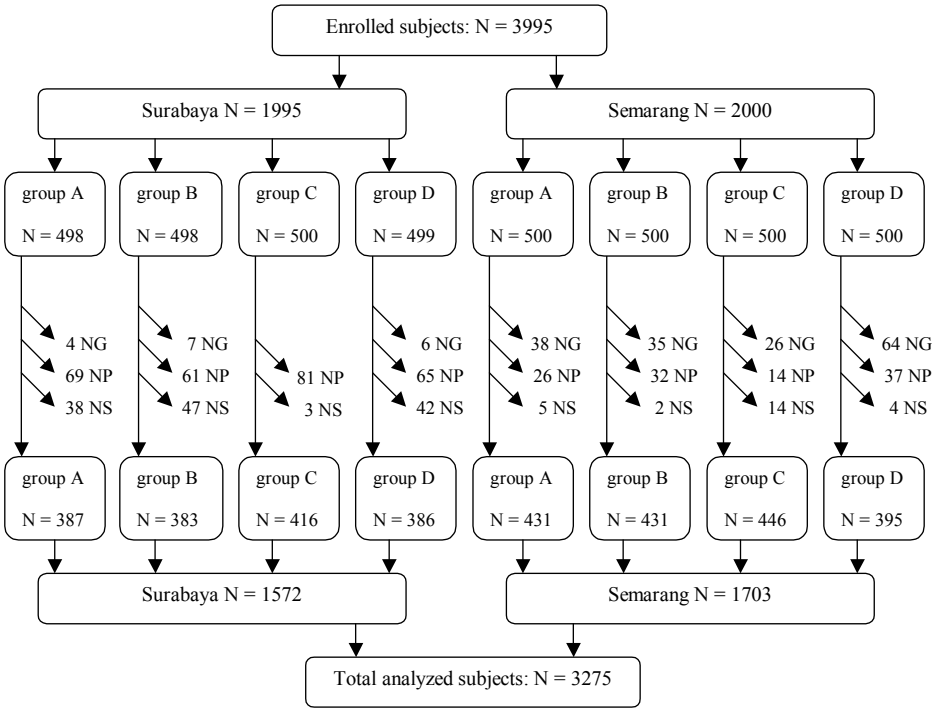

Figure 1. Flow chart with numbers of enrolled and analyzed subjects
Reasons for exclusion of enrolled subjects from analysis:
NG = no growth on agar plate
NP = no pink colonies on agar plate
NS = no complete susceptibility data

for analysis: 180 because there was no growth on the agar plate, 385 because no pink colonies were present in the culture, and 155 because of missing susceptibility data (Figure 1). No growth was observed significantly more frequently in Semarang (8%) than in Surabaya (1%, $P<0.001$). In Surabaya, no significant differences were observed between the groups, whereas in Semarang, the proportion with no growth varied from 5% in group B to 13% in group D ($P<0.001$). The proportion of pink colonies did not differ significantly between Surabaya and Semarang, or between the groups in Surabaya, but varied between 80% in group D and 92% in group B ($P<0.001$) in Semarang. Missing or incomplete susceptibility data occurred more frequently in Surabaya (8%) than in Semarang (1%, $P<0.001$). In Semarang, no significant differences were observed between the groups, whereas in Surabaya, the proportion with missing susceptibility data varied from 1% in group B to 11% in group C ($P<0.001$).

No significant differences in demographic, socioeconomic, disease-related, and healthcare-related variables were observed between the community and hospital populations, with the exception of age (Table 1). Additional information regarding

Table 1. Demographic characteristics of community and hospital populations.

	community n=2,494	hospital n=781	significant difference
Surabaya	1,186 (48)	386 (49)	NS
group A (admission)	818 (33)	-	-
group B (relatives)	814 (33)	-	-
group C (PHC)	862 (35)	-	-
group D (discharge)	-	781 (100)	-
Internal Medicine	197* (24)	192 (25)	NS
Surgery	203* (25)	204 (26)	NS
Obstetrics and Gynecology	217* (27)	209 (27)	NS
Pediatrics	201* (25)	176 (23)	NS
age above 16	2,032 (82)	558 (71)	*P*<0.001
female sex	1,548 (62)	460 (59)	NS
Javanese ethnicity	2,377 (95)	733 (94)	NS
urban provenance	1,615 (65)	497 (64)	NS
health insurance	641 (26)	219 (28)	NS
low income	1,084 (57)	360 (46)	NS
primary school completed	1,971 (79)	586 (75)	NS
employment	1,575 (63)	447 (83)	NS
crowding >8 persons per household	315 (13)	73 (9)	NS
nursing class III	679* (83)	615 (79)	NS
length of stay >8 days	-	394 (50)	-
clinical signs of infection	1,805 (72)	-	-
infection diagnosis in hospital	206* (32)	204 (26)	NS

Absolute numbers are shown, with percentages between brackets. 'NS' represents no significant differences were observed between the populations. * Only calculated for group A; percentages are proportions of patients in group A.

population characteristics can be found in Table S2 for the community and in Table S3 for the hospital.

Antimicrobial resistance

Of the 3,275 *E. coli* strains, 1,552 (47%) were susceptible to all tested antibiotics, 585 (18%) to a single antibiotic and 1,138 (35%) to two or more antibiotics (Table 2). In 69 strains (not shown in Table 2), resistance patterns were observed that occurred less than 8 times.

In the community, ampicillin resistance was observed most frequently (851 isolates, 34%), followed by trimethoprim-sulfamethoxazole resistance in 716 isolates (29%) and chloramphenicol resistance in 369 isolates (15%). Resistance to ciprofloxacin, gentamicin, and cefotaxime occurred less than 100 times. Single ampicillin resistance was observed in 236 isolates (9%) and single trimethoprim-sulfamethoxazole resistance

Table 2. Resistance patterns.

number of isolates (%)	ampicillin	chloramphenicol	gentamicin	cefotaxime	ciprofloxacin	trimethoprim-sulfamethoxazole
1,552 (47.4)	S	S	S	S	S	S
361 (11.0)	R	R	S	S	S	R
321 (9.8)	R	S	S	S	S	S
316 (9.6)	R	S	S	S	S	R
185 (5.6)	S	S	S	S	S	R
94 (2.9)	R	R	S	S	S	S
59 (1.8)	R	R	S	S	R	R
41 (1.3)	S	R	S	S	S	S
37 (1.1)	R	S	S	S	R	R
28 (0.9)	R	R	R	S	R	R
22 (0.7)	R	R	R	S	S	R
21 (0.6)	R	R	R	R	R	R
20 (0.6)	R	R	R	R	S	R
19 (0.6)	S	S	S	S	R	S
19 (0.6)	S	S	R	S	S	S
17 (0.5)	R	S	S	S	R	S
17 (0.5)	R	S	R	S	R	R
16 (0.5)	S	R	S	S	S	R
13 (0.4)	R	S	R	R	R	R
11 (0.3)	R	S	S	R	S	R
10 (0.3)	R	S	R	S	S	S
10 (0.3)	R	S	R	R	S	R
9 (0.3)	S	S	S	S	R	R
8 (0.2)	R	S	R	R	R	S

The number of times a given resistance pattern was found is shown in the first column, with the prevalence between brackets. Resistance is represented by an R, susceptibility by an S.

in 162 isolates (6%), whereas single chloramphenicol, gentamicin, and ciprofloxacin resistance were observed less than 100 times. Single cefotaxime resistance was not present in any of the isolates.

In hospitalized patients, ampicillin resistance was also observed most frequently (570 isolates, 73%), followed by trimethoprim-sulfamethoxazole resistance in 434 isolates (56%), chloramphenicol resistance in 334 isolates (43%), ciprofloxacin resistance in 173 isolates (22%) and gentamicin resistance in 141 isolates (18%). Cefotaxime resistance was observed less than 100 times. In hospitalized patients, single resistance was observed for less than 100 subjects for all tested antibiotics and single cefotaxime resistance was not present in any of the isolates.

Antibiotic use

Antibiotic use results are summarized in Table 3. In the community (2,494 individuals), 367 antibiotic courses were prescribed in the month preceding the study, while for 781 hospitalized individuals, 1,084 antibiotic courses were prescribed. Penicillins ranked first and accounted for 71% of antibiotic use in the community and 40% in hospitals. In the community, tetracyclines (10%), sulphonamides (7%), and amphenicols (7%) were the other frequently used antibiotics. In the community, 93% of antibiotic use concerned the use of a single antibiotic. In the 2,125 individuals in the community who received no antibiotic treatment, the carriage rate of multiple resistances (resistance to more than one antibiotic) was 24%, in the 347 patients receiving one antibiotic 38% and in the 22 patients receiving more than one antibiotic 46%.

In hospitalized patients, cephalosporins (22%) and quinolones (10%) ranked second and third, respectively. Single antibiotic use was observed in 33% of cases. In the 127 hospitalized patients who received no antibiotic treatment, the carriage rate of multiple resistances was 33%, in the 159 patients receiving one antibiotic 64% and in the 495 patients receiving more than one antibiotic 71%.

Table 3. Total and single antibiotic use in community and hospital populations.

	community		hospital	
	total use (n)	single use (%)	total use (n)	single use (%)
tetracycline	37	86	5	20
penicillins	261	97	440	51
amphenicols	24	75	52	15
cephalosporins	0	0	239	30
carbapenems	0	0	3	0
sulphonamides	26	88	39	15
macrolides	10	60	26	15
aminoglycosides	2	100	92	2
quinolones	3	100	114	34
metronidazole	4	100	69	0
others	0	0	5	0
total	367	93	1,084	33

Total use (n) is the number of antibiotic prescriptions; single use (%) is single antibiotic use as percentage of total number of prescriptions.

Determinants of resistance in the community (groups A, B, and C)

Analysis of determinants for resistance in the community was performed with resistance to any of the tested antibiotics, single ampicillin resistance, and single trimethoprim-sulfamethoxazole resistance, because more than 100 cases were available for these resistance groups.

Any antibiotic use was associated with carriage of *E. coli* with resistance to any of the tested antibiotics (odds ratio (OR) 1.8, 95% confidence interval (95%CI) 1.52.3), single ampicillin resistance (OR 1.6, 95%CI 1.12.3), and single trimethoprim-sulfamethoxazole resistance (OR 1.8, 95%CI 1.22.8). Prior use of penicillins was associated with carriage of *E. coli* resistant to any of the tested antibiotics (OR 1.8, 95%CI 1.42.4) and single ampicillin resistance (OR 1.8, 95%CI 1.22.7). Prior use of amphenicols was associated with carriage of *E. coli* resistant to any of the tested antibiotics (OR 3.1, 95%CI 1.37.5). Prior use of sulphonamides was associated with carriage of *E. coli* resistant to any of the tested antibiotics (OR 5.5, 95%CI 2.114.8) and single trimethoprim-sulfamethoxazole resistance (OR 7.5, 95%CI 2.028.0).

Logistic regression analysis performed with only single antibiotic use did not change the findings significantly; in most cases, the same antibiotics were associated with resistance when used as a single antibiotic drug or combined with other antibiotics (data not shown).

Socioeconomic variables were not associated with carriage of resistant *E. coli* in the community. Neither were demographic variables, except for age: adults were less likely to be carriers of *E. coli* with resistance to any of the tested antibiotics (OR 0.4, 95%CI 0.30.5) and single ampicillin resistance (OR 0.6, 95%CI 0.40.9) than children. The same analysis with children of less than two years old versus people of more than two years of age yielded similar results (data not shown). Admission to hospital (group A) was associated with carriage of *E. coli* resistant to any of the tested antibiotics (OR 2.4, 95%CI 2.03.0) and single ampicillin resistance (OR 2.7, 95%CI 1.94.0, group B = reference category). Susceptibility patterns of groups A and B did not correlate, although individuals from these groups were included as pairs (Pearson's correlation coefficient = 0.014). Diarrhoea was associated with carriage of *E. coli* resistant to any of the tested antibiotics (OR 1.9, 95%CI 1.32.7).

Determinants of resistance in hospitalized patients (group D)

Analysis of determinants for resistance in hospitalized patients was only performed with resistance to any of the tested antibiotics, because single resistance was observed for less than 100 subjects for all tested antibiotics.

The use of any antibiotic (OR 2.5, 95%CI 1.63.9), penicillins (OR 3.2, 95%CI 2.2-4.8), amphenicols (OR 3.9, 95%CI 1.212.8), quinolones (OR 6.8, 95%CI 3.015.1) and metronidazole (OR 2.9, 95%CI 1.17.6) was associated with carriage of *E. coli* with resistance to any of the tested antibiotics.

Logistic regression analysis with only single antibiotic use changed the findings significantly for carriage of *E. coli* with resistance to any of the tested antibiotics: any (single or combined) cephalosporin use was not associated with resistance, but single cephalosporin use was associated with less carriage of *E. coli* with resistance to any

of the tested antibiotics (OR 0.2, 95%CI 0.10.5). Single use of other antibiotics was not associated with carriage of *E. coli* with resistance to any of the tested antibiotics (data not shown).

Having no health insurance was associated with less carriage of *E. coli* with resistance to any of the tested antibiotics (OR 0.6, 95%CI 0.40.9). Discharge from the hospital in Semarang was associated with carriage of *E. coli* with resistance to any of the tested antibiotics (OR 2.2, 95%CI 1.53.3). Discharge from the Department of Pediatrics (OR 4.3, 95%CI 1.710.7), rather than from Internal Medicine (reference category) was associated with carriage of *E. coli* with resistance to any of the tested antibiotics. Significant differences were observed between several individual nursing wards, but for most wards the numbers of patients were too small to draw any conclusions from these data (data not shown).

DISCUSSION

This study shows that antibiotic use is the most important albeit not the only determinant of carriage of resistant *E. coli.* In the non-hospitalized population, age under 17 and diarrhoea were independent determinants. Individuals screened upon admission to hospital carried resistant *E. coli* more often than patients who visited a PHC and healthy relatives who accompanied patients at admission to hospital. In hospitalized patients screened upon discharge, having health insurance was associated with carriage of resistant *E. coli,* as were several healthcare-related determinants: hospitalization in Semarang and admission to the Gynecology and Obstetrics or Pediatric Departments.

In concordance with our hypothesis we observed that, for most antibiotic classes, most resistance was present in the group most exposed to antibiotics and least resistance in the group least exposed to antibiotics. In the community, direct associations were observed between the use of specific antibiotics and resistance to those antibiotics, namely between β-lactam antibiotics and ampicillin resistance and sulphonamide use and trimethoprim-sulfamethoxazole resistance. Here, the majority of antibiotic therapy consisted of single therapy.

For hospitalized patients two-thirds of antibiotic treatments were combined therapies. The use of penicillins, amphenicols, quinolones, and metronidazole was associated with resistance to any of the tested antibiotics. Epidemiologically one can assume that it represents a greater exposure to antibiotics, since most patients took more than one antibiotic. Indeed there was a high rate of multiple resistances. In the subset of hospitalized patients treated with a single antibiotic, single use of a cephalosporin was associated with less resistance to any of the tested antibiotics. It is

unlikely that cephalosporins actually protect against resistance. In a hospital population, where 84% of the patients took antibiotics during admission, single β-lactam use might reflect a relatively healthy population with a relatively low susceptibility to infections and exposed to relatively low quantities of antibiotics (e.g. as prophylaxis).

Several other determinants, although independent from antibiotic use in the analysis, can still be explained by a relatively high exposure to antibiotics. Health insurance increased the probability of carriage of resistant *E. coli*. This is most likely, at least partly, due to the different consumption pattern of antibiotics. Individuals with health insurance consumed antibiotics more frequently, took longer antibiotic courses and different antibiotic classes, namely cephalosporins, macrolides, and quinolones, than people without health insurance.

In the community, more children than adults carried resistant *E. coli*. Several factors may have contributed to carriage of resistant *E. coli* in children. Young children generally tend to receive antibiotics more frequently than adults (24). The AMRIN study confirmed that more children than adults received antibiotics. Apart from antibiotic use, children might acquire resistant bacteria more easily than adults, because of the greater exposure through unhygienic behaviour.

With regard to clinical signs and symptoms, we observed that individuals who reported diarrhoea had a higher probability of carriage of resistant *E. coli* than individuals with other or no complaints. We must interpret these data carefully, since diarrhoea often occurs during antibiotic use and patients may have incorrectly reported diarrhoea as a symptom instead of an adverse reaction to an antibiotic.

Our results indicate that the hospital, the department, and the nursing ward to which a patient is admitted are determinants of carriage of resistant *E. coli* in hospitalized patients. In hospitals, transmission of resistant bacteria contributes to the problem of antibiotic resistance, probably much more so than in the community (29, 30). Further investigations are needed to show whether transmission of resistant strains of *E. coli* explains the differences between the two hospitals, the departments, and the wards.

There are several limitations to the study. Antibiotic use in the community was self-reported. We may have missed determinants for carriage of resistant *E. coli*, because, since quantitative analysis was not feasible with the amount of variables analyzed, we dichotomized the variables for the purpose of analysis. The design of the study is not useful for making statements about mechanisms causing resistance, although it is helpful for making recommendations for further research. Finally, care must be taken in generalization of our results to the general Javanese population, as the majority of participants was in contact with healthcare institutions, in varying levels. The community population consisted of several subgroups, with group B being most representative of the general Javanese population. The hospital population was approximately representative of urban Javanese government hospitals, with a tendency

towards longer than average hospital admissions. However, the design proved useful to show that the more intensively individuals are in contact with healthcare institutions, the more prone they are to carriage of resistant *E. coli*.

In conclusion, antibiotic use was the most important determinant for carriage of resistant *E. coli* in our study. Most antibiotic classes were associated with carriage of resistant *E. coli*. An aberrant antibiotic consumption pattern of people with health insurance may explain the role of health insurance. Children, regardless of more frequent antibiotic use, were at greater risk of carriage of resistant *E. coli* than adults, perhaps because of the greater exposure to (resistant) microorganisms. Differences between and within hospitals point to transmission of resistant bacteria within hospitals.

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Table S1. Model of selection of candidate variables for final logistic regression model and model for analysis of antibiotic use.

group	model	interim analyses (selection of candidate variables)						significant variables	antibiotic use	
		all candidate variables	excluded*	demographic variables	socioeconomic adults†	socioeconomic all ages†	healthcare & disease-related		any antibiotic use	single antibiotic use
community (groups A, B, and C)	any resistance versus completely susceptible (n=2,494)	origin sex **age** ethnicity provenance health insurance income crowding **group** fever symptoms **diarrhoeal symptoms** respiratory symptoms other symptoms no symptoms **antibiotic use y/n**	infection diagnosis on admission*	origin sex **age** ethnicity provenance	health insurance income crowding education employment	health insurance income crowding	**group** fever symptoms **diarrhoeal sympt** respiratory sympt other symptoms no symptoms	group age diarrhoeal sympt antibiotic use y/n Goodness-of-fit: Nagelkerke R2: 0.137	tetracyclins penicillins amphenicols sulphonamides macrolides aminoglycosides quinolones metronidazole	tetracyclins penicillins amphenicols sulphonamides macrolides aminoglycosides quinolones metronidazole
	single ampicillin resistance (n=1,640)	origin sex **age** ethnicity provenance health insurance income crowding **group** fever diarrhoeal sympt respiratory sympt other symptoms no symptoms **antibiotic use y/n**	infection diagnosis on admission*	origin sex **age** ethnicity provenance	health insurance income crowding education employment	health insurance income crowding	**group** fever symptoms diarrhoeal symptoms respiratory symptoms other symptoms no symptoms	group age antibiotic use y/n Goodness-of-fit: Nagelkerke R2: 0.058	tetracyclins penicillins amphenicols sulphonamides macrolides metronidazole	tetracyclins penicillins amphenicols sulphonamides macrolides metronidazole

group	model	interim analyses (selection of candidate variables)						significant variables	antibiotic use	
		all candidate variables	excluded*	demographic variables	socioeconomic adults†	socioeconomic all ages†	healthcare & disease-related		any antibiotic use	single antibiotic use
	single sulfamethoxazole-trimethoprim resistance (n=1,566)	origin sex age ethnicity **provenance** health insurance income crowding group fever diarrhoeal sympt respiratory sympt other symptoms no symptoms **antibiotic use y/n**	infection diagnosis on admission*	origin sex age ethnicity **provenance**	health insurance income crowding education employment	health insurance income crowding	group fever symptoms diarrhoeal symptoms respiratory symptoms other symptoms no symptoms	provenance antibiotic use y/n Goodness-of-fit: Nagelkerke R2: 0.008	tetracyclins penicillins amphenicols sulphonamides macrolides metronidazole	tetracyclins penicillins amphenicols sulphonamides macrolides metronidazole
hospital (group D)	any resistance versus completely susceptible (n=781)	**origin** sex age ethnicity provenance **health insurance** income crowding group **departments** nursing wards nursing class length of stay **antibiotic use y/n**	infection diagnosis on discharge*	**origin** sex age ethnicity provenance	**health insurance** income crowding education employment	**health insurance** income crowding	**departments** nursing class length of stay	origin departments health insurance antibiotic use y/n Goodness-of-fit: Nagelkerke R2: 0.015	tetracyclins penicillins amphenicols cephalosporins carbapenems sulphonamides macrolides aminoglycosides quinolones metronidazole other antibiotics	tetracyclins penicillins amphenicols cephalosporins sulphonamides macrolides aminoglycosides quinolones metronidazole

All candidate variables that were significant in one or more of the interim analyses are printed in bold. Candidate variables that were excluded from the analyses due to very small dispersion (logistic regression could not be performed because of sparse data) are indicated by an asterisk. † Interim analysis of socioeconomic variables was carried out separately for all ages and for adults, since the variables education and employment could not be analyzed for children.

Table S2. Distribution of variables and resistance patterns in the community (groups A, B, and C).

	any resistance* (n=2,492, n resistant =1,090)			ampicillin resistance** (n=1,640, n resistant =236)			SXT resistance*** (n=1,566, n resistant =162)		
Variable	resis-tant	suscep-tible	*P*	resis-tant	suscep-tible	*P*	resis-tant	suscep-tible	*P*
origin (Surabaya)	516	670	0.850	93	670	0.018	92	670	0.029
sex (female)	645	903	0.009	148	903	0.635	97	903	0.265
age (over 16)	783	1249	0.000	188	1,249	0.000	140	1,249	0.334
ethnicity (Javanese)	1,036	1,341	0.661	225	1,341	0.719	154	1,341	0.131
provenance (urban)	724	891	0.125	138	891	0.143	117	891	0.027
health insurance (yes)	274	367	0.570	59	367	0.712	49	367	0.262
income (below poverty line)	476	608	0.840	110	608	0.344	63	608	0.282
crowding (>=8 per household)	142	173	0.593	28	173	0.843	21	173	0.815
group: A (admission)	499	319	0.000	98	319	0.000	49	319	0.087
B (family)	250	564		54	564		62	564	
fever symptoms	265	201	0.000	47	201	0.026	21	201	0.640
diarrhoeal symptoms	130	56	0.000	18	56	0.013	7	56	0.838
respiratory symptoms	349	417	0.208	79	417	0.243	38	417	0.097
other symptoms	415	560	0.367	104	560	0.226	68	560	0.607
no symptoms	840	964	0.000	183	964	0.006	109	964	0.721
any antibiotic use past month	223	145	0.000	42	145	0.001	28	145	0.007
any tetracycline use	20	17	0.201	3	17	0.938	4	17	0.187
any penicillin use	148	113	0.000	32	113	0.006	20	113	0.063
any amphenicol use	17	7	0.007	1	7	0.879	0	7	0.368
any sulphonamide use	21	5	0.000	1	5	0.874	4	5	0.001
any macrolide use	6	4	0.298	1	4	0.720	0	4	0.496
any aminoglycoside use	2	0	0.108	-			-		
any quinolone use	3	0	0.049	-			-		
any metronidazole use	2	2	0.799	0	2	0.562	0	2	0.631
single tetracycline use	17	15	0.280	3	15	0.782	4	15	0.123
single penicillin use	143	111	0.000	32	111	0.004	20	111	0.053
single amphenicol use	13	5	0.014	1	5	0.874	0	5	0.447
single sulphonamide use	19	4	0.000	1	4	0.720	4	4	0.000
single macrolide use	3	3	0.756	1	3	0.545	0	3	0.556
single aminoglycoside use	2	0	0.108	-			-		
single quinolone use	3	0	0.049	-			-		
single metronidazole use	2	2	0.799	0	2	0.562	0	2	0.631
only calculated for adults†	*(n=1,976, n resistant =754)*			*(n=1,403, n resistant =181)*			*(n=1,356, n resistant =134)*		
no education	82	124	0.607	20	124	0.709	7	124	0.067
only calculated for adults††	*(n=1,968, n resistant =751)*			*(n=1,395, n resistant =178)*			*(n=1,350, n resistant =133)*		
no fixed employment	150	243	0.997	35	243	0.924	27	243	0.927
only calculated for group A†††	*(n=818, n resistant =499)*			*(n=417, n resistant =98)*			*(n=368, n resistant =49)*		

Dept. of Surgery	95	108	0.000	21	108	0.005	15	108	0.476
Obstetrics and Gynecology	98	119		30	119		15	119	
Pediatrics	169	32		15	32		8	32	
nursing class III	420	259	0.521	78	259	0.658	43	259	0.499
admission diagnosis infection	199	61	0.000	23	61	0.348	7	61	0.417

In this table, the numbers of resistant and susceptible isolates are shown for each variable (e.g., in the first columns the numbers of resistant and susceptible isolates from Surabaya are shown, respectively). From this table, crosstabulations can be constructed for each variable. Corresponding *P*-values result from chi-square testing for each combination of variable and resistance pattern.

Resistance patterns: * resistance to any of the tested antibiotics (ampicillin, chloramphenicol, gentamicin, cefotaxime, ciprofloxacin and/or trimethoprim-sulfamethoxazole), ** single ampicillin resistance and *** single trimethoprim-sulfamethoxazole (SXT) resistance.

† Different denominators (1,976 for resistance to any of the tested antibiotics, 1,403 for single ampicillin resistance and 1,356 for single trimethoprim-sulfamethoxazole resistance), because this population characteristic could only be analyzed for the adult population. †† Different denominators, because this population characteristic could only be analyzed for the adult population and data were missing for eight subjects. ††† Different denominators, because these population characteristics could only be analyzed in subjects upon admission to hospital (group A).

Table S3. Distribution of variables and resistance patterns in the hospital (group D).

	any resistance* *(n=781,n resistant =633)*		
variable	resistant	susceptible	*P*-value
origin (Surabaya)	341	54	0.000
sex (female)	256	65	0.439
age (over 16)	443	115	0.061
ethnicity (Javanese)	595	138	0.106
provenance (urban)	403	93	0.972
health insurance (yes)	164	55	0.006
income (below poverty line)	297	63	0.339
crowding (8 or more per household)	56	17	0.321
dept: Surgery	157	47	0.000
Obstetrics and Gynecology	177	32	
Pediatrics	159	17	
roomclass in hospital: class III	502	113	0.686
length of stay 9 days or more	323	71	0.504
discharge diagnosis infection	168	36	0.581
any antibiotic use during hospitalization	559	95	0.000
any tetracycline use	5	0	0.278
any penicillin use	386	54	0.000
any amphenicol use	49	3	0.012
any cephalosporin use	199	40	0.295
any carbapenem use	2	1	0.524
any sulphonamide use	37	2	0.024
any macrolide use	21	5	0.970
any aminoglycoside use	79	13	0.209
any quinolone use	107	7	0.000
any metronidazole use	64	5	0.009
any other antibiotic use	5	0	0.278
single tetracycline use	1	0	0.628
single penicillin use	189	35	0.133
single amphenicol use	6	2	0.661
single cephalosporin use	46	25	0.000
single sulphonamide use	5	1	0.886
single macrolide use	4	0	0.332
single aminoglycoside use	2	0	0.494
single quinolone use	35	4	0.155
only calculated for adults†	*(n=547,n resistant =434)*		
no education	41	7	0.276
only calculated for adults††	*(n=542,n resistant =431)*		
no fixed employment	75	20	0.879
†††	*(n=711,n resistant =572)*		

Ward: internal medicine I Surabaya	18	8	0.005
internal medicine II Surabaya	12	6	
internal medicine female Surabaya	12	7	
tropical diseases male Surabaya	6	5	
tropical diseases female Surabaya	10	5	
surgery A Surabaya	16	8	
surgery B Surabaya	3	1	
surgery C Surabaya	2	2	
surgery D Surabaya	11	2	
surgery F Surabaya	4	0	
surgery G Surabaya	18	6	
surgery H Surabaya	19	7	
obstetrics Surabaya	69	15	
gynecology Surabaya	11	7	
medium care gynecology Surabaya	3	1	
pediatrics Surabaya	72	14	
internal medicine class 2 Semarang	11	2	
internal medicine class 3 Semarang	69	19	
surgery A2 Semarang	32	8	
surgery A3 Semarang	37	7	
obstetrics Semarang	35	2	
gynecology Semarang	30	4	
medium care gynecology Semarang	11	1	
pediatrics class 2 Semarang	32	1	
pediatrics class 3 Semarang	29	1	

In this table, the numbers of resistant and susceptible isolates are shown for each variable (e.g., in the first columns the numbers of resistant and susceptible isolates from Surabaya are shown, respectively). From this table, crosstabulations can be constructed for each variable. Corresponding *P*-values result from chi-square testing for each combination of variable and resistance pattern.
Resistance pattern: * resistance to any of the tested antibiotics (ampicillin, chloramphenicol, gentamicin, cefotaxime, ciprofloxacin and/or trimethoprim-sulfamethoxazole).
† Different denominator (547), because this population characteristic could only be analyzed for the adult population.
†† Different denominator, because this population characteristic could only be analyzed for the adult population and data were missing for eight subjects.
††† Different denominator, because for 70 subjects, these date were missing.

Chapter 10

Fluoroquinolone-resistant *Escherichia coli*, Indonesia

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ABSTRACT

In a recent, population-based survey of 3,995 persons in Indonesia, fluoroquinolone (FQ)-resistant *Escherichia coli* was prevalent in the fecal flora of 6% of patients at hospital admission and 23% of patients at discharge, but not among healthy relatives or patients visiting primary health centers (2%). Molecular typing showed extensive genetic diversity with only limited clonality among isolates. This finding suggests that independent selection of resistant mutants occurs frequently. FQ-resistant isolates exhibited a higher rate of spontaneous mutation, but sparser virulence profiles, than FQ-susceptible isolates from the same population. The resistant isolates belonged predominantly to phylogenetic groups A (57%) and B1 (22%) but also to the moderately virulent group D (20%). Hypervirulent strains from the B2 cluster were underrepresented (1%). Because FQ-resistant *E. coli* can cause disease, especially nosocomial infections in immunocompromised patients, spread of such strains must be stopped.

INTRODUCTION

Escherichia coli is a common constituent of the gastrointestinal flora of most vertebrates, including humans, and may be isolated from a variety of environmental sources. While most strains are non-pathogenic, certain ones can cause a variety of intestinal and extraintestinal infections. Pathogenicity is largely determined by the presence of genes encoding virulence factors (VFs), such as adhesins, toxins, and polysaccharide surface coatings (6). Phylogenetic analysis showed that most *E. coli* strains fall into four main phylogenetic groups, designated A, B1, B2, and D (5). *E. coli* strains that cause extraintestinal infections derive predominantly from group B2 and, to a lesser extent, group D. Strains of groups A and B1 represent most commensal strains and are largely devoid of virulence determinants (14). Although strains harboring a robust extraintestinal VF repertoire cluster predominantly in groups B2 and D, isolates within each phylogenetic group can be further classified as extraintestinal pathogenic *E. coli* (ExPEC) or non-ExPEC depending on whether specific virulence traits are present (7, 15).

The fluoroquinolones (FQs) are potent antimicrobial agents used for the treatment and prophylaxis of infections caused by Gram-negative bacteria, including *E. coli*. FQ-resistant *E. coli* has been reported increasingly during the last decade in both the hospital environment and the community, which may ultimately limit the utility of these broad-spectrum antibiotics (1, 4, 18). Moreover, FQ-resistant *E. coli* strains often show resistance to other antibiotics, such as ampicillin, tetracycline, chloramphenicol, trimethoprim-sulfamethoxazole, and gentamicin (4, 10). Recent reports have suggested that clinical FQ-resistant *E. coli* actually tends to be less virulent than susceptible isolates. FQ-resistant *E. coli* from hospitalized Dutch patients derived predominantly from the low-virulence phylogenetic groups A and B1. None of the 13 invasive isolates derived from phylogenetic group B2 (9). In addition, evidence suggests that clinical FQ-resistant *E. coli* isolates from humans in Iowa were associated with a shift toward non-B2 phylogenetic groups and to a lower overall virulence genotype (7). FQ resistance may also be associated with strains that intrinsically have a higher overall mutation rate, since the resistance to FQs in *E. coli* involves the accumulation of multiple spontaneously occurring point mutations in several genes (10, 13). These associations, however, may depend on the strains' geographic or clinical origin.

In our study, we investigated these putative associations in a well-defined collection of isolates from Indonesia. A population-based survey of almost 4,000 people in two cities on the island of Java (Surabaya and Semarang) was initiated in 2000 by the Antimicrobial Resistance in Indonesia, Prevalence and Prevention (AMRIN) study group to investigate the level of carriage of resistant microorganisms. FQ-resistant *E. coli* was prevalent in the fecal flora of 6% of patients at hospital admission and

23% of patients at discharge, but not among healthy relatives or patients visiting a primary health center (2% in both groups) (11). In our study, we analyzed these FQ-resistant *E. coli* isolates to elucidate their molecular epidemiology and virulence. To define clonal relatedness, we performed enterobacterial repetitive intergenic consensus (ERIC) polymerase chain reactions (PCRs). The phylogenetic background and virulence profile of these isolates were determined by PCR methods and compared with similar data for FQ-susceptible *E. coli* isolated from the same population. Finally, we examined the link between FQ resistance and the intrinsic mutation rate.

MATERIALS AND METHODS

Strains

The AMRIN surveillance program was initiated to determine the prevalence of antimicrobial resistance in Indonesia. Four different groups of persons in Surabaya and Semarang were studied for carriage of resistant microorganisms in their stools. The four groups were patients on the day of admission to the hospital (group 1), patients on the day of discharge after five or more days of hospitalization (group 2), patients visiting a primary health center (group 3), and healthy relatives or household members of group 1 patients (group 4). In groups 1 and 2, rectal swabs were taken from patients in the internal medicine, surgery, gynecology and obstetrics, or pediatrics departments. The specimens were collected from July to October 2001 in Surabaya and from January to May 2002 in Semarang. Further details on the methods of culturing have been published elsewhere (11). A total of 5,535 *E. coli* isolates from 3,284 individuals were cultured. Antimicrobial susceptibility testing was performed for one isolate per patient. The overall by-isolate prevalence of resistance to ciprofloxacin as determined by disk diffusion was 8%. The prevalence of resistance was highest among patients on the day of discharge (18% in Surabaya and 27% in Semarang) and lowest among patients visiting primary health centers and among family members of patients admitted to the hospital (2% in both groups). The prevalence of FQ-resistant *E. coli* among patients who were tested on the day of admission was 8% in Surabaya and 4% in Semarang. We studied 196 FQ-resistant isolates in more detail. Seventy-five (38%) of these were from Surabaya (19, 48, 4, and 4 isolates from stated population groups 1, 2, 3, and 4, respectively) and 121 (62%) from Semarang (13, 92, 11, and 5, respectively). The FQ-resistant isolates were recovered from patients from all four hospital departments in both cities. In Semarang, 43% of these isolates were from the surgery department and 41% were from the internal medicine department. In Surabaya, 43% of the isolates were from the internal medicine department.

All 196 ciprofloxacin-resistant *E. coli* and 200 ciprofloxacin-susceptible *E. coli* (20 randomly chosen isolates from groups 1, 2, and 3, and 40 from group 4, from each city) were confirmed by Vitek® 2 (bioMérieux, Marcy-l'Etoile, France) according to the manufacturer's instructions and included in the molecular analyses.

DNA isolation

Bacterial DNA was isolated by using the MagNA Pure LC™ with the MagNA Pure LC DNA Isolation Kit III for bacteria and fungi (standard protocol; Roche Molecular Biochemicals, Mannheim, Germany). DNA concentration was assessed spectrophotometrically. Samples were frozen at -20°C until further use.

Bacterial typing by ERIC-PCR

ERIC-PCR was conducted with primers ERIC-1R and ERIC-2 as described previously (18-20). The amplification products were subjected to electrophoresis in a 1% agarose gel and were stained with ethidium bromide (50 μg/mL). The ERIC-PCRs were performed by a single technician within a period of one month. Profiles were visually analyzed by two microbiologists. Single-band differences in profiles among strains led to the definition of separate genotypes. Ambiguous isolates were retested and analyzed by two other microbiologists.

Phylogenetic analysis and virulence typing

Isolates were assigned to one of the four main *E. coli* phylogenetic groups (A, B1, B2, and D) according to an established triplex PCR assay, in which the four phylogentic groups yield distinct combinations of three possible PCR products, *chuA* (heme transport), *yjaA* (unknown function gene from *E. coli* K-12 genome), and TSPE4. C2 (anonymous fragment identified by subtractive hybridization) (2, 5). All isolates were screened for five ExPEC-defining virulence markers, *papA/papC*, *sfa/focDE*, *afa/draBC*, *kpsM* II, and *iutA*. Based on previous statistical analyses of similar data, from collections within which each isolate's ExPEC status could be inferred based on ecological source or experimental virulence, isolates were classified as ExPEC if positive for two or more of these five defining virulence markers (7). All isolates were also screened for *hlyD* (hemolysin), another ExPEC-associated VF. Subsequently, all isolates that satisfied molecular criteria for ExPEC were screened for the following 32 additional virulence markers of ExPEC: *papEF* (P fimbrial tip pilins), *papG* (P adhesin), *papG* alleles I, II, and III, *sfaS* (S fimbriae), *focG* (F1C fimbriae), *iha* (putative adhesin-siderophore), *bmaE* (M fimbriae), *gafD* (G fimbriae), *F17a* (F17a fimbriae), *clpG* (CS31A adhesin), *afaE8* (afimbrial adhesin VIII), *fimH* (type 1 fimbriae), *cnf1* (cytotoxic necrotizing factor), *cdtB* (cytolethal distending toxin), *ireA* (siderophore receptor), *sat* (secreted autotransporter toxin), *astA* (S-like enterotoxin), *iroN* (sidero-

phore receptor), *fyuA* (yersiniabactin receptor), *kpsM II*, K1, and K2 (*kpsM* II variants; group 2 capsule), *kpsM* III (group 3 capsule), *rfc* (O4 lipopolysaccharide synthesis), *cvaC* (colicin V), *traT* (serum-resistance associated outer membrane protein), *ibeA* (invasion of brain endothelium), *ompT* (outer membrane protease T), *iss* (increased serum survival), *usp* (uropathogenic specific protein), *malX* (marker for pathogenicity-associated island from strain CFT073), and H7 *fliC* variant (flagellin). These virulence genes were detected by a combination of multiplex PCR and dot-blot hybridization with primers specific for internal or flanking sequences and probes generated and labeled with these primers; this method was previously validated by using dot-blot hybridization with defined control strains (8). A VF score was calculated for each strain as the sum of all VF genes for which the strain tested positive. In all of these PCR assays, the identity of the PCR products was deduced by comparing their size to molecular size standards in ethidium bromide-stained agarose gels. Appropriate positive and negative controls were included in each run.

Mutation rate analysis

The mutation rate was determined for 20 randomly selected isolates from phylogenetic group A (10 FQ-susceptible and 10 FQ-resistant) by monitoring the isolates' capacity to generate mutations conferring resistance to rifampicin, as described previously (10, 12). Forty independent cultures of each of the 20 strains were set up in Luria broth. After overnight incubation, equal concentrations of cultures were suspended in 0.85% NaCl. The suspensions were spread on Luria agar plates containing 100 µg/mL rifampicin and incubated overnight. For each strain, the proportion of cultures giving no resistant mutants was used to calculate the mutation rate per cell per generation according to the fluctuation test of Luria and Delbrück. To avoid confounding by variation in phylogenetic background, only phylogenetic group A isolates were investigated. For comparisons of results, we used the relative mutation rate, which was defined as the rate relative to the rate for *E. coli* strain Nu14 (5×10^{-9} per cell per generation) (10).

Statistical analysis

All data were analyzed by using the statistic software packages SPSS version 10.0 (SPSS, Chicago, IL, USA) and EpiInfo version 5.00 (Centers for Disease Control and Prevention, Atlanta, GA, USA). Chi-square or Fisher's exact tests (two-tailed) were used when appropriate for comparisons of proportions. Comparisons involving VF scores and relative mutation rates were analyzed using the Mann-Whitney *U* test. The criterion for statistical significance was a *P* value of <0.05.

RESULTS

Spread of FQ-resistant *E. coli*

Genetic heterogeneity among the 196 FQ-resistant *E. coli* was assessed by ERIC-PCR. We documented 158 different patterns, designated types 1 to 158, which indicated a genetically diverse collection of strains. Twenty pairs of isolates with identical profiles were identified, and nine distinct multiple-isolate clones were represented by isolates from three patients each. The limited number of shared genotypes were mainly recovered from group 2 patients, i.e. patients at the time of discharge from the hospital, 49 (73%) of 67 isolates. Among the total number of 140 isolates from group 2, we identified 119 different ERIC-PCR profiles.

Type 37 occurred in three patients from the internal medicine department in Surabaya: all three patients were present within this department on the same day. The finding of this unique isolated cluster can be explained by patient-to-patient transmission or a nonpatient-associated environmental source. This explanation was not further examined in this study. Type 90 was isolated from two patients on the day they were discharged from the internal medicine department in Semarang. Samples were collected on consecutive days. An isolate with an identical ERIC-PCR pattern was found in the same period in the same hospital in a pediatric patient at discharge. No further obvious clustering in time and place was observed among isolates from the nine multiple-strain clusters.

Phylogenetic analysis

PCR-based phylotyping showed that the 200 FQ-susceptible isolates were predominantly from phylogenetic groups A (52%) and B1 (30%) (Table 1). The 196 FQ-resistant isolates also mainly derived from phylogenetic groups A (57%) and B1 (22%), but some derived from the moderately virulent phylogenetic group D (20%). Hypervirulent strains from the B2 cluster were underrepresented (1%). Eighteen (67%) of the 27 isolates from the nine distinct clones that were represented by three isolates each belonged to group A.

Table 1 shows that the resistant isolates were significantly depleted for phylogenetic group B2 and enriched for group D, when compared with the susceptible isolates. These shifts in phylogenetic distribution were significant both overall and specifically in Semarang, whereas a similar but nonsignificant trend was observed in Surabaya.

The phylogenetic distribution of all 396 isolates among the two cities was highly similar (data not shown). Comparisons of the distributions among the four population groups showed that group D isolates were more often obtained from patients sampled on the day of discharge than from other population groups (37 of the 180 group 2 isolates belonged to group D (21%) versus 25 of the 216 nongroup 2 isolates (12%),

Table 1. Distribution of phylogenetic groups and virulence factors among 196 FQ-resistant *E. coli* and 200 FQ-susceptible *E. coli* isolates from human feces in Indonesia.

Group or factor	All isolates (n=396)	FQ-susceptible (n=200)	FQ-resistant (n=196)	*P* values
A	215 (54)	103 (52)	112 (57)	NS
B1	102 (26)	59 (30)	43 (22)	NS
B2	17 (4)	15 (8)	2 (1)	0.001
D	62 (16)	23 (12)	39 (20)	0.02
papA	28 (7)	27 (14)	1 (1)	< 0.001
papC	29 (7)	28 (14)	1 (1)	< 0.001
sfa/focDE	8 (2)	8 (4)	0	0.007
afa/draBC	11 (3)	11 (6)	0	0.001
iutA	131 (33)	61 (31)	70 (36)	NS
hlyD	20 (5)	20 (10)	0	< 0.001
kpsM II	56 (14)	54 (27)	2 (1)	< 0.001
ExPEC	44 (11)	40 (20)	4 (2)	< 0.001

Screening for ExPEC was performed for 199 FQ-susceptible and 195 FQ-resistant isolates. Data are no. (%) of isolates. ExPEC: Extra-intestinal Pathogenic *E. coli*. NS: not significant.

P=0.01). Stratification showed, however, that this association was due to the excess prevalence of FQ-resistant group D isolates among the group 2 patients. Furthermore, B2 isolates were significantly more prevalent in group 3, i.e. patients visiting public health centers (7 of the 55 group 3 isolates belonged to group B2 (13%) versus 10 of the 341 nongroup 3 isolates (3%), *P*=0.004).

Virulence typing

All *E. coli* isolates were tested for a set of virulence factors to allow an inference as to their pathogenic potential. The overall prevalence of the five defining ExPEC VFs ranged from 2% (*sfa/focDE*) to 33% (*iutA*) (Table 1). The FQ-resistant isolates were significantly depleted for *papA*, *papC*, *sfa/focDE*, *afa/draBC*, *hlyD*, and *kpsM II*, when compared with the susceptible isolates (Table 1). Accordingly, 40 FQ-susceptible *E. coli* isolates (20%), but only four FQ-resistant isolates (2%), were classified as ExPEC, as they exhibited two or more of the five key ExPEC VFs (*P*<0.001). Thus, FQ resistance was associated with reduced inferred virulence. All FQ-resistant *E. coli* isolates from the nine distinct clones that were represented by three patients each were found to be non-ExPEC.

The distribution of the six screening VFs was also analyzed in relation to the four phylogenetic groups (Table 2). Each VF was broadly distributed, occurring in three or more phylogenetic groups. However, *papA*, *papC*, *kpsM II*, *hlyD*, and *sfa/focDE* were all significantly associated with phylogenetic group B2. Accordingly, 53% of the phylogenetic group B2 isolates qualified as ExPEC versus 9% of the non-B2 isolates (*P*<0.001) (Tables 2 and 3).

Table 2. Distribution of virulence factors among 394 isolates from phylogenetic group A, B1, B2, and D.

		Phylogenetic group, no. (%)			
Virulence factor	All isolates (n=394)	A (n=215)	B1 (n=101)	B2 (n=17)	D (n=61)
papA	28 (7)	7 (3)	11 (11)	8 (47)*	2 (3)
papC	29 (7)	8 (4)	11 (11)	8 (47)*	2 (3)
iutA	131 (33)	66 (31)	32 (32)	8 (47)	25 (41)
kpsMII	56 (14)	19 (9)	23 (23)	11 (65)*	3 (5)
hlyD	20 (5)	8 (4)	7 (7)	5 (29)*	0
sfa/focDE	8 (2)	2 (1)	4 (4)	2 (12)*	0
afa/draBC	11 (3)	6 (3)	2 (2)	1 (6)	2 (3)

* $P<0.05$.

Table 3. Distribution of (phylogenetic) groups and virulence factors among 44 ExPEC isolates from human feces in Indonesia.

	Prevalence of associated characteristic, no. (%)			
Characteristic	All isolates (n=44)	FQ-susceptible (n=40)	FQ-resistant (n=4)	*P* values
group A	16 (36)	14 (35)	2 (50)	NS
group B1	14 (32)	14 (35)	0	NS
group B2	9 (21)	9 (23)	0	NS
group D	5 (11)	3 (8)	2 (50)	NS
Surabaya	23 (52)	22 (55)	1 (25)	NS
Semarang	21 (48)	18 (45)	3 (75)	NS
Admission	13 (30)	12 (30)	1 (25)	NS
Discharge	16 (36)	14 (35)	2 (50)	NS
PHC	10 (23)	9 (23)	1 (25)	NS
Relatives	5 (11)	5 (13)	0	NS
iha	25 (58)	25 (64)	0	0.025
sat	25 (58)	25 (64)	0	0.025
fyuA	35 (81)	35 (90)	0	0.001
ibeA	3 (7)	1 (3)	2 (50)	0.019
malX	26 (60)	26 (67)	0	0.019

Extended virulence typing was performed for 39 FQ-susceptible isolates and 4 FQ-resistant isolates. Only those virulence factors are shown for which the comparison of FQ-resistant ExPEC to FQ-susceptible ExPEC was statistically significant. ExPEC: Extra-intestinal Pathogenic *E. coli*. NS: not significant. PHC: primary health center.

The 44 ExPEC isolates were studied in more detail (Table 3). The ExPEC isolates derived mainly from phylogenetic groups A (36%) and B1 (32%), with the four FQ-resistant ExPEC isolates belonging to groups A (n=2) and D (n=2). Many ExPEC isolates originated from patients on the day of discharge (36%). Both of the FQ-resistant ExPEC isolates from group 2 were from patients in the surgical department in Semarang. Again, no evidence for clonality was seen. The four resistant ExPEC

isolates exhibited sparse VF profiles, when compared with the susceptible ExPEC isolates. These isolates lacked classic ExPEC VFs such as *focG*, *hlyD* and *cnf1*. Four other VFs, *iha*, *sat*, *fyuA* and *malX*, were more prevalent among susceptible, rather than resistant, ExPEC isolates. Only *ibeA* was more prevalent among the resistant isolates. The VF *iutA* was detected in all FQ-resistant ExPEC isolates and in 27 of the 40 FQ-sensitive isolates (68%). This difference was not significant. Aggregate VF scores were lower among FQ-resistant ExPEC isolates (median 6, range 4-8) than among the 40 FQ-susceptible ExPEC isolates (median 10, range 3-16; P=0.024).

Mutation rate

The link between mutation rate and resistance to FQs was studied, as the rate of mutation accumulation might be a factor in the development of FQ-resistance. The 10 FQ-susceptible isolates had relative mutation rates of ≤0.52 (median rate 0.32, range 0.03-0.52), whereas the 10 FQ-resistant *E. coli* exhibited relative mutation rates of ≥0.55 (median rate 0.97, range 0.55-4.58) (P<0.001) (Figure 1).

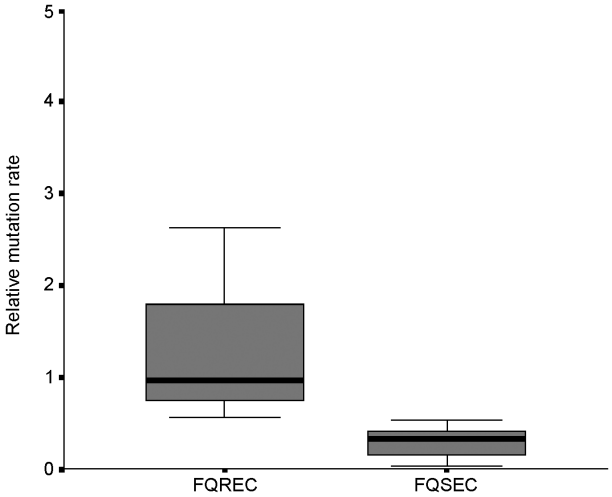

Figure 1. Box plot of relative mutation rate of 10 FQ-resistant (FQREC) and 10 FQ-sensitive (FQSEC) *E. coli*.

DISCUSSION

In this study, we investigated the epidemiology and virulence characteristics of FQ-resistant *E. coli* collected during a large, population-based survey of almost 4,000 people in two cities in Indonesia (Surabaya and Semarang). The overall prevalence of resistance to ciprofloxacin was 8%, but in the fecal flora of patients at time of discharge from the hospital the prevalence was 23%.

Dissemination of FQ-resistant *E. coli* and mutation rate

Three possible explanations for the high prevalence of FQ-resistant *E. coli* among patients that had been hospitalized for five or more days must be considered: transferable resistance, clonal spread, and mutation-based selection of resistance fostered by the use of antimicrobial agents. Transferable plasmid-mediated quinolone resistance has been described recently in *E. coli* from China (21). Wang et al. found that six of 78 ciprofloxacin-resistant *E. coli* strains from a hospital in Shanghai contained *qnr* (8%). However, from the present study we cannot draw any conclusion about the contribution of this mechanism in Indonesia. As for clonal spread, molecular typing showed extensive genetic diversity among FQ-resistant isolates in Indonesia. We identified a few distinct multiple-isolate clones in the hospital environment. Although all these clonal strains were shown to be non-ExPEC, they may still pose a health threat, especially to immunocompromised patients in hospital settings. Nosocomial outbreaks of infections caused by disseminating FQ-resistant clones have already been described (18). However, in our study, limited clonality among isolates was found, which suggests that other factors contribute more to the high prevalence of FQ-resistant *E. coli* among hospitalized patients.

To determine whether mutation-based resistance fostered by selection pressure contributed to the prevalence of FQ-resistant *E. coli* in Indonesia, we performed a mutation rate analysis of selected isolates. We found a strong correlation between resistance and an elevated mutation rate. This finding agrees with a recent report from Komp Lindgren et al., in which high mutation rates of *E. coli* strains from urinary tract infections were strongly associated with FQ resistance (10). To demonstrate that this mutation-based resistance was selected for by the use of FQs, we must know the consumption figures of the quinolones. In other reports, evidence suggests that the use (and misuse) of ciprofloxacin in human and animal medicine may predispose to an increase in infections with resistant *E. coli* (18). As information on the use of FQs in Indonesia is currently not available, we cannot draw any conclusions on a potential link between antimicrobial drug use, selection pressure, and mutation-based resistance. Thus, based on the large clonal diversity of the FQ-resistant *E. coli* and the slightly elevated mutation rates of resistant isolates compared with those of FQ-sensitive isolates, independent emergence of new resistant mutants likely occurs regularly in this setting.

Phylogenetic typing and virulence profiling

Phylogenetic typing and virulence profiling were performed to investigate whether a potential clinical hazard was associated with the presence of these isolates. Our data on the distribution of phylogenetic groups among the 396 *E. coli* isolates are consistent with most other studies. In an examination of human commensal *E. coli*

strains, the frequencies of B2 strains were found to be 2% (1 of 55) in Mali, 11% (6 of 56) in France, and 19% (11 of 57) in Croatia (3). In our study, 4% of the isolates overall were of B2 origin. However, the results from a report by Zhang et al. do not agree with our data (22). B2 strains accounted for 48% (42 of 88) of commensal rectal strains from healthy college-aged women in Michigan. Likewise, Sannes et al. noted a high prevalence of group B2 among rectal isolates from hospitalized, elderly, male veterans in Minnesota (16). Differences may be due to geographic variation, differences in host population characteristics, or differences in strain characteristics such as antimicrobial resistance.

We did not observe a significant shift toward low-virulence phylogenetic groups for resistant isolates, as was reported by Johnson et al. (9). However, we confirmed that the isolates were notably depleted for phylogenetic group B2 and enriched for group D. We also confirmed that FQ-resistant *E. coli* exhibited sparser virulence profiles. The most prevalent VF was *iutA*, which was detected in 36% of the resistant isolates; however, this VF is less common in virulent group B2 strains (6). Accordingly, only 2% of the resistant isolates were found to be ExPEC. These four isolates also lacked the VFs *iha*, *sat*, *fyuA,* and *malX* as compared to the FQ-susceptible ExPEC. Whether ExPEC strains cause infection in humans depends on several other factors, including susceptibility of the host. Therefore, that many (36%) of the 44 ExPEC isolates were from group 2 patients who had been hospitalized for five or more days is of concern. When patients become colonized with FQ-resistant ExPEC strains in the hospital, they presumably will have an increased risk for acquiring a nosocomial infection and, when discharged with such a strain, also for community-acquired infection; in such case, an optimal therapy will be more difficult to select. Of note, a relationship has recently been shown to exist between ciprofloxacin-resistance in *E. coli* and the production of extended-spectrum β-lactamases, which would further limit therapeutic options (17).

Concluding remarks

Our observations provide insight into the epidemiology and virulence characteristics of FQ-resistant *E. coli* from stools of patients and healthy persons in Indonesia. The high prevalence of FQ-resistant *E. coli* in the hospital environment seems to be primarily due to a combination of limited clonal spread and the spontaneous emergence of resistant strains, possibly fostered by selection pressure. Transferable resistance, however, cannot be ruled out as an additional explanation in the present study, and will be the subject of future investigations. Although the resistant isolates mainly belong to phylogenetic groups A and B1 and show a low virulence profile, similar strains have caused disease in humans (9, 14). The data support the need to implement strict infection control measures in hospitals and to promote and monitor

the prudent use of antimicrobial drugs. Continued surveillance of the changes of resistance patterns and virulence profiles of clinical and nonclinical *E. coli* isolates is warranted.

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Chapter 11

Quinolone resistance mechanisms in commensal *Escherichia coli* isolated in a population-based survey in Indonesia

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ABSTRACT

Objectives. To investigate quinolone resistance mechanisms in commensal *Escherichia coli* isolated in a population-based survey in Indonesia.

Methods. Seventy-eight *E. coli* with ciprofloxacin MICs ranging from <0.00375 to >128 mg/L were analyzed for mutations in the quinolone resistance determining regions (QRDRs) of DNA gyrase genes (*gyrA* and *gyrB*) by PCR and sequencing, for the presence of efflux pump mechanisms by an efflux pump inhibitor microdilution assay, and for the presence of the *qnrA*, *qnrB*, *qnrS*, and *aac(6')-Ib*-cr genes by PCR.

Results. Resistance to ciprofloxacin was most frequently caused by mutations in the QRDR of *gyrA*. Two amino acid changes were present in 96.8% of strains with ciprofloxacin MIC ≥2 mg/L, and in none of ciprofloxacin-susceptible strains. Efflux pump activity was found in 3.8% of isolates. Four isolates (5.1%) carried the *qnrA* gene, *qnrB* and *qnrS* were absent, and *aac(6')-Ib*-cr was detected in 13 strains (16.7%).

Conclusion. All four quinolone resistance mechanisms - target modification, decreased intracellular drug accumulation, target protection, and enzymatic drug modification - were present in different strains of commensal *E. coli* from Indonesia.

INTRODUCTION

The fluoroquinolones (FQs) are potent antimicrobial agents effective in the treatment of infections caused by a variety of pathogens, including *Escherichia coli*. Their antibacterial activity is due to inhibition of DNA gyrase and topoisomerase IV leading to inhibition of DNA replication. For many Gram-negative bacteria DNA gyrase is the primary target, whereas topoisomerase IV is the primary target for many Gram-positive bacteria (1, 2). Resistance in *E. coli* is increasingly reported from many parts of the world. Four mechanisms have been described that can increase MICs in *E. coli*: target modification, decreased intracellular drug accumulation, target protection, and enzymatic drug modification (1). The most important of these is target modification by mutations in the quinolone resistance determining regions (QRDRs) of the DNA gyrase (*gyrA* and *gyrB*) and topoisomerase IV genes (*parC* and *parE*). In clinical *E. coli*, the most frequent mutations described are located in the *gyrA* gene at amino acid position S83 and D87. Decreased intracellular drug accumulation is due to overexpression of multidrug efflux pumps, which may be chromosomally or plasmid-encoded (6), and decreased expression of outer membrane proteins (1). Resistance due to target protection is conferred by the plasmid-mediated *qnr* genes (A, B, C, and S) (6). Qnr proteins are able to bind to DNA gyrase and topoisomerase IV, thereby protecting them from the inhibitory activity of quinolones. Another transferable quinolone resistance mechanism is the aminoglycoside-modifying enzyme AAC(6')-Ib-cr, which confers resistance to kanamycin and is able to modify ciprofloxacin and norfloxacin by N-acetylation of the piperazinyl amine (9). Acquisition of high-level FQ-resistance appears to be a process involving different, simultaneously expressed mechanisms.

Commensal *E. coli* are frequently used as an indicator of antibiotic resistance in populations. We previously reported that FQ-resistant *E. coli* was prevalent in the fecal flora of patients at hospital admission (6%) and of patients at discharge after a hospitalization of five or more days (22%), but not among healthy persons and persons visiting a public health center in Indonesia (2%) (5). The high prevalence in hospitalized patients seemed to be due to a combination of limited clonal spread and the spontaneous emergence of resistant strains, since molecular typing revealed extensive genetic diversity and the FQ-resistant isolates exhibited an elevated mutation rate compared to FQ-susceptible isolates from the same population (4). In the present study, we used a random set of these *E. coli* isolates with ciprofloxacin MICs ranging from <0.00375 to >128 mg/L to investigate the prevalence of the various resistance mechanisms in Indonesian *E. coli*.

MATERIALS AND METHODS

Strains

Seventy-eight strains, collected by the Antimicrobial Resistance in Indonesia, Prevalence and Prevention (AMRIN) study group in 2001/2002, were included. In the AMRIN study, 3,995 patients and healthy persons from Surabaya and Semarang were screened for carriage of resistant Gram-negative bacteria in their fecal flora (5). Previous use of antibiotics was recorded. A total of 5,535 *E. coli* from 3,284 persons were collected. Susceptibility testing of ciprofloxacin was performed by disk diffusion (5). For the present study, we randomly selected 39 *E. coli* from each city with a variety of ciprofloxacin zone diameters.

MICs

MICs of ciprofloxacin and nalidixic acid were determined by the broth microdilution method as described by the CLSI (7). Twofold serial dilutions of ciprofloxacin from 0.00375 to 8 mg/L for ciprofloxacin-susceptible and ciprofloxacin-intermediately resistant strains and from 0.06 to 128 mg/L for ciprofloxacin-resistant *E. coli* were used. For nalidixic acid, twofold serial dilutions ranged from 0.06 to 128 mg/L. *E. coli* ATCC 25922 was used for quality control. Antimicrobial susceptibility to additional antibiotics was determined using the Vitek® 2 system (bioMérieux, Marcy-l'Etoile, France).

Detection of efflux pump mechanisms

MIC determinations were performed in parallel in the presence of 20 mg/L of the efflux pump inhibitor phenyl-arginine-naphthylamide (PAβN) in the broth microdilution assay (10).

Amplification and sequencing of QRDRs of *gyrA* and *gyrB*

DNA was extracted with the MagNA Pure LC DNA Isolation Kit III (Bacteria, Fungi) using the MagNA Pure LC Instrument (Roche Diagnostics, Almere, The Netherlands) according to the manufacturer's instructions. The QRDRs of *gyrA* and *gyrB* were amplified by PCR using primers described by Komp Lindgren et al (3). Amplification conditions were: initial denaturation at 95°C for 10 min; 40 cycles of 94°C for 1 min, 42°C for 1 min and 72°C for 1 min; and a final elongation at 72°C for 10 min. Products resulting from amplifications were subjected to sequencing using a 3100 ABI Prism genetic analyzer (Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands). Analysis and comparisons of nucleotide and amino acid sequence data were carried out using MegAlign software (DNAStar Inc., Madison, USA) and programs available at the National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov).

Detection of plasmid-mediated resistance

The presence of the *qnrA*, *qnrB*, *qnrS*, and *aac(6')-Ib* genes was determined by PCR (9, 11). Each *aac(6')-Ib* PCR product was sequenced and aligned as described above to identify the cr variant.

Statistical analysis

Statistical analyses were performed with appropriate tests using the statistical software packages SPSS Version 15.0 (SPSS, Chicago, IL, USA). $P<0.05$ was considered significant.

RESULTS AND DISCUSSION

The 78 *E. coli* strains included in the analysis exhibited ciprofloxacin MICs ranging from <0.00375 to >128 mg/L and nalidixic acid MICs from 1 to >128 mg/L. Seventeen ciprofloxacin-resistant isolates were from Surabaya and 13 from Semarang. The distribution of the quinolone resistance mechanisms among the isolates with different combinations of MICs of ciprofloxacin and nalidixic acid are shown in Table 1. The number of isolates resistant to tetracycline and chloramphenicol is included in the table, since this may be an indication of the reduced expression of outer membrane proteins (1).

Thirty isolates with ciprofloxacin MIC ≥2 mg/L had two amino acid changes in *gyrA* (30/31, 96.8%), and one isolate had a single amino acid substitution. Three nalidixic acid-resistant, ciprofloxacin-susceptible isolates showed one amino acid mutation each. The most frequent mutations were Ser83Leu and Asp87Asn, which is in agreement with previous reports (1). In the *gyrB* QRDR, no mutations were detected, except for some silent mutations (data not shown).

None of the strains exhibited more than a two-step dilution reduction of MICs of ciprofloxacin upon exposure to the efflux pump inhibitor PAβN. A four-step dilution reduction of nalidixic acid MICs was observed in the presence of PAβN for three isolates, indicating the presence of efflux pump mechanisms. In the high-level-resistant isolates we could not detect any reduction in MIC, due to the concentration ranges tested in this study.

Four isolates (5.1%) carried the *qnrA* gene: 3/30 (10.0%) of FQ-resistant strains and 1/47 (2.1%) of FQ-susceptible strains. The FQ-susceptible strain also carried the *aac(6')-Ib*-cr gene and had a Ser83Ala mutation, and exhibited a ciprofloxacin MIC of 0.25 mg/L and a nalidixic acid MIC of 64 mg/L. Both the presence of *qnr* genes and *gyrA* mutations facilitate the selection of additional resistance mutations, because of an increase in the Mutant Prevention Concentration (MPC) (6, 8). All four *qnrA*-

Table 1. Distribution of quinolone resistance mechanisms among 78 commensal *E. coli* from Indonesia with different combinations of MICs of ciprofloxacin and nalidixic acid.

MIC (mg/L)		Amino acid mutations in QRDR of *gyrA*					*qnrA*	*aac(6')-Ib*-cr	Efflux pump[b]	No. of isolates	No. of isolates resistant to	
CIP[a]	NAL[a]	Ser-83Leu	Ser-83Ala	Asp-87Asn	Asp-87Tyr	Asp-87Ala					TET[a]	CHL[a]
>128	>128	+		+				+		7	7	7
>128	>128	+		+						2	1	2
>128	>128	+			+		+			1	1	1
>128	>128	+						+		1	1	1
128	>128	+		+						5	4	3
128	>128	+		+				+		3	3	2
64	>128	+		+						4	4	2
64	>128	+		+				+		1		1
32	>128	+		+			+			1		
32	>128	+			+					1	1	1
16	>128	+		+						2	2	1
16	>128	+		+			+			1		
8	>128	+		+						1	1	
2	>128	+				+				1		
0.5	>128	+								1		1
0.25	128	+								1	1	1
0.25	64		+				+	+		1	1	1
0.03	4									2		1
0.03	1									1	1	
0.015	16								+	1		
0.015	8								+	1	1	
0.015	8									1		
0.015	4									9	5	1
0.015	4								+	1	1	1
0.015	2									9	7	3
0.015	1									1		
0.0075	4									3	2	1
0.0075	2									11	6	1
0.0075	1									2	1	
<0.00375	1									2	2	

[a] CIP, ciprofloxacin; NAL, nalidixic acid; TET, tetracycline; CHL, chloramphenicol.

[b] The presence of an efflux pump mechanism was considered when a fourfold reduction in MIC was observed in the presence of PaβN.

positive strains in our study had one or more amino acid substitutions in *gyrA* as well. None of the isolates harbored *qnrB* or *qnrS*.

aac(6')-Ib-cr was detected in 13 strains: 12/30 (40.0%) of FQ-resistant strains and 1/47 (2.1%) of FQ-susceptible strains. The presence of *aac(6')-Ib*-cr by itself may

not result in large increases in MIC values for quinolones, but it is known to favor the selection of chromosomal mutants in topoisomerase QRDR sequences leading to a higher resistance level (6). Nevertheless, among our ciprofloxacin-resistant isolates with the Ser83Leu and Asp87Asn mutation, ciprofloxacin MICs for isolates with *aac(6')-Ib*-cr were significantly higher than those for strains without *aac(6')-Ib*-cr (P=0.012, Mann-Whitney test). This suggests that *aac(6')-Ib*-cr has an additional effect on the ciprofloxacin MIC.

The 78 *E. coli* in our study had been cultured from 19 patients on admission, 43 patients on discharge, and 16 persons in the community. Forty-two study subjects had taken an antibiotic in the month preceding the culture, and 15 of these had used ciprofloxacin. All of these 15 patients were cultured on discharge after being hospitalized for at least 5 days and carried an *E. coli* with a ciprofloxacin MIC ≥16 mg/L. Within the total group of discharged patients from whom *E. coli* were included in this analysis, carriage of a ciprofloxacin-resistant *E. coli* was associated with previous use of ciprofloxacin (P<0.001, Fisher's exact test).

Most studies that investigated the different mechanisms of quinolone resistance in *E. coli* have focused on bacteria with particular phenotypes of resistance, such as an extended-spectrum beta-lactamase phenotype or quinolone MICs ≥2 mg/L (6, 8). In the present study, we analyzed commensal *E. coli* isolates from Indonesia with a wide range of quinolone MICs, without a predefined resistance background. Target modification, target protection, and enzymatic drug modification were all present in both ciprofloxacin-resistant and -susceptible isolates. Indications of an efflux pump mechanism were only found in ciprofloxacin-susceptible isolates.

For future research, it would be interesting to culture *E. coli* from the fecal flora of patients at admission, and subsequently monitor their intestinal *E. coli* during therapy with quinolones in order to study the emergence of the different resistance mechanisms within a patient with respect to the doses and dosing frequencies of certain quinolones.

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Chapter 12

Fecal carriage of extended-spectrum β-lactamase-producing Enterobacteriaceae among patients and healthy persons from Java, Indonesia

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ABSTRACT

Objective: To characterize commensal *Escherichia coli* and other Enterobacteriaceae with reduced susceptibility to cefotaxime that were collected in a large survey carried out among 3,995 patients and healthy persons in two urban regions on the island of Java, Indonesia, in 2001-2002.

Setting: Two academic hospitals and three public health centers in Semarang and Surabaya, Indonesia.

Methods: The putative ESBL-producing Enterobacteriaceae were analyzed using the double-disk synergy test, isoelectric focusing, PCR assays, DNA sequencing and pulsed-field gel electrophoresis (PFGE).

Results: On the day of discharge after five or more days of hospitalization, at least 95 of 999 (9.5%) patients carried ESBL-positive Enterobacteriaceae as dominant fecal flora. Six patients were simultaneously colonized with *E. coli* and *K. pneumoniae* isolates with ESBL activity. On admission, only 6 of 998 (0.6%) patients were colonized. Fecal carriage of ESBL-producing Enterobacteriaceae among healthy persons or persons visiting a public health center was not detected. The 107 ESBL-positive strains included 68 *E. coli*, 35 *K. pneumoniae*, 3 *Enterobacter cloacae*, and 1 *Citrobacter freundii*. CTX-M-15 was the most prevalent ESBL in both *E. coli* (47.1%) and *K. pneumoniae* (45.7%). Other ESBL types found were: SHV-2, -2a, -5, -12, CTX-M-3, -9, -14, and TEM-19. PFGE revealed extensive genetic diversity among the isolates, but some clonality was observed among CTX-M-15-positive *K. pneumoniae* from Semarang.

Conclusion: In 2001-2002, fecal carriage of ESBL-producing Enterobacteriaceae as dominant flora in Indonesia was almost exclusively hospital-associated. We assume that this was the result of nosocomial exposure to extended-spectrum cephalosporins and subsequent selection of these strains.

INTRODUCTION

In the past two decades, extended-spectrum β-lactamase (ESBL)-producing Enterobacteriaceae have increasingly been reported worldwide, causing outbreaks as well as sporadic infections (1, 30). Until the late 1990s, these ESBL-producing bacteria were mainly isolated from hospitalized patients and were almost exclusively of the TEM and SHV enzyme variants. Recent studies have revealed a worrisome increase in the number of community-acquired isolates, especially from patients suffering from urinary tract infections, along with the emergence of the CTX-M type enzymes in both the hospital and community (4, 5).

The human intestinal tract has shown to be an important reservoir for ESBL-producing bacteria and colonized persons are at risk for subsequent infection (3, 25, 31). Among colonized patients recently discharged from the hospital, ESBL-producing *Escherichia coli* and *Klebsiella pneumoniae* often persist in stool cultures for ≥3 months (2). Such carriers may subsequently transmit their resistant pathogen to their household contacts (36). The influx of ESBLs from the community into the hospital has already been described in a recent study from Israel (3). In the hospital, as a consequence of patient-to-patient transmission of these bacteria, other patients become carriers as well (3, 14, 20). For infection control teams it is necessary to know the epidemiology of ESBLs among patients on admission, during hospitalization and at discharge, in order to be able to promote effective control practices.

Hospitals in Indonesia are also affected by the spread of ESBL-genes. In a survey at the Dr. Soetomo Hospital in Surabaya, Indonesia, in 2005, the estimated prevalence of ESBLs among clinical *E. coli* and *K. pneumoniae* was 20.1% for *E. coli* and 27.9% for *K. pneumoniae*. $bla_{\text{CTX-M-15}}$ was present in clonal and non-clonal clinical *E. coli* strains, while different SHV- and CTX-M-type ESBLs co-resided in *K. pneumoniae* strains (J. A. Severin et al., submitted for publication). Only few data are available on fecal carriage of ESBL-producing bacteria in Indonesia. In 2001-2002, the 'Antimicrobial Resistance in Indonesia: Prevalence and Prevention' (AMRIN) study group investigated rectal carriage of resistant bacteria among 3,995 people in two cities on the island of Java (Surabaya and Semarang) (21). *E. coli* strains resistant to third-generation cephalosporins were found to be highly prevalent in the fecal flora of patients at discharge after a hospital stay of ≥5 days (12.5%), but not among healthy relatives or patients visiting primary health centers (0.7% and 0.9%, respectively) (21). However, in that particular study the presence, type, and expression of ESBLs was not studied. The present study was conducted in order to further characterize these commensal cefotaxime-resistant *E. coli*. Other Enterobacteriaceae with reduced susceptibility to cefotaxime that were collected during the AMRIN study were included as well. Isolates were studied phenotypically by the double-disk synergy test for the

presence and expression of ESBL-genes. ESBL-positive isolates were characterized by isoelectric focusing (IEF), PCR and sequencing. To define clonal relatedness among ESBL-producing strains pulsed-field gel electrophoresis was performed.

MATERIALS AND METHODS

Study setting

The AMRIN study was carried out in two governmental teaching hospitals (Dr. Soetomo Hospital in Surabaya, Dr. Kariadi Hospital in Semarang) and three primary health centers (two in Surabaya and one in Semarang). A total of 3,995 individuals were studied for intestinal carriage of resistant microorganisms using rectal swab cultures. These individuals were patients on the day of admission to the hospital (admission group, n=998), patients on the day of discharge after at least five days of hospitalization (discharge group, n=999), patients visiting a primary health center (PHC group, n=1,000), and healthy relatives or household members of admission group patients (relatives group, n=998). Individuals could be included only once in the study. Admission group patients were included in the study within 24 hours after admission. The following departments were involved: Internal Medicine, Surgery, Gynecology and Obstetrics, and Pediatrics (admission and discharge group). Individuals were excluded from the study if they were transferred from another hospital, if they were not accompanied by a family member (admission group), or if they had been admitted to a hospital within the previous three months (admission, PHC, and relatives group). The specimens were collected from July to October 2001 in Surabaya and from January to May 2002 in Semarang. More details about the study have been published elsewhere (10, 21).

Bacterial isolates, antimicrobial susceptibility testing, and detection of ESBL

Rectal swabs were cultured on CHROMagar Orientation (Becton Dickinson, Heidelberg, Germany) for *E. coli* (pink colonies) and other Enterobacteriaceae (blue colonies) (11). From each swab, two colonies representing the dominant growth in the fecal flora were collected. Susceptibility to cefotaxime was determined using disk diffusion according to the guidelines by the Clinical and Laboratory Standard Institution (CLSI, formerly NCCLS) (27). All non-susceptible (i.e. resistant and intermediately susceptible) isolates were further analyzed. Phenotypic confirmation of ESBL-production was performed by the double-disk synergy test (DDST) using four indicator antibiotics (cefotaxime, ceftazidime, cefepime, and aztreonam) which were placed 20 mm (edge to edge) away from an amoxicillin/clavulanic disk (Oxoid, Basingstoke, UK) (9, 16). Additional antibiotic susceptibility testing was carried out by the Vitek® 2 system using

AST-N041 cards (bioMérieux, Marcy-l'Etoile, France). These cards contain, among others, the following antibiotics: ampicillin, amoxicillin-clavulanic acid, cefazolin, cefepime, cefotaxime, cefoxitin, ceftazidime, piperacillin, piperacillin-tazobactam, imipenem, gentamicin, amikacin, ciprofloxacin, trimethoprim-sulfamethoxazole, nitrofurantoin, and tetracycline. Breakpoints as defined by CLSI were applied (8). AST-N041 also contains a specific test for ESBL-detection. ESBL-positive isolates, either confirmed by DDST or as indicated by the Vitek® 2 system, were identified using the Vitek® 2 system and further genetically characterized. *E. coli* strain ATCC 25922 was used for quality control purposes.

Isoelectric focusing

IEF was used to visualize the number and nature of the β-lactamases expressed in these bacteria. IEF was performed as described previously with minor modifications (24). Briefly, a suspension of the tested strain was incubated overnight at 37°C in brain heart infusion broth containing 100 μg/mL of ampicillin. β-lactamases were subsequently extracted with lysozyme and subjected to IEF (PhastGel 3-9; Pharmacia AB, Uppsala, Sweden). Gels were run on a PhastSystem apparatus (Pharmacia). Presence of β-lactamase activity was detected by staining with nitrocefin (0.5 mg/ml). Control strains containing a TEM-1 (isoelectric point [pI] 5.4) or SHV-1 (pI 7.6) β-lactamase were used for pI comparisons.

Molecular characterization of β-lactamases

DNA was isolated by suspending bacteria in Aqua dest and subsequently boiling the suspension for 10 min. The debris was spun down at 10,000 x *g* for 5 min. PCR assays to determine the presence of bla_{TEM}, bla_{SHV}, and $bla_{OXA\text{-}1\text{-}like}$ genes were carried out using primers and conditions described previously (19, 22, 28). A multiplex PCR assay was used for the detection of genes encoding CTX-M-type β-lactamases (39). Isolates that tested positive for the CTX-M-1 group or CTX-M-9 group β-lactamase underwent additional amplification with a separate set of primers. For the CTX-M-1 group the primer set was CTX-M-1m fw (5'- AAA AAT CAG TGC GCC AGT TC -3') and CTX-M-1 multi-r (5'- TTA CAA ACC GTC GGT GAC GA -3') with the following amplification conditions: initial denaturation at 94°C for 5 min; 35 cycles of 94°C for 2 min, 60°C for 1 min and 72°C for 2 min; and a final elongation at 72°C for 10 min (38). For the CTX-M-9 group we used primers CTX-M-9m fw (5'- CAA AGA GAG TGC AAC GGA TG -3') and CTX-M-9 multi-r (5'- CCT TCG GCG ATG ATT CTC -3') with the following amplification conditions: initial denaturation at 94°C for 5 min; 35 cycles of 94°C for 30 s, 54°C for 90 s and 72°C for 1 min; and a final elongation at 72°C for 5 min. All PCRs were conducted with a GeneAmp® PCR System 9700 (Perkin-Elmer Applied Biosystems, Foster City, CA, USA) or a T3000 Thermocycler (Biometra, Goettingen,

Germany). Products resulting from amplifications were subjected to sequencing using a 3100 ABI Prism genetic analyzer (Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands), with an additional internal primer for the CTX-M-1 group (5'- AAC GTG GCG ATG AAT AAG CT-3') and CTX-M-9 group (5'- GGT GAT GAA CGC TTT CCA A -3'). The nucleotide and deduced amino acid sequences were analyzed using MegAlign software (DNAStar Inc., Madison, USA) and programs available at the National Centre for Biotechnology Information website (http://www.ncbi.nlm.nih.gov).

Discrepancy analysis

Isolates that were phenotypically confirmed as ESBL-producers by DDST, but which were negative for bla_{TEM}, bla_{SHV}, and bla_{CTX-M} ESBL-genes, were further analyzed by detailed review of the Vitek® 2 report and were subjected to additional ESBL E-tests containing ceftazidime, cefotaxime, and cefepime, all with and without clavulanate (AB Biodisk, Solna, Sweden). The E-test method was carried out according to the manufacturer's instructions.

Pulsed-field gel electrophoresis

All ESBL-positive *E. coli* and *K. pneumoniae* isolates were characterized by macrorestriction analysis of genomic DNA with *Xba*I. DNA fragments were analyzed by pulsed-field gel electrophoresis (PFGE) in a CHEF-DR III system (Bio-Rad, Hercules, CA, USA) as described previously (37). Relatedness among the PFGE profiles was evaluated with Bionumerics software (version 3.0; Applied Maths, Ghent, Belgium). Isolates with PFGE profiles showing ≥85% similarity were considered to be clonally related.

Phylogenetic grouping

A selection of *E. coli* isolates was assigned to one of the four main *E. coli* phylogenetic groups (A, B1, B2, and D) according to an established triplex PCR assay (7).

Statistical analysis

Data were analyzed using statistical software packages SPSS Version 15.0 (SPSS, Chicago, IL, USA) and EpiInfo version 5.00 (Centers for Disease Control and Prevention, Atlanta, GA, USA). Chi-square or Fisher's exact tests (two-tailed) were used when appropriate for comparisons of proportions. $P<0.05$ was considered significant.

RESULTS

Bacterial isolates

A total of 3,995 individuals were screened for fecal carriage of resistant Enterobacteriaceae. This resulted in 5,535 *E. coli* isolates from 3,284 individuals and 1,637 other Enterobacteriaceae from 1,422 subjects. Susceptibility to cefotaxime by disk diffusion was determined for one *E. coli* per person and 482 other Enterobacteriaceae (most dominant growth). In total, 264 *E. coli* and 83 other Enterobacteriaceae were non-susceptible to cefotaxime. ESBL-production was phenotypically confirmed in 68 *E. coli* and 39 other Enterobacteriaceae. Phenotypic confirmatory testing was negative for 130 *E. coli* and 33 other Enterobacteriaceae. The remaining isolates were lost during storage (n=71, mainly from the discharge group) or did not belong to the Enterobacteriaceae (n=6).

ESBL-producing *E. coli*

Molecular analysis revealed the presence of a variety of SHV- and CTX-M-type enzymes among the ESBL-positive *E. coli* strains (Table 1). TEM-type ESBLs were not found. Sixty-five *E. coli* isolates carried a single bla_{ESBL} and one isolate carried multiple bla_{ESBL} genes (Table 1). Two additional isolates showed a typical ESBL phenotype in the discrepancy analysis and were thus considered "truly" ESBL-positive, but did not contain a TEM-, SHV-, or CTX-M-type ESBL-enzyme by neither PCR nor IEF. The majority of these 68 ESBL-positive *E. coli* were isolated from patients at discharge

Table 1. Occurrence of *bla* types among 68 ESBL-producing *E. coli* collected as dominant fecal flora from Indonesian people.

Type of ESBLs	No. of isolates (%)	No. of isolates with additional $bla_{non\text{-}ESBL}$		City[a]		Study group		Department[b]			
		TEM-1	OXA-1	Sem	Sby	admission	discharge	Int	Surg	Gyn	Ped
CTX-M-15	32 (47.1)	19	22	25	7	1	31	9	16	3	4
SHV-5	13 (19.1)	8	4	9	4	1	12	1	2	1	9
CTX-M-14	7 (10.3)	6	4	1	6	1	6	3	3	1	
CTX-M-9	5 (7.4)				5		5	1	2	2	
SHV-2	4 (5.9)	2		1	3	1	3		1	1	2
SHV-2a	3 (4.4)	1	1	2	1	1	2	1		1	1
SHV-12	1 (1.5)	1		1			1				1
CTX-M-9 + SHV-2	1 (1.5)				1		1		1		
Uncharacterized	2 (2.9)	2			2		2		1		1
Total	68 (100)	39	31	39	29	5	63	15	26	9	18

[a] Sem, Semarang; Sby, Surabaya.

[b] Int, Internal Medicine; Surg, Surgery; Gyn, Gynecology/Obstetrics; Ped, Pediatrics.

after a hospital stay of at least 5 days (63/68 strains; 92.6%). Five ESBL-positive strains were isolated from patients at hospital admission. No confirmed ESBL-positive *E. coli* were cultured as dominant fecal flora from individuals in the PHC or relatives group.

The bla_{CTX-M}-type ESBL enzymes were the most prevalent (66.2%). These were CTX-M-15 (n=32), CTX-M-14 (n=7), and CTX-M-9 (n=6). Fourteen CTX-M-15-positive *E. coli* were isolated from patients on the day of discharge from the department of Surgery in Semarang. A bla_{SHV}-type ESBL gene was detected in 22 *E. coli* strains (32.4%). Of these, SHV-5 was the most frequently found enzyme (13 strains). The non-ESBL bla_{TEM-1} and bla_{OXA-1} genes were demonstrated in 57.4% and 45.6%, respectively, of *E. coli* isolates.

The phylogenetic group was determined for 26 ESBL-positive *E. coli*. They belonged to phylogenetic group A (50.0%), B1 (26.9%), and D (23.1%).

PFGE identified 51 different types, of which 42 were represented by a single isolate and 9 included more than one isolate. Among the 14 CTX-M-15-positive isolates from patients on the day of discharge from the department of Surgery in Semarang 12 PFGE types were found. PFGE analysis of 5 isolates revealed band-smearing patterns.

The frequencies of resistance among the ESBL-positive *E. coli* isolates were: tetracycline, 85.3%; gentamicin, 73.5%; trimethoprim-sulfamethoxazole, 63.2%; ciprofloxacin, 45.6%; nitrofurantoin, 16.2%; and amikacin, 11.8%. The highest co-resistance rates were observed in CTX-M-15-positive isolates for tetracycline (100%), gentamicin (93.8%), and ciprofloxacin (65.6%). All isolates were susceptible to imipenem.

ESBL-producing other Enterobacteriaceae

The 39 ESBL-producing other Enterobacteriaceae included 35 *K. pneumoniae*, 3 *Enterobacter cloacae*, and 1 *Citrobacter freundii*. Most of these were from Semarang (76.9%). All except one of the 39 isolates had been isolated from patients on the day of their discharge from the hospital. Among *K. pneumoniae* isolates, the bla_{SHV}-type was the most prevalent ESBL (54.3%): SHV-2a (n=6), SHV-12 (n=5), SHV-5 (n=5), and SHV-2 (n=3) (Table 2). bla_{CTX-M} type enzymes were detected in 18 *K. pneumoniae* isolates (51.4%), and these were predominantly CTX-M-15 (n=16). All CTX-M-positive *K. pneumoniae* originated from Semarang. ESBL-positive *K. pneumoniae* isolates from Surabaya contained exclusively SHV-type ESBL genes. One strain from Semarang produced TEM-19. Another strain from Semarang exhibited a classical ESBL phenotype, but we were unable to identify the responsible ESBL gene. The non-ESBL bla_{TEM-1}, bla_{SHV-1} and bla_{OXA-1} genes were detected in 22.9%, 20.0%, and 42.1%, respectively, of *K. pneumoniae* isolates. Two *E. cloacae* isolates carried $bla_{CTX-M-9}$ and one carried $bla_{CTX-M-15}$ in combination with bla_{OXA-1}. The *C. freundii* isolate produced SHV-12 in combination with TEM-1.

Table 2. Occurrence of *bla* types among 39 ESBL-producing non-*E. coli* Enterobacteriaceae collected as dominant fecal flora from Indonesian people.

Type of ESBLs	No. of isolates (%)	No. of isolates with additional $bla_{non\text{-}ESBL}$			City[a]		Study group		Department[b]			
		TEM-1	SHV-1	OXA-1	Sem	Sby	admis-sion	dis-charge	Int	Surg	Gyn	Ped
CTX-M-15[c]	14 (35.9)	1	7	13	14			14	5	5	1	3
SHV-2a	5 (12.8)	1			3	2		5	3	1	1	
SHV-5	5 (12.8)				3	2	1	4	3	2		
SHV-2	3 (7.7)	2			1	2		3		2		1
SHV-12[d]	3 (7.7)	2			1	2		3		1		2
CTX-M-9[e]	2 (5.1)				2			2		1	1	
CTX-M-15 + SHV-12	2 (5.1)	1		2	2			2		1		1
CTX-M-15 + SHV-2a	1 (2.6)			1	1			1		1		
CTX-M-9 + SHV-12	1 (2.6)	1			1			1	1			
CTX-M-3	1 (2.6)				1			1		1		
TEM-19	1 (2.6)				1			1				1
Uncharacterized	1 (2.6)	1				1		1		1		
Total	39 (100)	9	7	16	30	9	1	38	12	16	3	8

[a] Sem, Semarang; Sby, Surabaya.

[b] Int, Internal Medicine; Surg, Surgery; Gyn, Gynecology/Obstetrics; Ped, Pediatrics.

[c] These included 13 *K. pneumoniae* and 1 *E. cloacae*.

[d] Two *K. pneumoniae* and 1 *C. freundii*.

[e] Both strains were *E. cloacae*.

PFGE analysis revealed extensive genetic heterogeneity among the isolates. Among *K. pneumoniae* 27 different profiles were observed, of which 23 were unique, one PFGE type was represented by four isolates, one by three isolates, and two by two isolates each. The 11 clonal isolates originated from discharge group patients from Semarang and were all CTX-M-15-positive except for one. One *K. pneumoniae* was untypeable. The *E. cloacae* isolates revealed three unique banding patterns.

Most of the 39 ESBL-positive isolates showed decreased susceptibility to non-related classes of antibiotics such as nitrofurantoin (87.2%), gentamicin (74.4%), trimethoprim-sulfamethoxazole (71.8%), tetracycline (66.7%), ciprofloxacin (38.5%), and amikacin (7.7%). Resistance to imipenem was not observed.

Six patients from the discharge group were simultaneously colonized with *E. coli* and *K. pneumoniae* strains with ESBL activity. The combinations are shown in Table 3.

Overall epidemiology

Fecal carriage of ESBL-producing Enterobacteriaceae was most prevalent among patients on the day of discharge after ≥5 days of hospitalization. At least 95 of the 999 patients cultured at discharge (9.5%) carried one or more ESBL-positive microorganisms as dominant fecal flora at that specific moment compared to 0.6% of patients

Table 3. Combinations of *E. coli* and *K. pneumoniae* with ESBL activity simultaneously found in one patient each.

Patient number	bla_{ESBL} in *E. coli*	bla_{ESBL} in *K. pneumoniae*
61049	CTX-M-15	CTX-M-15
62104	CTX-M-15	CTX-M-3
22033	CTX-M-9 + SHV-2	SHV-2
64086	SHV-12	CTX-M-15 + SHV-12
61079	CTX-M-15	CTX-M-9 + SHV-12
22076	uncharacterized	uncharacterized

on admission (P<0.0001). Of the 101 ESBL-positive microorganisms found in the discharge group, the majority (n=42; 41.6%) had been isolated from patients that were discharged from the department of Surgery (28 from Semarang and 14 from Surabaya).

DISCUSSION

Fecal carriage of ESBLs among patients and healthy individuals has been studied in several settings. Most studies have been carried out during nosocomial outbreaks or in highly specialized departments, such as the neonatal intensive care unit. In Spain, intestinal colonization of ESBL-producing isolates was studied in 1991 and 2003 during nonoutbreak situations (35). The prevalence among hospitalized patients and outpatients increased dramatically from 0.3% and 0.7%, respectively, in 1991, to 11.8% and 5.5%, respectively, in 2003. In the present Indonesian study, we have shown that fecal carriage of ESBL-producing Enterobacteriaceae was most prevalent among patients on the day of discharge after ≥5 days of hospitalization (9.5%). This prevalence is comparable to the prevalence found among hospitalized patients in 2003 in Spain. In Indonesia, ESBLs were only occasionally found in isolates from patients screened on the day of admission (0.6%), which is again similar to the data from Spain. These results are, however, in contrast to the data of Ben-Ami et al. who demonstrated a fecal carriage rate of ESBL-producing isolates on admission of 10.8%, with CTX-M-2, CTX-M-16-like, and SHV-12 as the ESBL enzymes detected in true community-acquired strains (3). The drawback of their study is that a majority of the patients had been hospitalized recently and strains may thus not be "truly" community-acquired, given the observation that ESBL-producing *E. coli* and *K. pneumoniae* often persist in stool cultures for ≥3 months (2). In our study, admission group patients were not included if they had been hospitalized in the preceding 3 months. This probably explains the lower prevalence of ESBL carriage in patients on admission in our study, in addition to differences in antibiotic use. Among healthy persons in Indonesia we did not find

any ESBL-producing Enterobacteriaceae. In studies performed in Spain, Bolivia, Peru, India, China, and Saudi Arabia, ESBL carriage rates from 0.1% in Bolivia and Peru in 2002 to 13.1% in Saudi Arabia were documented for healthy individuals (15, 17, 29, 32, 34, 35). Our data show that in 2001-2002 fecal carriage of ESBLs in Indonesia was almost exclusively hospital-associated, and influx from the community into the hospital was negligible. Most of the 101 ESBL-positive microorganisms had been isolated from patients that were discharged from the departments of Surgery of both hospitals. Antibiotic use, and especially the use of third-generation cephalosporins, has been described as a risk factor for the acquisition of ESBL-producing bacteria (30). In the two hospitals where our study was carried out, the third-generation cephalosporins were the second most prescribed antibiotics in the hospitals, and most of these were administered in the departments of Surgery (12). Due to lack of statistical power no significant correlation between the use of cephalosporins and carriage of a cefotaxime-resistant *E. coli* could be established, but it is reasonable to assume that ESBL-producing microorganisms as dominant intestinal flora in the discharged patients reflects exposure to extended-spectrum cephalosporins and subsequent selection of these strains (10). In addition, none of the individuals from the admission, PHC, and relatives group had been treated with (third-generation) cephalosporins (13).

The key question is whether the ESBL-producing Enterobacteriaceae that appear as dominant intestinal flora during hospitalization are nosocomially acquired or not. Strong evidence for nosocomial acquisition would be provided when isolates are genetically indistinguishable by a typing method. The isolates in the present study were genetically highly diverse, indicating unrelatedness, and, thus, another mode of acquisition must be considered. One of the possibilities is dissemination of certain mobile genetic elements in the hospital, which could even take place between different species of Enterobacteriaceae (23). We found six patients to be simultaneously colonized with *E. coli* and *K. pneumoniae* strains with ESBL activity and in three of these identical ESBL genes were found, which could suggest transfer of genetic elements within a patient. However, plasmid analysis was not performed. In case of SHV and TEM, *de novo* mutation could be an alternative mode of acquisition. Another possibility could be that susceptible microorganisms in the intestines were killed upon exposure to third-generation cephalosporins which was followed by selection and subsequent enrichment of resistant Enterobacteriaceae. Previous studies demonstrated the dramatic effect of parenteral extended-spectrum cephalosporins on the human intestinal flora. Enterobacteriaceae were eliminated or strongly suppressed (33). In pigs, it has been shown that a 3-days treatment with third- or fourth-generation cephalosporins resulted in selection of CTX-M-producing *E. coli* in their intestinal flora (6). If this scenario is indeed applicable in the Indonesian setting, than

this would imply that ESBL-positive isolates were already present in low numbers in the intestinal flora of patients when admitted to the hospital. We demonstrated in our study that the rectal cultures from patients on admission only rarely revealed ESBL-producing Enterobacteriaceae, but this omission could be due to the applied culture method, i.e. rectal swabs were directly plated on the CHROMagar Orientation and only the dominant flora was stored and analyzed. A culture method using a selective enrichment broth would probably have a higher sensitivity for detecting ESBL-positive Enterobacteriaceae in rectal cultures when only a small subpopulation of the intestinal flora is ESBL-positive (26).

It is of utmost importance to elucidate the exact mechanisms of emergence and spread of ESBLs in the hospital, since this determines the focus of action to control these resistant bacteria. If indeed ESBL-positive Enterobacteriaceae are already present in low numbers in the intestines of healthy individuals, than a "search and destroy" policy would imply that all patients should be screened on admission and, when found to be colonized, isolated to prevent transmission of the specific clone to other patients. At the moment of an outbreak, surveillance cultures would identify (newly) colonized patients, but the source would be difficult to find. In case of transmission of plasmids or other mobile genetic elements, an outbreak would be even harder to recognize and control.

Characterization of the ESBL-genes revealed that CTX-M-15 was the most frequently found ESBL in both *E. coli* and *K. pneumoniae* isolates, which was associated with bla_{OXA-1} and, variably, bla_{TEM-1}. This combination of β-lactamases has also been described for plasmids of *E. coli* strains from India and the United Kingdom (19). In the UK, these *E. coli* are epidemic and belong to the virulent phylogenetic group B2. Fecal carriage of ESBL-producing organisms confers an increased risk for subsequent invasive infection with the same organism. However, the virulence of *E. coli* is dependent on the phylogenetic group and numerous virulence factors (3, 18). The ESBL-positive *E. coli* from our discharged patients belonged to the less virulent phylogenetic groups A, B1, and D. Nevertheless, among clinical *E. coli* isolates collected in the Dr. Soetomo Hospital in Surabaya in 2005, CTX-M-15 was the most prevalent ESBL, but many of those isolates belonged to non-B2 groups (63.2%). Among clinical *K. pneumoniae* bla_{SHV}-type ESBLs were most prevalent. Indeed, commensal *K. pneumoniae* isolates from Surabaya contained exclusively SHV-type ESBL genes. Interestingly, CTX-M-14 and CTX-M-9 were found among the commensal isolates in the present study, but not among clinical isolates (J.A. Severin et al, submitted for publication).

Our study has some limitations. First, the estimates of the ESBL prevalence may be too low as the use of cefotaxime for screening might have biased the selection against ESBLs that preferably hydrolyse other oxyimino-cephalosporins. Also, only cefotaxime non-susceptible *E. coli* and *K. pneumoniae* (zone diameter ≤23 mm) were

subjected to confirmatory testing, instead of all isolates exhibiting zone diameters of ≤27 mm, which is now recommended by the CLSI (8). Second, our study was performed in a specific geographical location, and the results may not be taken to reflect the distribution of ESBLs throughout Indonesia. Third, these data were collected in 2001-2002, and colonization rates may have changed in the population since that time. However, the data provide insight in an early stage of the emergence of ESBLs in Indonesia, the fourth most populous country in the world.

In summary, we have shown that ESBL-producing Enterobacteriaceae were already present as intestinal flora of inhabitants of Indonesia in 2001-2002. They were found in up to 10% of patients at the time of discharge from hospital, but not among healthy individuals. Both prudent use of antibiotics, especially cephalosporins, and compliance with infection control measures are essential to reduce the selection and spread of ESBLs. Continued targeted surveillance of clinical and non-clinical Enterobacteriaceae is necessary to monitor the evolving epidemiology of ESBLs in this part of the world.

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Chapter 13

Molecular characterization of extended-spectrum β-lactamases in clinical *Escherichia coli* and *Klebsiella pneumoniae* isolates from Surabaya, Indonesia

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ABSTRACT

Background: Limited information regarding extended-spectrum β-lactamase (ESBL)-producing Enterobacteriaceae in Indonesia is available.

Methods: A survey was carried out to investigate the molecular epidemiology and genetic characteristics of clinical ESBL-producing *Escherichia coli* and *Klebsiella pneumoniae* isolates in the Dr. Soetomo Hospital in Surabaya, Indonesia, over a 4-month period (January to April 2005).

Results: We found 73 consecutive non-duplicate ESBL-positive *E. coli* and 72 *K. pneumoniae*. Three isolates of *E. coli* and 20 *K. pneumoniae* carried multiple ESBL genes. The prevalence of $bla_{\text{CTX-M-15}}$ was strikingly high in *E. coli* (94.5%). The gene was detected in both clonal and non-clonal isolates, as defined by pulsed-field gel electrophoresis (PFGE) and repetitive-sequence-based PCR (rep-PCR) using the DiversiLab™ system (bioMérieux). SHV-type ESBLs were detected in 7 *E. coli* strains. Among *K. pneumoniae* isolates, bla_{SHV}-type enzymes were the most prevalent β-lactamases (65.3%). These were SHV-5 (n=28), SHV-12 (n=13), and SHV-2 (n=6). CTX-M-type enzymes were detected in 58.3% of *K. pneumoniae* strains and were predominantly CTX-M-15. Of note, no TEM-type ESBLs were found.

Conclusion: We have shown that Indonesia is another country affected by the emergence and spread of bacterial isolates harbouring ESBL genes.

INTRODUCTION

The first clinical *Klebsiella* spp. isolates producing a plasmid-encoded β-lactamase capable of hydrolyzing the extended-spectrum cephalosporins were described in 1983, while the first clinical *Escherichia coli* isolate with an oximino-cephalosporin hydrolyzing β-lactamase was found in 1986 (20, 33). Since then, the number of variants of these extended-spectrum β-lactamase (ESBL) enzymes has increased rapidly, with worldwide dissemination (32). Presence of different ESBL-genes has been reported in many Gram-negative bacteria. However, ESBL-positive *Klebsiella* spp. and *E. coli* remain the species most frequently found in outbreaks, clinical reports, and surveillance culture data. Nosocomial infections with ESBL-producing Enterobacteriaceae have been associated with higher rates of mortality and morbidity, increased hospital costs, and longer hospital stays (37). In addition, antibiotic treatment options are increasingly scarce because ESBL-producing pathogens often possess additional genes encoding for resistance to other classes of antibiotics, including aminoglycosides and fluoroquinolones (2).

During the 1990s, TEM and SHV derivates were the most common ESBL types worldwide. In the present decade, this pattern has changed rapidly with the emergence of the CTX-M type enzymes. CTX-M type enzymes were first isolated in South America and Japan, but are now widespread in the European region as well (23). CTX-M positive isolates have also been described in some other Asian countries, but only limited data are available from the Southeast Asian region (14). Molecular characterization of clinical strains producing ESBLs has only been reported from Malaysia, Singapore, Thailand, and Vietnam (3, 4, 6, 11, 18, 19, 21, 29, 31, 38).

The information with regard to the level of resistance to broad-spectrum β-lactams in Indonesia is even more limited. To our knowledge, only one report suggested the presence of ESBL-enzymes in *E. coli* and *Klebsiella* in clinical isolates from Indonesia (22). In this survey from the late 1990s, 23.3% of *E. coli* and 33.3% of *Klebsiella* spp. had an ESBL-phenotype. However, neither confirmatory testing nor molecular characterization was performed.

The present study was conducted in order to investigate the prevalence of ESBL-positive isolates among clinical *E. coli* and *K. pneumoniae* in the 1,432-beds Dr. Soetomo Hospital in Surabaya, Indonesia. From January to April 2005 isolates were collected and studied phenotypically by disk diffusion for the presence and expression of ESBL-genes. ESBL-positive isolates were characterized by isoelectric focusing (IEF), PCR and sequencing. In addition, these isolates were characterized by pulsed-field gel electrophoresis (PFGE) and repetitive-sequence-based PCR (rep-PCR) in order to define clonal relatedness.

MATERIALS AND METHODS

Setting

This study was conducted at the Dr. Soetomo Hospital in Surabaya, capital of the province of East Java, Indonesia. It is the main referral hospital in the region, providing all publicly funded health care services to more than 5 million people. The hospital has 1,432 beds for approximately 41,000 admissions per year.

Bacterial isolates

From January 2005 until April 2005, all non-duplicate isolates of *E. coli* and *K. pneumoniae* from all clinical specimens from patients hospitalized at the Dr. Soetomo Hospital, Surabaya, were prospectively collected at the laboratory of clinical microbiology and screened for ESBL production using disk diffusion with ceftazidime and cefotaxime as recommended by the Clinical and Laboratory Standards Institute (CLSI) (8). Phenotypic confirmation of ESBL production was performed by disk diffusion using disks of ceftazidime with and without clavulanic acid (Oxoid, Basingstoke, UK) as described by the CLSI (8). Quality control was carried out by testing *K. pneumoniae* American Type Culture Collection (ATCC) strain 700603. Identification was performed using the Microbact™ System 12A (Medvet diagnostics, Thebarton, Adelaide, Australia). For the isolates that were further analyzed using molecular methods, identification testing was repeated using the Vitek® 2 system (bioMérieux, Marcy-l'Etoile, France) and, in case of an inconclusive Vitek® 2 result, the API 20E test (bioMérieux) was performed.

Antimicrobial susceptibility testing

Antimicrobial susceptibility of the following antibiotics was determined using the Vitek® 2 system: ampicillin, amoxicillin-clavulanic acid, cefazolin, cefepime, cefotaxime, cefoxitin, ceftazidime, piperacillin, piperacillin-tazobactam, imipenem, gentamicin, amikacin, ciprofloxacin, trimethoprim-sulfamethoxazole, and tetracycline. Results were interpreted according to the CLSI criteria (8). *E. coli* strain ATCC 25922 was used as a control.

Isoelectric focusing

IEF was used to visualize the number and nature of the β-lactamases expressed in these bacteria. IEF was performed as described by Mathew et al. with minor modifications (26). Briefly, a suspension of the tested strain was incubated overnight at 37°C in brain heart infusion broth containing 100 μg/mL of ampicillin. β-lactamases were subsequently extracted with lysozyme and subjected to IEF (PhastGel 3-9; Pharmacia AB, Uppsala, Sweden). Gels were run on a PhastSystem apparatus (Pharmacia).

Presence of β-lactamase activity was detected by staining with nitrocefin (0.5 mg/ml). Control strains containing a TEM-1 (isoelectric point [pI] 5.4) or SHV-1 (pI 7.6) β-lactamase were used for pI comparisons.

Molecular characterization of β-lactamases

The DNA for the PCR assays was obtained by suspending 2-3 fresh bacterial colonies in 200 µl of aqua dest, boiling the suspension for 10 min, followed by centrifugation for 5 min at 10,000 x *g*. PCR assays to determine the presence of bla_{TEM} and bla_{SHV} genes were carried out with a GeneAmp® PCR System 9700 (Perkin-Elmer Applied Biosystems, Foster City, CA, USA) using primers and conditions described previously (25, 30). The digestion of the SHV-amplicons by *Nbe*I was used to discriminate between $bla_{SHV\text{-}ESBL}$ and $bla_{SHV\text{-}non\text{-}ESBL}$ genes (30). A multiplex PCR assay was used for the detection of genes encoding CTX-M-type β-lactamases (42). Isolates that tested positive for the CTX-M-1 group or CTX-M-9 group β-lactamase underwent amplification with a separate set of primers (Table 1). Amplification conditions for the CTX-M-1 group PCR were: initial denaturation at 94°C for 5 min; 35 cycles of 94°C for 2 min, 60°C for 1 min and 72°C for 2 min; and a final elongation at 72°C for 10 min. For the CTX-M-9 group PCR conditions were: initial denaturation at 94°C for 5 min; 35 cycles of 94°C for 30 s, 54°C for 90 s and 72°C for 1 min; and a final elongation at 72°C for 5 min. Products resulting from amplifications were subjected to sequencing using a 3100 ABI Prism genetic analyzer (Applied Biosystems), with an additional primer for the CTX-M-1 and CTX-M-9 group (Table 1). Analysis and comparisons of nucleotide and amino acid sequence data were carried out using MegAlign software (DNAStar Inc., Madison, USA) and programs available at the National Centre for Biotechnology Information website (http://www.ncbi.nlm.nih.gov).

Table 1. Sequences of additional primers used to detect and sequence $bla_{CTX\text{-}M}$ genes.

Primer name	Oligonucleotide sequence	PCR target	Product size (bp)	Reference
CTX-M-1m fw	5′- AAA AAT CAG TGC GCC AGT TC -3′	$bla_{CTX\text{-}M}$ (CTX-M-1 group)	863	(42)
CTX-M-1 multi-r	5′- TTA CAA ACC GTC GGT GAC GA -3′			this study
CTX-M-1 multi (rv)f seq	5′- AAC GTG GCG ATG AAT AAG CT-3′			this study
CTX-M-9m fw	5′- CAA AGA GAG TGC AAC GGA TG -3′	$bla_{CTX\text{-}M}$ (CTX-M-9 group)	862	(42)
CTX-M-9 multi-r	5′- CCT TCG GCG ATG ATT CTC -3′			this study
CTX-M-9 multi rv-fw seq	5′- GGT GAT GAA CGC TTT CCA A -3′			this study

Discrepancy analysis

Isolates that were phenotypically confirmed as ESBL-producers by disk diffusion, but which were negative for bla_{TEM}, bla_{SHV}, and $bla_{CTX\text{-}M}$ ESBL-genes, were further analyzed by detailed review of the Vitek® 2 report and were subjected to additional ESBL E-tests containing ceftazidime, cefotaxime, and cefepime, all with and without clavulanate (AB Biodisk, Solna, Sweden). The E-test method was carried out according to the manufacturer's instructions.

Molecular typing

All ESBL-positive *E. coli* and *K. pneumoniae* isolates were characterized by macrorestriction analysis of genomic DNA with *Xba*I. DNA fragments were analyzed by pulsed-field gel electrophoresis (PFGE) in a CHEF-DR III system (Bio-Rad, Hercules, CA, USA) as described previously (12). Restriction patterns were compared visually and interpreted using the criteria established by Tenover et al. (39).

A subset of *E. coli* isolates was further analyzed using the repetitive-sequence-based PCR (rep-PCR) DiversiLab™ Microbial Typing System® (bioMérieux, Boxtel, The Netherlands) (15). Isolates with >95% identical profiles were considered to be closely related.

Phylogenetic grouping

A selection of *E. coli* isolates was assigned to one of the four main *E. coli* phylogenetic groups (A, B1, B2, and D) according to an established triplex PCR assay (7).

RESULTS

Bacterial isolates

During the 4-month study period, 1,910 clinical specimens, including 767 urines, 548 blood cultures, 355 wound specimens, 137 respiratory specimens, 85 stool specimens, and 18 cerebrospinal fluids, were received by the laboratory of clinical microbiology in the Dr. Soetomo Hospital. ESBL screening revealed presumptive ESBL production in 39.0% (157/403) of the *E. coli* strains and 51.5% (150/291) of the *K. pneumoniae* strains. ESBL production was phenotypically confirmed in 28.5% (n=115) of *E. coli* and 35.7% (n=104) of *K. pneumoniae* isolates. Of these, 98 *E. coli* and 100 *K. pneumoniae* were available for further characterization. Additional testing confirmed the identification of 86 *E. coli* and 85 *K. pneumoniae*.

ESBL-producing *E. coli*

An ESBL gene was detected in 73 *E. coli* isolates (Table 2). The thirteen other isolates were initially phenotypically confirmed as ESBL-positive, but appeared negative by molecular characterization. To rule out exotic β-lactamases the discrepant isolates were retested by Vitek® 2 and/or E-tests. All thirteen isolates appeared to be ESBL-negative using these additional phenotypic tests. As confirmed by molecular techniques, 73 of the 86 *E. coli* were ESBL-positive (Table 2). The majority of these ESBL-positive *E. coli* were isolated from urine specimens (Table 3).

Molecular analysis showed that 70 isolates carried a single bla_{ESBL} and three isolates carried multiple bla_{ESBL} genes (Table 2). CTX-M-15 was the most widespread encoded enzyme (69/73 strains, 94.5%), occurring in isolates recovered from 46 urines, 9 wound specimens, 8 stool specimens, 4 respiratory specimens and 2 blood cultures from all departments. Most of these CTX-M-15 positive isolates showed resistance to non-related classes of antibiotics such as ciprofloxacin, tetracycline, trimethoprim-sulfamethoxazole, and gentamicin, but not to amikacin (Table 2). Like most of the CTX-M enzymes, CTX-M-15 showed a preference for the cefotaxime substrate, with 67 isolates exhibiting an MIC of ≥64 mg/L. Also, MICs for ceftazidime above the CLSI-defined susceptibility breakpoint (8 mg/L) were observed for 67 isolates. Furthermore, cefepime seemed to be affected by the enzyme as well, since 18 isolates exhibited MICs of 32 mg/L and 25 isolates MICs of ≥64 mg/L. However, some of these isolates also contain a TEM-1 enzyme and therefore the susceptibility results should be regarded as the cumulative result of the activity of the presence of more than one mechanism of resistance. Interestingly, only three of the 69 isolates with a CTX-M-15 β-lactamase revealed a product in the CTX-M pI range using IEF.

Table 2. Occurrence of *bla* types among and antimicrobial susceptibility results of 73 ESBL-producing *E. coli* consecutively isolated from patients admitted to the Dr. Soetomo Hospital in Surabaya, Indonesia.

Type of ESBLs	No. of isolates (%)	No. of isolates in combination with TEM-1	% resistant to[a]:				
			GEN	AMK	CIP	SXT	TET
CTX-M-15	66[b] (90.4)	35	72.7	7.6	80.3	57.6	84.8
SHV-5	3[c] (4.1)	2	100	33.3	0	100	100
SHV-5 + CTX-M-15	2[d] (2.7)	1	100	100	50.0	100	100
SHV-12 + CTX-M-15	1 (1.4)	0	0	0	100	100	100
SHV-12	1 (1.4)	1	100	100	100	100	0
Total	73 (100)	39	74.0	12.3	76.7	61.6	84.9

[a] Rates of resistance include resistant as well as intermediately susceptible isolates. Abbreviations: AMK, amikacin; CIP, ciprofloxacin; GEN, gentamicin; SXT, trimethoprim-sulfamethoxazole; TET, tetracycline.
[b] PFGE: 36 types + 10 untypeable isolates; rep-PCR: 23 types.
[c] PFGE: 3 types.
[d] PFGE: 1 type + 1 untypeable isolate; rep-PCR: 2 types.

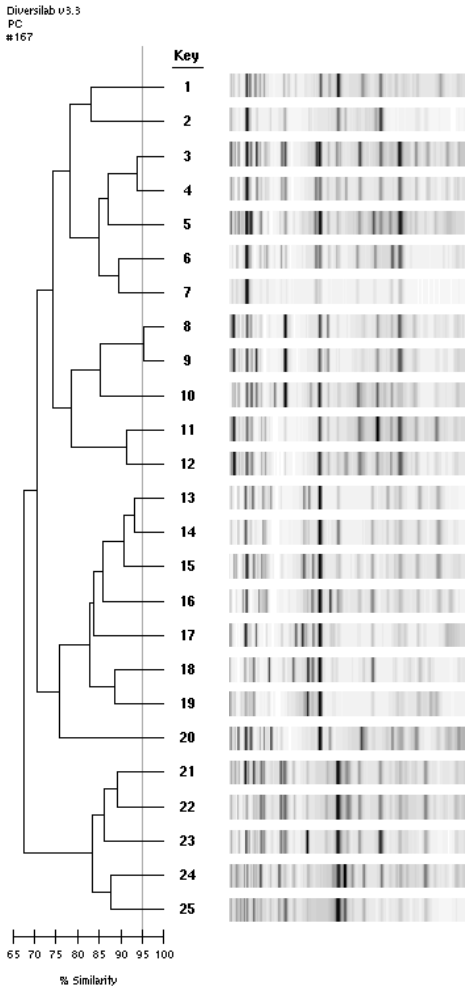
Table 3. Origin of ESBL-producing *E. coli* and *K. pneumoniae* strains consecutively isolated from patients admitted to the Dr. Soetomo Hospital in Surabaya, Indonesia.

Origin		*E. coli* (n=73)	*K. pneumoniae* (n=72)
Specimen type			
	urine	49	45
	wound specimen	9	9
	blood	2	12
	stools	9	2
	sputum / tracheal aspirate	4	3
	cerebrospinal fluid	0	1
Department			
	Internal Medicine	16	26
	Urology	18	9
	Pediatrics	17	8
	Surgery	11	11
	Intensive Care Unit	6	8
	Gynecology/ Obstetrics	3	2
	Neonatology	1	4
	Other[a]	1	4

[a] Burn unit, Neurology, Geriatric Medicine, and Pulmonology.

A bla_{SHV}-type ESBL gene was detected in seven *E. coli* strains (9.6%), three of which carried a bla_{CTX-M} as well (Table 2). Although 39 strains (53.4%) were positive for the bla_{TEM-1} gene, TEM-type ESBLs were not found. As expected, all of the ESBL-producing isolates remained susceptible to imipenem.

Molecular typing of the *E. coli* isolates by PFGE revealed extensive heterogeneity. Patterns were designated type 1 to 42. Most of the types represented unique isolates (34/42, 81.0%). PFGE type 22 was observed for 8 isolates from 4 departments, whereas type 17 was represented by 6 isolates from 3 departments. Since the PFGE analysis of 10 of the CTX-M-15 positive isolates revealed band-smearing patterns, an additional rep-PCR typing method using the DiversiLab™ Microbial Typing System® was performed for all 69 CTX-M-15 positive strains. A total of 25 profiles was found, designated rep-PCR type A to Y (Figure 1). Type G was the most prevalent type (n=16 isolates), and included all 8 PFGE type 22 isolates. Ten of the type G strains were isolated from urine cultures from 3 departments, and 5 from pus. Type E was represented by 7 isolates from 4 departments. In the PFGE analysis, these isolates showed 6 different profiles. Rep-PCR type D (n=7) included all PFGE type 17 isolates. The two CTX-M-15 positive isolates from blood were identical (PFGE type 17; rep-PCR type D), but were obtained from two different departments, the Intensive Care Unit and



M	1	1	24
N	1	1	42
R	1	1	NT
Q	3	2	38, NT
S	1	1	NT
P	2	1	29, NT
O	3	2	2, 3, 40
U	4	2	18, 20
V	6	4	1, 19, 25, NT
W	1	1	10
X	1	1	26
Y	1	1	15
A	2	1	8, NT
B	1	1	23
C	1	1	NT
D	7	3	17, 35
G	16	5	4, 22, 23, 27, 33, 34, NT
F	1	1	32
E	7	4	9, 11, 16, 21, 31, 36
T	1	1	14
H	1	1	30
I	1	1	41
L	2	2	28, 37
J	3	2	39, NT
K	1	1	6

Figure 1. Molecular characterization of 69 CTX-M-15-positive *E. coli* using the repetitive-sequence-based PCR (rep-PCR) DiversiLab Microbial Typing System®.
The 25 profiles, designated rep-PCR type A to Y, are shown in the dendrogram. Similarity analysis was performed with the DiversiLab software with the Pearson correlation coefficient to determine distance matrices and the unweighted-pair group method with arithmetic mean (UPGMA) to create the dendrogram. The scale on the bottom shows percentages of similarity. Further information is shown on the right side of the figure: the number of representative isolates of each rep-PCR type, the number of departments (dpt) affected by the specific putative clone, and the corresponding PFGE types. NT, not typeable by PFGE.

Internal Medicine. Rep-PCR typing and PFGE were thus concordant with PFGE being the method of highest resolution.

The phylogenetic group was determined for 68 CTX-M-15 positive *E. coli*. They belonged to phylogenetic group B2 (36.8%), A (33.8%), D (27.9%), and B1 (1.5%). All

rep-PCR type E and G isolates belonged to phylogenetic group B2, while all rep-PCR type D isolates belonged to phylogenetic group D.

ESBL-producing *K. pneumoniae*

In 69 *K. pneumoniae* isolates an ESBL gene was characterized (Table 4). Three additional isolates showed a typical ESBL phenotype in the discrepancy analysis and were thus considered "truly" ESBL-positive, but we did not succeed to characterize the responsible genes. Most of the 72 ESBL-positive *K. pneumoniae* were isolated from urine (n=45) and blood (n=12) (Table 3). Molecular analysis revealed the presence of SHV- and CTX-M-type enzymes among these strains (Table 4). TEM-type ESBLs were not found. The bla_{SHV}-type ESBL enzymes were the most prevalent (47/72, 65.3%): SHV-5 (n=28), SHV-12 (n=13), and SHV-2 (n=6). CTX-M-type enzymes were detected in 42 *K. pneumoniae* strains (58.3%). bla_{CTX-M} encoding CTX-M-15 and CTX-M-9 were found in 55.6% and 2.8% of isolates, respectively. Eleven of these revealed a product in the CTX-M pI range using IEF. Twenty strains carried multiple ESBL genes (27.8%). The $bla_{CTX-M-15}$ gene was encountered in combination with SHV-12 (n=10), SHV-5 (7), or SHV-2 (2), while the $bla_{CTX-M-9}$ gene occurred once with SHV-5. For two of

Table 4. Occurrence of *bla* types among and antimicrobial susceptibility results of 72 ESBL-producing *K. pneumoniae* consecutively isolated from patients admitted to the Dr. Soetomo Hospital in Surabaya, Indonesia.

Type of ESBLs	No. of isolates (%)	No. of isolates in combination with		% resistant to[a]:				
		TEM-1	SHV-1	GEN	AMK	CIP	SXT	TET
CTX-M-15	21[b] (29.2)	5	18	85.7	9.5	81.0	76.2	76.2
SHV-5	20[c] (27.8)	3	-	75.0	0	20.0	90.0	70.0
CTX-M-15 + SHV-type	19[d] (26.4)	4	-	78.9	52.6	68.4	94.7	73.7
SHV-2	4[e] (5.6)	1	-	0	25.0	75.0	75.0	50.0
SHV-12	3[f] (4.2)	1	-	66.7	33.3	66.7	66.7	66.7
Other[g]	2 (2.8)	0	1	50.0	0	0	100	50.0
Uncharacterized[h]	3 (4.2)	1	0	33.3	0	0	0	0
Total	72 (100)	15	19	72.2	19.4	54.2	81.9	68.1

[a] Rates of resistance include resistant as well as intermediately susceptible isolates. Abbreviations: AMK, amikacin; CIP, ciprofloxacin; GEN, gentamicin; SXT, trimethoprim-sulfamethoxazole; TET, tetracycline.
[b] PFGE: 19 types + 2 untypeable isolates.
[c] PFGE: 19 types.
[d] PFGE: 17 types.
[e] PFGE: 4 types.
[f] PFGE: 3 types.
[g] Other (combinations of) ESBL genes included CTX-M-9 (n=1) and CTX-M-9 + SHV-5 (1). PFGE types: 2 types.
[h] In two isolates we detected bla_{SHV-11}. This is a broad-spectrum β–lactamase, but not an ESBL.

the three isolates with an uncharacterized ESBL-mechanism a β-lactamase enzyme in the CTX-M pI range was observed by IEF. The other isolate did not express any β-lactamase enzyme.

The results of the susceptibility testing are summarized in Table 4. All of the ESBL-producing *K. pneumoniae* isolates were susceptible to imipenem (not shown), but resistance to trimethoprim-sulfamethoxazole, gentamicin, tetracycline, and ciprofloxacin was prevalent.

PFGE revealed a high level of genomic diversity among the ESBL-producing *K. pneumoniae* isolates. No identical types were observed except for 2 SHV-5-producing isolates from fecal flora from the department of Neonatology and 3 isolates from urine containing both an SHV-type ESBL gene and $bla_{\text{CTX-M-15}}$ (Table 4).

DISCUSSION

This is the first systematic survey on ESBL-enzymes from a large teaching hospital in East Java, Indonesia. Our data provide insights into the genetic characteristics and molecular epidemiology of ESBL-producing *E. coli* and *K. pneumoniae* in a highly populated part of the world wherefrom only few data have been generated so far.

The ESBL-positive *E. coli* isolates investigated encoded mainly CTX-M-15 enzymes (94.5%). Strains harboring this ESBL are a major problem worldwide, being the commonest CTX-M type in Europe and Canada, causing outbreaks as well as sporadic infections (2, 5, 34). It was first described in four *E. coli* and one *K. pneumoniae* from New Delhi, India, in 2000 (16). Nowadays, $bla_{\text{CTX-M-15}}$ is highly prevalent among ESBL-producing Enterobacteriaceae in India and, therefore, it has been suggested that the enzyme originated in India (14, 23). In clinical ESBL-producing *E. coli* from Thailand, this enzyme was detected in 37.2% of isolates (18). Our report is the first description of the dominant presence of CTX-M-15 in clinical ESBL-producing *E. coli* from Indonesia. Most of the isolates were resistant to ciprofloxacin, tetracycline, trimethoprim-sulfamethoxazole, and gentamicin. The association between ESBL production and fluoroquinolone resistance has been recognized before but the nature of this association is not yet understood (40). The CTX-M-15 gene was found in both clonal (PFGE type 22 / rep-PCR type G and PFGE type 17 / rep-PCR type D) and non-clonal *E. coli* which indicates that both clonal spread of epidemic strains and transfer of genetic elements between non-related strains have contributed to the spread of this specific ESBL-type. This is an interesting finding, since most previously reported nosocomial infections by CTX-M-producing *E. coli* were caused by clonally unrelated isolates (36, 41).

CTX-M-15 is known to be able to efficiently hydrolyse not only cefotaxime but also ceftazidime (17). The majority of our CTX-M-15 positive *E. coli* was indeed resistant to both cephalosporines. CTX-M enzymes in general confer variable levels of resistance to cefepime, which was also observed in our collection (1). CTX-M-15 was difficult to detect by IEF in our study probably due to low expression of the corresponding genes. Only three of the 69 isolates revealed an enzyme band in IEF. This phenomenon has been observed before for CTX-M-15 in the UK (17). SHV-type ESBLs were detected in only a minority of *E. coli* strains (9.6%). TEM-type ESBLs were not detected at all.

The epidemiology of ESBL enzymes found among *K. pneumoniae* differed in some aspects from that of *E. coli*. Among *K. pneumoniae*, there was more diversity of ESBL types, but again, no TEM-types were found. Multiple ESBL enzymes were present in at least 27.8% of strains. Also, unlike *E. coli*, there was only limited clonal spread. Furthermore, in *K. pneumoniae* bla_{SHV}-type enzymes were the most prevalent ESBLs (65.3%). The frequently occurring bla_{SHV-5} may be the result of spreading of SHV-5 containing plasmids among multiple strains. However, such a mode of spread has not been documented often (27, 35). *De novo* mutation and selection is probably a better explanation for this finding. Similar to *E. coli*, CTX-M-15 was the most prevalent bla_{CTX-M} as it was detected in as many as 55.6% of isolates. In clinical ESBL-producing *K. pneumoniae* from Thailand, this enzyme was detected in 38.9% of isolates (18).Overall, the picture of clinical ESBL-producing *E. coli* in Surabaya in 2005 is consistent with the current worldwide situation of CTX-M-producing *E. coli* being more prevalent than classic TEM- and SHV-variants. The CTX-M and SHV-type ESBL-genes found in our Indonesian *E. coli* and *K. pneumoniae* are also in agreement with reports from other Southeast Asian countries (Table 5). However, among nosocomial *K. pneumoniae* strains collected in 2004-2005 in Thailand, the bla_{CTX-M} was present in as many as 99.2% of isolates, while in the Indonesian isolates collected in the same period bla_{SHV} was the most prevalent bla_{ESBL} (18). It has been suggested that the insertion sequence (IS) element IS*Ecp*1 plays a role in the transfer of the CTX-M-1 group genes among Enterobacteriaceae. This element was detected in the majority of the Thai isolates (18). Whether this element is present in our CTX-M-15 isolates should be further investigated. Of note, CTX-M-14, one of the dominant CTX-M types in most Asian countries, was not found in our survey (14). Three *K. pneumoniae* isolates were resistant to broad-spectrum cephalosporins but did not give rise to any PCR product, suggesting the presence of other β-lactamases in these isolates. These are currently studied further.

During the study period, 767 urinary tract samples and 548 blood cultures were collected. Also, the majority of the ESBL-producing isolates in our survey were cultured from urine (n=96; 65.3% of all ESBL-producing *E. coli* and *K. pneumoniae*; 12.5% of all urines). This is in agreement with other surveys (5, 18). A possible explanation

Table 5. Occurrence of bla_{ESBL} types among clinical isolates of *E. coli* and *K. pneumoniae* in Southeast Asia.

Species	Country[a]	Year of collection	bla_{ESBL} types	Reference
E. coli	Malaysia	1998-2000	SHV-5	(38)
	Singapore	NA[b]	CTX-M-2, -20; Toho-1	(21)
	Thailand	1994/1996	SHV-5, -12	(6)
		1999	VEB-1[c]	(11)
		2004-2005	CTX-M-14; SHV-12	(29)
		2004-2005	CTX-M-14, -15, -27, -40, -55; SHV-12; VEB-1	(18, 19)
	Indonesia	2005	CTX-M-15; SHV-5, -12	this study
K. pneumoniae	Malaysia	1997-1998	SHV-5	(31)
	Singapore	NA[b]	CTX-M-9, -11, -15	(21)
	Thailand	1994/1996	SHV-2a, -5, -12	(6)
		1999	VEB-1	(11)
		2004-2005	CTX-M-14; SHV-12, -27, -28	(29)
		2004-2005	CTX-M-3, -14, -15, -27, -55; SHV-2a, -12, -27, -71, -75; VEB-1	(18, 19)
	Vietnam	1996	CTX-M-17	(3)
		2000-2001	CTX-M-14, -17; SHV-2; VEB-1	(4)
	Indonesia	2005	CTX-M-9, -15; SHV-2, -5, -12	this study

[a] There are no data in Pubmed from Brunei, Cambodia, East-Timor, Lao People's Democratic Republic, Myanmar, and the Philippines.
[b] NA, not availble.
[c] VEB, Vietnamese extended-spectrum β-lactamase.

for this phenomenon is that colonization of humans with ESBL-producing bacteria occurs primarily in the gut, which may, given the proximity of the anus to the urethral meatus, lead to urinary tract colonization and infection, especially in patients with risk factors such as indwelling catheters. Additional virulence factors of the bacteria may play a role as well. Recently, Carattoli et al. showed that most CTX-M-15-producing *E. coli* from urine specimens belonged to the virulent phylogenetic group B2 and contained uropathogenic pathogenicity islands (5). Apparently, CTX-M enzymes have successfully been introduced in uropathogenic lineages of *E. coli*. Indeed, the globally disseminated highly virulent ciprofloxacin-resistant clone B2-*E. coli* O25:H4-ST131 causes urinary tract infections and is associated with the CTX-M-15 pandemic (9). We have shown that 25 of 68 (36.8%) CTX-M-15-positive *E. coli* from Indonesia belong to phylogenetic group B2, 15 of which were ciprofloxacin-resistant, but whether this specific clone is present in Indonesia needs further research. The high prevalence of ESBL-producing microorganisms among urinary tract pathogens has important implications for the treatment of these infections, especially given the high co-resistance rates to other classes of antibiotics such as ciprofloxacin and trimethoprim-sulfamethoxazole. Bloodstream infections by ESBL-producing bacteria seem to be another important

clinical problem in the Dr. Soetomo Hospital. Of the 9 isolates with a preliminary identification of *E. coli* and 25 isolates with a preliminary identification of *K. pneumoniae* that were cultured from the 548 blood cultures, we identified 2 proven ESBL-producing *E. coli* and 12 proven ESBL-positive *K. pneumoniae*. In general, the main risk factors associated with nosocomial infections with ESBL-producing strains include hospital length of stay, severity of illness, presence of invasive medical devices for a prolonged duration, and previous exposure to antibiotics. Several studies have found a relationship between third-generation cephalosporin use and acquisition of an ESBL-producing bacterium (32). The present study was not designed to identify risk factors, but it has already been shown that the cephalosporins, and especially third-generation cephalosporins, are the second most prescribed antibiotics in at least two hospitals in Java, Indonesia, including the Dr. Soetomo Hospital in Surabaya (10, 13). Due to lack of statistical power no significant correlation between the use of cephalosporins and carriage of a cefotaxime-resistant *E. coli* could be established as yet.

Our study has certain limitations. First, the exact prevalence of ESBLs among clinical *E. coli* and *K. pneumoniae* cannot be calculated due to preliminary identification results for the phenotypically ESBL-negative isolates. However, if 88% of the preliminary identification results was correct for *E. coli*, and 85% for *K. pneumoniae*, then the estimated prevalence would be 73/363 (20.1%) for *E. coli* and 72/258 (27.9%) for *K. pneumoniae*. Although resistance rates should always be compared with a careful consideration of sampling bias, these possible rates from Surabaya are similar to those reported in comparable surveys from Rome, Italy in 2006 (17.2% in clinical *E. coli*) and from Bangkok, Thailand in 2005/2006 (27.1% in clinical *K. pneumoniae*) (5, 19, 24). Second, confirmatory testing was performed using only ceftazidime. Therefore, we could have missed some ESBL-strains, especially those with CTX-M β-lactamases, since these enzymes are much more active against cefotaxime as a substrate than against ceftazidime. Third, molecular analyses for ESBL resistance genes are always subject to limitations due to the selection of target bla_{ESBL}. At present, numerous ESBLs have been described worldwide, and it is likely that many more will be discovered in the future (28). Any negative PCR result in our study must be evaluated with this in mind. Fourth, our study was performed in a specific geographical location, and the results may not be taken to reflect the distribution of ESBLs throughout Indonesia.

In summary, we have shown that Indonesia is another country in the Southeast Asian region affected by the emergence and spread of bacterial isolates harbouring ESBL genes. Our findings support the need for a national surveillance program and the implementation of strict antibiotic policies to limit the use of cephalosporins. Transmission of ESBL-producing bacteria must be prevented by adhering to infection control measures. Finally, empirical treatment of infections potentially caused by ESBL-producing microorganisms may need to be reconsidered.

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Part V

Discussion

Chapter 14

General discussion and conclusion

INTRODUCTION

In this thesis, we have studied antimicrobial resistance among potential pathogens in the commensal microflora of representative cohorts of people in two major urban centers in Indonesia as part of the Antimicrobial Resistance in Indonesia: Prevalence and Prevention (AMRIN) study. In addition to determining the prevalence of resistance against the major classes of antibiotics, we have evaluated the accuracy of antimicrobial susceptibility testing performed in this low-resource setting and analyzed the genetic basis of some important types of resistance. The determinants of antibiotic resistance among *Staphylococcus aureus* and *Escherichia coli* inside and outside hospitals have been investigated as well. In this chapter, the main findings are summarized and discussed on the basis of the five research questions posed in chapter 2 of this thesis. Furthermore, suggestions for further research on the different topics are presented.

RESEARCH QUESTION 1 - ANTIMICROBIAL SUSCEPTIBILITY TESTING

Is the agar diffusion method using antibiotic impregnated disks an appropriate method for antimicrobial susceptibility testing in a surveillance study in Indonesia?

There are several methods that can be used for susceptibility testing, but in the present study the Kirby Bauer disk diffusion test on Mueller Hinton agar as described by the National Committee on Clinical Laboratory Standards (NCCLS) / Clinical and Laboratory Standards Institute (CLSI) was employed, since it is less costly compared to other methods, and has a long history of reliable use around the world (22). It was performed in four separate laboratories in Indonesia, one in Semarang and three in Surabaya. Inhibition zones were measured manually (21). The results of 4,098 drug-organism combinations were compared to results from standardized and validated susceptibility methods performed in the Netherlands. After the discrepancy analysis, the estimated accuracy of the disk diffusion method with manual zone measurements in Indonesia was found to be at least 83%, compared to an accuracy of 85% using disk diffusion with automated zone measurements in the Netherlands. This method can thus reliably be applied for antimicrobial susceptibility testing of medically relevant bacteria in low-resource settings, such as in Indonesia. However, in one of the four participating laboratories in Indonesia, the visual reading, interpretation or recording of inhibition zones met with inconsistencies that were ascribed to the fact that in that particular laboratory testing had not been assigned to qualified and experienced technicians but, rather, was performed by several, relatively inexperienced, personnel.

In all four laboratories, quality control was performed twice weekly with in general acceptable results, but whenever an unacceptable result was found, the source of the error was not investigated. In every laboratory that performs antimicrobial susceptibility testing, for surveillance purposes or direct patient care, an internal quality control system should be implemented in daily routine (14). Participation in an external quality assessment (proficiency testing) scheme allows laboratories to compare their performances with that of other laboratories.

In conclusion, the agar diffusion method using antibiotic impregnated disks is an appropriate method for antimicrobial susceptibility testing in a surveillance study in Indonesia or any low-resource country. However, given an accuracy rate of 83%, there is room for improvement. Firstly, the disk diffusion method should be performed by well-trained, experienced, and dedicated technicians. Secondly, an internal quality control system should be fully implemented. Finally, the use of an automated zone reader will also reduce the number of errors.

The final aim of the AMRIN study was to develop a self-assessment program for Indonesian hospitals for antimicrobial resistance, antibiotic usage, and prevention and control of hospital infections. The disk diffusion method has become part of this self-assessment tool that was published under the auspices of the Directorate General of Medical Care of the Ministry of Health, Republic of Indonesia, which was presented during a conference in Bandung in 2005 (1).

RESEARCH QUESTION 2 – PREVALENCE OF RESISTANCE

What is the prevalence of resistance among commensal S. aureus *and* E. coli *isolates carried in the Indonesian population inside and outside hospitals?*

A total of 3,995 individuals were screened for *S. aureus* nasal carriage and *E. coli* as dominant rectal colonization. These individuals were patients on the day of admission to the hospital (admission group, n=998), patients on the day of discharge after five or more days of hospitalization (discharge group, n=999), patients visiting a primary health center (PHC group, n=1,000), and healthy relatives or household members of admission group patients (relatives group, n=998).

The rate of *S. aureus* nasal carriage was only 9%, which is much lower than the 20% or higher levels generally found among populations in other countries, including the neighbouring country Malaysia (15, 23). However, the finding is in agreement with a report from the Yogyakarta area, where 14% of individuals carried *S. aureus* (3). These were all methicillin-sensitive. In our study, we found two methicillin-resistant *S. aureus* (MRSA), both from patients cultured at discharge from the hospital in Surabaya,

indicating that, although the prevalence of MRSA was low, it is present in the hospital setting in Surabaya. Resistance rates to tetracycline were high in all groups (25%), especially in the admission and discharge group in Surabaya (40% and 34%, respectively). Overall, resistance rates of isolates from patients at discharge from the hospital were not higher than on admission, suggesting that resistance in *S. aureus* was mainly community derived.

E. coli as dominant intestinal flora was cultured from 3,284 individuals. Resistance towards gentamicin, cefotaxime, and ciprofloxacin was low on admission to the hospital (4%, 2%, and 6%, respectively). However, high rates of resistance against these three antibiotics were found among *E. coli* cultured from patients at the time of discharge from the hospital (18%, 13%, and 22%, respectively). Among all *E. coli*, resistance rates towards chloramphenicol (range 8-43%), trimethoprim-sulfamethoxazole (20-56%), and ampicillin (20-73%) were substantial. Therefore, resistance in *E. coli* appeared to be both hospital and community derived. Commensal *E. coli* have also been studied in two other Southeast Asian countries. Nys et al. studied faecal *E. coli* from healthy adult volunteers from the Philippines (24). Compared to our data, much higher resistance rates were found for ampicillin (81%), chloramphenicol (64%), ciprofloxacin (49%), and gentamicin (33%). In Thailand, *E. coli* from swine and chicken farm workers were also highly resistant to ampicillin, chloramphenicol, and nalidixic acid (10). This latter observation may be a reflection of what is occurring in the animal industry in Southeast Asia.

There is an increasing interest in the surveillance of antimicrobial resistance in the human commensal microflora. This bacterial population is continuously exposed to the selective pressure generated by antimicrobial chemotherapy and may become a potential reservoir of resistant strains that can cause infections, and of resistant mobile elements that can be transferred to pathogenic bacteria. In our study, we for the first time presented data on resistance patterns of commensal *S. aureus* and *E. coli* isolates from Indonesia. We clearly demonstrated that antimicrobial resistance is rather prevalent among these isolates.

RESEARCH QUESTION 3 – DETERMINANTS OF RESISTANCE

What are the determinants of carriage of resistant S. aureus *and* E. coli *in the Indonesian population inside and outside hospitals?*

Many factors may contribute to the emergence of resistance among microorganisms, but the use of antimicrobial agents is considered the most important determinant of resistance (18, 19, 22). The use of antibiotics in the hospitalized patients in the

AMRIN study was strikingly high, 84% of the patients were treated with one or more courses of antibiotics during their hospital stay, mainly with aminopenicillins (54%) and cephalosporins (17%). When evaluated by experts, only 21% of the prescriptions were considered definitely appropriate, 15% were inappropriate regarding either choice of agent, dosage or duration, and, strikingly, 42% of prescriptions, many of which were for surgical prophylaxis or for episodes of fever without evidence of infection, were deemed unnecessary (9). In the community, antibiotic use was much lower (16-25%) and the most frequently used agents were the aminopenicillins (71%) and the tetracyclines (9%). The median duration of these antibiotic treatments was 3 days. We analyzed potential determinants of resistance among *S. aureus* and *E. coli*. These determinants included recent antibiotic use as well as demographic, socioeconomic, healthcare- related and disease-related variables. Patients included at admission, their relatives, and patients seen when visiting a primary health center were analyzed as one group, the so-called community group. Patients included on the day of discharge were analyzed separately. Individuals carrying resistant strains were compared with individuals carrying bacteria susceptible to all tested antibiotics.

Of the 361 *S. aureus*, 245 (68%) were susceptible to all antibiotics tested. In the hospital (98 patients), the use of aminopenicillins was associated with resistance to any of the tested antibiotics and with resistance to tetracycline in combination with other antibiotics. Whether this observation reflects the exposure to antibiotics in hospitalized patients, or an association of penicillin use/exposure with a diversity of resistance patterns including tetracycline resistance is uncertain. One could hypothesize that the patients treated with aminopenicillins in the hospital had recently used tetracycline or another antibiotic before admission. However, genes encoding penicillin and tetracycline resistance may co-exist and co-evolve in *S. aureus* and spread in special epidemiologic populations (17). In the community (263 individuals), no associations between antibiotic use and resistance patterns were observed. This could be due to the rather small proportion of individuals carrying nasal *S. aureus* and the low number of resistant *S. aureus*, which limited the statistical power to detect associations. The short treatment courses in the community, however, may have contributed to a relatively low selective pressure and consequently low resistance rates (7). Furthermore, isolates that were intermediate susceptible according to the CLSI criteria were categorized as susceptible. However, intermediate susceptible isolates may already have gained some resistance mechanisms after a certain level of selective pressure.

In addition to recent antibiotic use, demographic, socioeconomic, disease-related, and health care-related variables were analyzed. As stated before, resistance rates of isolates from patients at discharge from the hospital were not higher than on admission. However, patients upon admission to a hospital were more often colonized with *S. aureus* resistant to any antibiotic and to tetracycline, which strengthens the sugges-

tion that resistance in *S. aureus* was mainly community derived. In the community, crowding and low income were associated with multidrug-resistant *S. aureus*. It is possible that certain strains that are effective colonists and, coincidently, resistant, may spread more easily in certain environments.

Of the 3,275 *E. coli*, 1,552 (47%) were susceptible to all antibiotics tested. Among hospitalized patients (781 individuals), 81% carried *E. coli* resistant to one or more antibiotics. The use of any antibiotic, penicillins, amphenicols, quinolones and metronidazole was associated with carriage of *E. coli* resistant to any of the tested antibiotics. In the non-hospitalized population (2,494 individuals), 43% carried resistant *E. coli*. Antibiotic use was, again, the most important independent determinant of carriage of resistant *E. coli*. Direct associations were observed between the use of β-lactam antibiotics and ampicillin resistance and between sulphonamide use and resistance to trimethoprim-sulfamethoxazole.

Other variables were analyzed as well. In hospitalized patients screened upon discharge, having a health insurance was associated with carriage of resistant *E. coli*. A different antibiotic consumption pattern of people with a health insurance may explain the role of health insurance. In non-hospitalized persons, children, regardless of more frequent antibiotic use, were at greater risk of carriage of resistant *E. coli* when compared to adults, perhaps because of their greater exposure to (resistant) microorganisms when living and playing under less sanitary conditions.

Interestingly, patients upon discharge in Semarang had a higher risk of carriage of resistant *E. coli,* but a lower risk of carriage of resistant *S. aureus* compared with patients in Surabaya. Since the proportion of patients treated with antibiotics was similar in the two hospitals, other factors may determine this difference.

In conclusion, antibiotic use was the most prominent albeit not the only determinant of carriage of resistant bacteria inside hospitals, and for *E. coli*, also outside hospitals. We confirmed the well-recognized association between antibiotic use and resistance, but now for the first time in the commensal flora of Indonesian people.

RESEARCH QUESTION 4 – GENETIC CHARACTERISTICS OF *S. AUREUS*

What are the genetic characteristics of the S. aureus *isolates carried in the Indonesian population?*

Nasal carriage of *S. aureus* plays a key role in the epidemiology and pathogenesis of *S. aureus* infections (15, 30). Essentially any *S. aureus* that is able to colonize a human being is also able to cause (invasive) disease. Of special concern are MRSA strains,

because infections caused by these can only be treated with usually less effective and generally more expensive antibiotics. Until the 1990s, MRSA strains were mainly restricted to hospitals, but since then, virulent community-associated MRSA (CA-MRSA) clones have emerged on all continents. Nowadays, these "CA-MRSA" may also be found in some hospitals. The classical CA-MRSA clones are genetically characterized by staphylococcal cassette chromosome *mec* (SCC*mec*) IV or V, and the presence of the Panton–Valentine leukocidin (PVL) genes. PVL is an exotoxin that causes tissue necrosis and leukocyte destruction by forming pores in cellular membranes. Strains enriched for PVL are associated with skin infections and necrotizing pneumonia. Although the epidemiological linkage between PVL and CA-MRSA is compelling, the role of PVL has recently been questioned, because animal models failed to demonstrate virulence of PVL in prevalent CA-MRSA strains (4). Interestingly, we found a low prevalence of MRSA (1%) but a high prevalence of PVL-positive methicillin-sensitive *S. aureus* (MSSA) (11%) in our collection of Indonesian carriage strains. The SCC*mec* of the two MRSA strains were identified as type III and type V. The strain with SSC*mec* type V did not contain the *pvl* genes. Molecular typing by pulsed-field gel electrophoresis (PFGE) showed extensive genetic diversity among both PVL-positive and PVL-negative strains. In Surabaya, however, we identified a cluster that was strongly associated with the presence of the PVL locus. The high prevalence of PVL-positive strains among commensal *S. aureus* is in contrast with the low PVL frequencies in carriage isolates reported from the Netherlands (1%), France (0%), and Germany (1%), but in agreement with data from the Yogyakarta area: 16% of the nasal MSSA strains were PVL-positive (3, 20, 27, 29).

As determined by high-throughput amplified fragment length polymorphism (AFLP), PVL-positive strains were found in each major staphylococcal genome cluster, indicating that PVL has been introduced in distinct phylogenetic subpopulations of *S. aureus*. However, multilocus sequence typing showed that most PVL-positive strains belonged to sequence type (ST) 188/clonal complex (CC) 1 and ST121/CC121, while most PVL-negative isolates belonged to ST45/CC45. AFLP cluster IVb (similar to ST121), was significantly enriched with PVL compared with the other clusters. Interestingly, one of the five major clusters, CC30, was almost completely absent in our Indonesian collection.

In conclusion, PVL-positive MSSA are successful commensals in Indonesia. The high prevalence of PVL in *S. aureus* strains is, however, of concern since these strains may cause severe infections in the community and in hospitals. When the SCC*mec* transfers from other staphylococci into these PVL-positive MSSA, putative virulent MRSA lineages may emerge. This should be monitored closely, since the epidemiology of *S. aureus* can rapidly change.

In our surveillance study, we unexpectedly cultured *Staphylococcus sciuri*, a coagulase negative *Staphylococcus* spp. (CoNS) that resembles *S. aureus*, from the noses of 55 individuals. *S. sciuri* is a common commensal of animals, and only rarely involved in colonization or infection in humans. The majority of the strains in our analysis was from Semarang (n=54). The explanation for this may be found in slight differences in culture methods. In Surabaya, a coagulase test was often performed before an isolate was stored as "putative *S. aureus*".

CoNS are believed to constitute a reservoir of resistance genes and SCC*mec* elements for *S. aureus* (11). More specifically, *S. sciuri* is described to ubiquitously contain a *S. sciuri mecA* that may be the evolutionary precursor of the *mecA* in MRSA. Some *S. sciuri* strains also carry another *mecA* gene, identical to *S. aureus mecA* (32). In our study, the *S. aureus mecA* gene was detected in 22 *S. sciuri* isolates (43%), whereas the *S. sciuri mecA* was found in 33 isolates (65%). The SCC*mec* regions of *S. aureus mecA*-positive *S. sciuri* isolates were not typeable by the current classification scheme as defined for *S. aureus*, but most of the strains contained elements of the classical SCC*mec* types II and/or III.

Carriage of *S. sciuri* was associated with hospitalization. Although antibiotic consumption was not a risk factor for *S. sciuri* carriage, it was associated with carriage of an *S. sciuri* that harbored the *S. aureus mecA* gene. In addition, the *S. aureus mecA*-positive *S. sciuri* strains were more resistant to non-β-lactam antibiotics than *S. aureus mecA*-negative strains. Hospitalized patients in Semarang with recent antibiotic use are, thus, at risk of becoming colonized with a multidrug-resistant *S. sciuri*. Colonization with resistant *E. coli* alike, carriage of *S. sciuri* seems to be associated with health care exposure. The presence of *S. sciuri* in a nosocomial setting where a selective antibiotic pressure exists can perhaps provide a source for new MRSA lineages.

Our study was not designed to investigate the rate of *S. sciuri* carriage. We did not speciate all CoNS found in the survey and we did not use selective media specifically for the isolation of *S. sciuri*. The actual prevalence in Indonesia may thus be higher than the 55/3,995 (1%) observed in our study.

In conclusion, *S. sciuri* is a colonizer of the nares of people in Indonesia and carriage with this particular species of staphylococci is associated with hospitalization. However, further research is needed to investigate the clinical significance, if any, of this staphylococcal species in Indonesia.

RESEARCH QUESTION 5 – RESISTANCE MECHANISMS IN *E. COLI*

What is the genetic basis of resistance towards ciprofloxacin and cefotaxime, two worldwide emerging phenomenons, in (commensal) E. coli *from Indonesia?*

Although the highest levels of resistance among *E. coli* were found against ampicillin, trimethoprim-sulfamethoxazole, and chloramphenicol, the resistance rates to ciprofloxacin, a fluoroquinolone (FQ), and cefotaxime, a third-generation cephalosporin, were of most concern, especially in the hospital, because these limit the options for empirical therapy when such strains cause nosocomial infections. Therefore, strains with resistance to these antibiotics were analyzed in more detail.

Ciprofloxacin

The prevalence of FQ resistance in *E. coli* was highest among patients on the day of discharge (18% in Surabaya and 27% in Semarang) and lowest among persons visiting a primary health center and family members accompanying patients on admission (2%). Molecular typing using enterobacterial repetitive intergenic consensus (ERIC) polymerase chain reactions (PCR) showed considerable genetic heterogeneity among 196 FQ-resistant *E. coli*. Among the total number of 140 isolates from discharge group patients, we identified 119 different ERIC-PCR profiles. Some isolates with indiscriminate ERIC-PCR patterns were found with an obvious clustering in time and place. Type 37 occurred in three patients from the Internal Medicine department in Surabaya; all 3 patients were present within this department on the same day. Type 90 was isolated from two patients on the day they were discharged from the Internal Medicine department in Semarang. Samples were collected on consecutive days. The finding of these small clusters can be explained by inadvertent patient-to patient transmission, either directly or via healthcare workers, or by contact with an environmental source.

Phylogenetic typing and virulence profiling were performed to investigate whether a potential clinical hazard was associated with the presence of these FQ-resistant isolates (13). The results were compared with similar data of FQ-susceptible isolates from the same collection. The 200 FQ-susceptible isolates were predominantly from phylogenetic groups A (52%) and B1 (30%). These two phylogenetic groups consist of strains with low virulence for man. The 196 FQ-resistant isolates also mainly belonged to phylogenetic groups A (57%) and B1 (22%), but some were derived from the moderately virulent phylogenetic group D (20%). Hypervirulent strains from the B2 cluster were underrepresented (1%) in this collection. Although strains harboring a robust extraintestinal virulence factor repertoire cluster predominantly in groups B2 and D, isolates within each phylogenetic group can be further classified as extraint-

estinal pathogenic *E. coli* (ExPEC) or non-ExPEC based on the presence or absence of specific virulence traits. Only 4 FQ-resistant *E. coli* (2%) could be classified as ExPEC, and even these exhibited sparse virulence profiles. Twenty percent of the FQ-susceptible isolates were ExPEC. Thus, FQ resistance was associated with reduced inferred virulence.

FQ resistance may also be associated with strains that intrinsically have a higher overall mutation rate, since the resistance to FQs in *E. coli* usually involves the accumulation of multiple spontaneously occurring point mutations in several topoisomerase genes (16). We studied the mutation rate in 10 FQ-susceptible and 10 FQ-resistant *E. coli* isolates. Indeed, the FQ-resistant isolates exhibited significantly higher relative mutation rates.

From all this we conclude that the emergence of FQ resistance among *E. coli* predominantly regards the less virulent but highly adaptable strains within this species, basically assuring their survival as part of the commensal microflora of man, even in the face of regular antimicrobial challenges by antimicrobial agents percolating through their niches.

Besides the presence of mutations in the quinolone resistance determining regions (QRDRs) of the topoisomerase genes (DNA gyrase and topoisomerase IV), leading to modification of the target of the quinolones, three other mechanisms have been described that can increase quinolone MICs in *E. coli*: decreased intracellular drug accumulation, target protection, and enzymatic drug modification (2). The latter two mechanisms are exclusively plasmid-mediated. Although we had demonstrated that FQ-resistant *E. coli* exhibited elevated mutation rates compared to FQ-susceptible isolates, we had not confirmed that these isolates had mutations in the QRDRs of the topoisomerase genes. Furthermore, transferable resistance could not be ruled out as an additional explanation for the high prevalence of FQ resistance in *E. coli* in hospitalized patients. Therefore, we decided to investigate these resistance mechanisms in a subset of 78 *E. coli* with ciprofloxacin MICs ranging from <0.00375 to >128 mg/L. The presence of an efflux pump was investigated as well.

Resistance to ciprofloxacin was most frequently caused by mutations in the QRDR of *gyrA*, which is in agreement with the international literature (12). Two amino acid changes were present in 97% of strains with ciprofloxacin MIC ≥2 mg/L, and in none of the ciprofloxacin-susceptible strains. Four isolates (5%) carried the plasmid-mediated *qnrA* gene. Qnr proteins are able to bind to DNA gyrase and topoisomerase IV, thereby protecting them from the inhibitory activity of quinolones. The plasmid-mediated *aac(6')-Ib*-cr was detected in 13 strains (17%). This gene encodes an aminoglycoside-modifying enzyme that is also able to modify ciprofloxacin and norfloxacin. The presence of *aac(6')-Ib*-cr is of concern due to the possibility of hori-

zontal transfer and co-selection by exposure to antibiotics other than quinolones, such as aminoglycosides. Efflux pump activity was found in three FQ-susceptible isolates.

In conclusion, the high level of quinolone resistance in hospitals in Indonesia may not only be explained by limited clonal spread and spontaneously occurring point mutations leading to amino acid changes in gyrase A, but also by plasmid-mediated resistance mechanisms. For a full appreciation of the combinations of resistance mechanisms in association with certain MIC levels, mutations in the two genes identified in topoisomerase IV, *parE* and *parC*, need to be studied too, as well as the permeability of bacterial cell membranes. This will be the subject of future investigations.

Clinical use of FQs is considered an important risk factor in the emergence of FQ-resistance in the hospital setting (12). Among the hospitalized patients that were colonized with *E. coli* in our study, the quinolones were the third most used class of antibiotics, but most of these were used in combination with another antibiotic (6). Quinolone use was a risk factor for carriage of *E. coli* with any resistance, but a clear-cut association between FQ use and FQ resistance could not be identified due to lack of statistical power. Within the total group of discharged patients from whom *E. coli* were included in this molecular sub-analysis, carriage of a ciprofloxacin-resistant *E. coli* was associated with previous use of ciprofloxacin.

Not only the use of quinolones in humans, but also the quinolone use in animals may be responsible for the rapidly increasing quinolone resistance in *E. coli* (12). In Asia, several quinolones, including fluoroquinolones licensed for humans (ciprofloxacin, ofloxacin, and norfloxacin), have been approved for animal use (31). To the best of our knowledge, reports on the antibiotic use among animals in Indonesia are not available.

Cefotaxime

The prevalence of cefotaxime resistance in *E. coli* was highest among the fecal flora of patients on the day of discharge (13%) and lowest among persons visiting a primary health center and relatives accompanying patients on admission (<1%). Whenever an *E. coli* isolate is found to be resistant or intermediate susceptible to cefotaxime or another third-generation cephalosporin, a confirmation test should be performed to demonstrate the expression of an extended-spectrum β-lactamase (ESBL). ESBLs are plasmid-mediated enzymes that have the ability to hydrolyse and cause resistance to penicillins, narrow- and extended-spectrum cephalosporins, and aztreonam. In the clinical microbiology laboratory its accurate detection is crucial for the management of patients, as inappropriate antimicrobial therapy in the initial stages of (bloodstream) infections caused by ESBL-producing bacteria significantly increases morbidity and mortality. In addition, ESBL detection is essential for surveillance and infection prevention purposes. Molecular analyses are necessary to fully understand the epidemiology of ESBL-producing bacteria.

We studied the presence and expression of ESBLs in the commensal *E. coli* with reduced susceptibility to cefotaxime and characterized the genes encoding the ESBL enzymes. Other Enterobacteriaceae with reduced susceptibility to cefotaxime that were collected during our surveillance in 2001-2002 were included as well.

At the time of discharge almost 10% of the patients carried confirmed ESBL-positive Enterobacteriaceae as dominant fecal flora. On admission, only 6 of 998 (1%) patients were colonized. Fecal carriage of confirmed ESBL-producing Enterobacteriaceae among healthy persons or persons visiting a primary health center was not detected. The 107 ESBL-positive strains included 68 *E. coli*, 35 *K. pneumoniae*, 3 *Enterobacter cloacae*, and 1 *Citrobacter freundii*. $bla_{\text{CTX-M-15}}$ was the most prevalent ESBL in both *E. coli* (47%) and *K. pneumoniae* (46%). Other ESBL types found were: SHV-2, -2a, -5, -12, CTX-M-3, -9, -14, and TEM-19. ESBL-positive *E. coli* belonged to phylogenetic groups A (50%), B1 (27%), and D (23%). PFGE revealed extensive genetic diversity among the isolates, but some clonality was observed among CTX-M-15-positive *K. pneumoniae* from Semarang.

Most of the ESBL-positive microorganisms had been isolated from patients who were discharged from the departments of Surgery of both hospitals. Antibiotic use, and especially the use of third-generation cephalosporin, has been described as a risk factor for the acquisition of ESBL-producing bacteria (25). In the two hospitals where our study was carried out, the third-generation cephalosporins were the second most prescribed antibiotics in the hospitals, and most of these were administered in the departments of Surgery (9). Due to lack of statistical power no significant correlation between the use of cephalosporins and carriage of a cefotaxime-resistant *E. coli* could be established, but it is reasonable to assume that ESBL-producing microorganisms as dominant intestinal flora in the discharged patients reflects exposure to extended-spectrum cephalosporins and subsequent selection of these strains (6).

The key question is whether the ESBL-producing Enterobacteriaceae that appear as dominant intestinal flora during hospitalization are nosocomially acquired or not. Since molecular typing only provided evidence for some limited spread, we hypothesize that ESBL-positive isolates were already present in low numbers in the intestinal flora of patients when admitted to the hospital, which were subsequently selected after exposure to extended-spectrum cephalosporins. However, this needs further investigation, using enrichment broths. Another possibility is transfer of genetic elements between non-related strains, but given the large variety of bla_{ESBL} genes in these commensal bacteria this cannot explain the whole picture.

In conclusion, fecal carriage of ESBL-producing Enterobacteriaceae as dominant flora in Indonesia was almost exclusively hospital-associated. Although colonized persons are at risk for subsequent infection, whether ESBL-producing isolates were present in clinical cultures was unknown. Therefore, we studied clinical ESBL-

producing Enterobacteriaceae that were prospectively collected in 2005 in the Dr. Soetomo Hospital in Surabaya.

Overall, the epidemiology of clinical ESBL-producing *E. coli* in Surabaya was consistent with the worldwide situation of CTX-M-producing *E. coli* being more prevalent than classic TEM- and SHV-variants. The gene encoding CTX-M-15 was found in as many as 95% of *E. coli*, which is a much higher prevalence than found in the commensal isolates. The CTX-M-15-positive clinical *E. coli* isolates were partly clonal and partly non-clonal as demonstrated by PFGE and repetitive-sequence-based PCR (rep-PCR). This finding suggests that clonal spread of epidemic strains was present, but may not be the only explanation for the epidemiology of CTX-M-15-positive clinical *E. coli* in Surabaya.

Regarding the worldwide dissemination of CTX-M-positive *E. coli*, it is not yet understood whether spreading of highly virulent clones or epidemic plasmids between non-related strains is most important in this pandemic. Although previous reports on nosocomial infections often showed heterogeneity in CTX-M-positive *E. coli*, recent publications describe the spread of a highly virulent CTX-M-15-positive ciprofloxacin-resistant B2-*E. coli* O25:H4-ST131 that is associated with urinary tract infections. Pitout et al. recently demonstrated that the rep-PCR DiversiLab™ system successfully identified this multidrug-resistant *E. coli* clone ST131 (26). We showed that 37% of CTX-M-15-positive *E. coli* from Surabaya belonged to phylogenetic group B2. Fifteen of these were ciprofloxacin-resistant, of which only two had reduced susceptibility to amikacin, while most CTX-M-15-positive *E. coli* ST131 isolates are resistant to amikacin. The 15 isolates, however, exhibited identical rep-PCR profiles (type G) and it would be interesting to compare our rep-PCR images to results obtained in other laboratories throughout the world via a public database as is available for multilocus sequence typing (http://www.mlst.net). To our knowledge, such a DiversiLab™ database does not exist as yet, but is urgently required.

Similar to *E. coli*, CTX-M-15 was the most prevalent bla_{ESBL} in *K. pneumoniae* (56%), but unlike *E. coli*, there was no clonality among CTX-M-positive isolates.

In conclusion, we have shown that Indonesia is another (developing) country in the Southeast Asian region affected by the spread of bacterial isolates harbouring ESBL genes. CTX-M-15 is the most prevalent ESBL in both commensal and clinical Enterobacteriaceae. We hypothesize that various bla_{ESBL} genes are introduced in the hospital by low-level intestinal carriers, which may be selected by exposure to third-generation cephalosporins. Plasmids containing $bla_{\text{CTX-M-15}}$ are subsequently easily spread to non-related, more virulent *E. coli* in other patients, and those *E. coli* will cause infections. This needs to be further investigated. Continued targeted surveillance of clinical and non-clinical Enterobacteriaceae is necessary to monitor the evolving epidemiology of ESBLs in this part of the world.

RECOMMENDATIONS FOR CONTROL MEASURES

Although emergence of resistance to antimicrobials is a natural biological phenomenon, certain human practices can stop or at least slow down the process. Prudent use of antibiotics and an active infection control programme to prevent the selection and dissemination of resistant bacteria inside healthcare institutions are the critical determinants in managing the further emergence of antimicrobial resistance. In the second phase of the AMRIN study, which lasted from February 2003 to May 2005, intervention studies were performed to promote the prudent use among patients with fever upon admission to the hospital, to improve antimicrobial prophylaxis for surgery, and to increase adherence to standard precautions. The results were recently presented in the theses by dr. Hadi and dr. Duerink (5, 8). As is observed in other developed and developing countries, the implementation of guidelines on antimicrobial therapy and infection control policies proved to be difficult.

Hadi et al. showed that the absence of adequate microbiological diagnostics was an important drawback to the prudent use of antibiotics in the Dr. Soetomo Hospital in Surabaya. Therefore, priority should be given to the improvement of microbiological laboratory services in Indonesia, for both surveillance and clinical purposes. Quality assurance should be implemented in each laboratory. On a national level, an accreditation system for laboratories could be organized. Overall resistance data should be reported regularly to the clinicians. However, such reports will only reflect resistance among strains clinically detected. When clinicians only order cultures in cases of repeated failure of empiric treatments, as is the case in Indonesian health care today, the strains collected at the level of the microbiology laboratory will be heavily biased towards nosocomially acquired, multi-resistant strains. Such summary data on antimicrobial resistance patterns can, therefore, not be used to make rational choices for patients presenting with infection at the time of admission. Rather, Indonesian clinicians should be encouraged to perform cultures as part of the first diagnostic work up at the time of presentation in order to make proper diagnoses and allow the microbiology laboratory to generate susceptibility reports that are more useful in daily practice.

Infection control in a resource-limited country, as is Indonesia, differs substantially from that in the developed world. To stop the dissemination of resistance in the hospital, priority should be given to hand hygiene. Duerink et al. found that alcohol-based hand rub was poorly accepted by health care workers. MRSA needs to be controlled primarily through infection control measures. The “search-and-destroy” policy that is proven to be successful in the Netherlands to stop any spread of MRSA, should be adapted to the Indonesian health care setting.

RECOMMENDATIONS FOR FUTURE RESEARCH

S. aureus has been a neglected pathogen in developing countries. We showed that PVL-positive MSSA are successful commensals in Indonesia. Whether they are also successful pathogens needs to be corroborated by analyzing clinical isolates. Clinical isolates of *S. aureus* infections in both the community and the hospital should be prospectively collected and analyzed for the presence of both methicillin-resistance and PVL. Resistance to vancomycin should be monitored as well.

Our study was carried out in 2001-2002 and the actual prevalence of MRSA among carriage isolates in patients is likely to have risen since then as a "search-and-destroy" policy has not been implemented in Indonesian health care institutions. Therefore, a surveillance study, specifically focused on MRSA, should be repeated in health care settings in Indonesia, in combination with the implementation of infection control measures that are suitable for the local situation. The rate of nasal carriage of *S. aureus* in our study was rather low compared to published literature from other parts of the world. The mechanisms leading to *S. aureus* nasal carriage are multi-factorial (28). Bacterial, environmental, and host susceptibility factors play a role as well as co-colonization with other bacterial species. Of these, bacterial interference has been postulated to be a major determinant of the *S. aureus* non-carrier state. When an ecological niche is already occupied by certain bacteria, other bacteria do not seem to have the means to replace this resident bacterial population. We unexpectedly found *S. sciuri* as a nasal commensal in Indonesia. It would be interesting to investigate the bacterial interference between *S. sciuri* and *S. aureus*.

The epidemiology of resistance, especially of β-lactamases, in Gram-negative bacteria is complex. We found a high prevalence of $bla_{\text{CTX-M-15}}$ in commensal and clinical isolates of *E. coli* and *K. pneumoniae*, but we could not fully elucidate the routes of transmission. A survey of resistance plasmids circulating in hospitalized patients and the hospital environment is needed to better understand this process. It would also be interesting to culture the fecal flora of patients at admission, and then subsequently monitor their intestinal flora during and after therapy with broad-spectrum antibiotics.

FINAL REMARKS

We showed that resistant pathogenic bacteria are prevalent in Java, Indonesia, especially in hospitals. Not surprisingly, antibiotic use was the most prominent determinant of carriage of a resistant microorganism. The epidemiology of the resistant bacteria was analyzed using a variety of molecular techniques. Knowledge of the molecular epidemiology and genetic characteristics of resistant bacteria is not only of value for

fundamental research purposes, but above all of utmost importance to get insight into the emergence and the routes of dissemination of resistant bacteria.

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Chapter 15

Summary

In this thesis we present studies on the prevalence, risk factors, molecular epidemiology, and mechanisms of antimicrobial resistance among commensal bacteria from almost 4,000 patients and healthy persons in Semarang and Surabaya, two urban regions on the island of Java, Indonesia. The studies have been performed as part of the Antimicrobial Resistance in Indonesia: Prevalence and Prevention (AMRIN) study.

Part I provides some background information on the problem of antimicrobial resistance in Southeast Asia and the activities that the AMRIN study group has set up to address this problem on the island of Java, Indonesia. **Chapter 2** presents an overview of peer-reviewed literature on antimicrobial resistance among pathogenic bacteria in Southeast Asia from January 1995 to January 2007. The general conclusion is that antimicrobial resistance in Southeast Asia is on the rise, especially among *Streptococcus pneumoniae*, *Neisseria gonorrhoeae*, and pathogens causing diarrheal diseases. For many other medically important bacteria, such as *Staphylococcus aureus*, a surveillance system for antimicrobial resistance in this part of the world is lacking, and accurate data are sparse, both in time as well as in geography. In **chapter 3**, a detailed description of the design and rationale of the AMRIN study is given. It is the first population-based study of antimicrobial resistance among common pathogenic bacteria in the commensal microflora of several groups of individuals in Indonesia.

In **chapter 4** of part II we explored the possibility of using the agar diffusion method with antibiotic impregnated disks for antimicrobial susceptibility testing in Indonesia by comparing the accuracy of disk diffusion zone diameters obtained by manual zone measurements in Indonesia to that by automated zone measurements (Oxoid aura image system) in the Netherlands. Discrepancies were analyzed by the microdilution broth method. The results of this study indicate that the disk diffusion method with manual zone measurements, if properly applied and controlled, can provide reliable data on antimicrobial susceptibilities of medically relevant bacteria in low-resource settings, such as Indonesia. The use of an automated zone reader will reduce the number of errors, and thus improve the accuracy of susceptibility test results. **Chapter 5** reports the prevalence of antimicrobial resistance among commensal isolates carried in the Indonesian population inside and outside hospitals. A total of 3,995 individuals were screened for nasal carriage of *S. aureus* and rectal colonization with *Escherichia coli*. These individuals were patients on the day of admission to the hospital (Dr. Kariadi Hospital in Semarang and Dr. Soetomo Hospital in Surabaya), patients on the day of discharge after at least five days of hospitalization, patients visiting a primary health center, and healthy relatives or household members of admission group patients. The rate of *S. aureus* nasal carriage was 9%. Two methicillin-resistant *S. aureus* (MRSA) were found, both from patients cultured at the time of

discharge from the hospital in Surabaya. Twenty-five percent of *S. aureus* isolates were resistant to tetracycline, but this was not associated with hospital stay. *E. coli* as dominant intestinal aerobic flora was cultured from 3,284 individuals. Resistance rates towards gentamicin, cefotaxime, and ciprofloxacin were low among *E. coli* cultured from patients at the time of admission (4%, 2%, and 6%, respectively), but high at the time of discharge from the hospital (18%, 13%, and 22%, respectively). Resistance rates towards ampicillin, trimethoprim-sulfamethoxazole, and chloramphenicol were substantial in all groups (43%, 35%, and 21% overall, respectively).

Part III consists of three studies focused on *Staphylococcus* spp. **Chapter 6** describes an analysis of possible associations of recent antibiotic use as well as demographic, socioeconomic, disease-related and healthcare-related determinants with nasal carriage of resistant *S. aureus* in the Indonesian population inside and outside hospitals. In hospitalized patients, the use of aminopenicillins was associated with carriage of a strain resistant to any of the tested antibiotics, but in the community there was no association between antibiotic use and resistance. In the community, crowding and low income were associated with multidrug-resistant *S. aureus*. The molecular epidemiology and population structure of the *S. aureus* carriage isolates is described in **chapter 7**. Although the prevalence of MRSA was low, we found a high prevalence of Panton-Valentine leukocidin (PVL)-positive methicillin-sensitive *S. aureus* (MSSA). This is of concern, since PVL is an exotoxin that is associated with severe infections. As determined by high-throughput amplified fragment length polymorphism (AFLP), the Indonesian *S. aureus* strains clustered within the previously defined AFLP clusters, although one of the five major clusters, clonal complex 30, was almost completely absent in Indonesia. PVL-positive strains were found in each major staphylococcal genome cluster, indicating that PVL has been introduced in distinct phylogenetic subpopulations of *S. aureus* in Indonesia.

In our surveillance study, we unexpectedly cultured *Staphylococcus sciuri*, a coagulase negative *Staphylococcus* spp. that resembles *S. aureus*, from a number of individuals (**chapter 8**). *S. sciuri* has commonly been described as a commensal of animals. We showed that hospitalized patients in Semarang with recent antibiotic use were at risk of becoming colonized with a multidrug-resistant *S. sciuri*. The clinical significance of this staphylococcal species is yet unknown.

Part IV comprises five studies devoted to Enterobacteriaceae, especially *E. coli*. An analysis of possible determinants for carriage of resistant *E. coli* in the Indonesian population inside and outside hospitals is presented in **chapter 9**. Antibiotic use was the most important independent determinant of carriage of (multidrug-) resistant *E. coli*. In the non-hospitalized population, direct associations were observed between

the use of β-lactam antibiotics and ampicillin resistance and between sulphonamide use and resistance to trimethoprim-sulfamethoxazole. Furthermore, hospital admission, diarrhoeal symptoms, and age under 16 were associated with carriage of *E. coli* resistant to any of the tested antibiotics. For hospitalized patients, having no health insurance was associated with less resistance. Although the highest levels of resistance among *E. coli* were found against ampicillin, trimethoprim-sulfamethoxazole, and chloramphenicol, the resistance rates to the fluoroquinolones (FQs) and third-generation cephalosporins were of most concern, especially in the hospital, because these limit the options for empirical therapy when such strains cause nosocomial infections. Therefore, strains with resistance to these antibiotics were analyzed in more detail. **Chapter 10** and **chapter 11** describe the studies on the fluoroquinolone-resistant *E. coli*. Evidence was provided that the high level of quinolone resistance among *E. coli* in hospitals in Indonesia was the result of limited clonal spread, spontaneously occurring point mutations leading to amino acid changes in gyrase A, and the presence of plasmid-mediated resistance mechanisms. The emergence of FQ resistance among commensal *E. coli* predominantly regarded the less virulent but highly adaptable strains within this species.

Chapter 12 reports on the commensal *E. coli* and other Enterobacteriaceae with reduced susceptibility to cefotaxime. At the time of discharge almost 10% of the patients carried confirmed extended-spectrum β-lactamase (ESBL)-positive Enterobacteriaceae as dominant fecal aerobic flora. On admission, only 1% of the patients was colonized. Fecal carriage of confirmed ESBL-producing Enterobacteriaceae among healthy persons or persons visiting a primary health center was not detected. The ESBL-positive strains were identified as *E. coli* (n=68), *K. pneumoniae* (n=35), *Enterobacter cloacae* (n=3), and *Citrobacter freundii* (n=1). $bla_{\text{CTX-M-15}}$ was the most prevalent ESBL in both *E. coli* (47%) and *K. pneumoniae* (46%). Subsequently, clinical ESBL-producing Enterobacteriaceae were prospectively collected in the Dr. Soetomo Hospital in Surabaya during a 4-month period in 2005 (**chapter 13**). Among the 73 ESBL-positive *E. coli*, the gene encoding CTX-M-15 was highly prevalent (95%). Among the 72 ESBL-producing *K. pneumoniae*, $bla_{\text{CTX-M-15}}$ was found in 56% of isolates. We found some clonality among ESBL-positive strains, indicating patient-to-patient transmission, but this could not fully elucidate the epidemiology of ESBLs in the hospital.

In Part V, **chapter 14**, the main results are summarized and discussed and suggestions for further research are provided.

Nederlandse samenvatting
Dutch summary

Resistentie van bacteriën voor antimicrobiële middelen is wereldwijd een toenemend probleem. Infecties met resistente bacteriën gaan gepaard met een verhoogde ziektelast en sterfte ten opzichte van infecties met gevoelige bacteriën. Dit proefschrift beschrijft de prevalentie (aanwezigheid), risicofactoren, moleculaire epidemiologie en de mechanismen van antimicrobiële resistentie van bacteriën in de neus- en darmflora van bijna 4000 patiënten en gezonde personen in Semarang en Surabaya, twee grote steden op het eiland Java in Indonesië. De onderzoeken zijn uitgevoerd als onderdeel van een groter onderzoek, de AMRIN-studie (Antimicrobiële Resistentie in Indonesië: Prevalentie en Preventie).

In Deel I wordt achtergrondinformatie gegeven over het probleem van antimicrobiële resistentie in Zuidoost-Azië en de initiatieven die de AMRIN studiegroep ondernomen heeft om dit probleem aan te pakken op het eiland Java, Indonesië. In **hoofdstuk 2** wordt een overzicht gegeven van resultaten uit studies over antimicrobiële resistentie van pathogene bacteriën in Zuidoost-Azië, gepubliceerd tussen januari 1995 en januari 2007. De algemene conclusie is dat antimicrobiële resistentie in Zuidoost-Azië aan het toenemen is, in het bijzonder bij *Streptococcus pneumoniae*, *Neisseria gonorrhoeae* en bacteriën die darminfecties veroorzaken. Voor veel andere medisch belangrijke bacteriën, zoals *Staphylococcus aureus*, bestaat in dit deel van de wereld geen enkel systeem van surveillance en zijn betrouwbare gegevens dus schaars. In **hoofdstuk 3** wordt een gedetailleerde beschrijving van de opzet van de AMRIN-studie gegeven. Het is het eerste populatiegebaseerde onderzoek naar antimicrobiële resistentie van bacteriën in de neus- en darmflora, zogenaamde commensale bacteriën, van verschillende groepen mensen in Indonesië.

Hoofdstuk 4 van deel II beschrijft onderzoek naar de vraagstelling of de agardiffusietechniek met gebruik van met antibiotica geïmpregneerde schijfjes een betrouwbare methode is voor het bepalen van antimicrobiële gevoeligheden in laboratoria in Indonesië. Daartoe werden de diameters van de remmingszones, zoals handmatig opgemeten in Indonesië, vergeleken met de diameters van de zones die met een automatische methode (Oxoid aura image system) waren opgemeten in Nederland. Discrepanties werden geanalyseerd met de standaard bouillonverdunningsmethode. De resultaten van dit onderzoek laten zien dat de agardiffusiemethode met handmatig

opmeten van diameters van de remmingszones een bruikbare methode is voor het bepalen van antimicrobiële gevoeligheden van medisch relevante bacteriën in een land met beperkte middelen, zoals Indonesië, mits de methode juist wordt uitgevoerd en er een systeem van kwaliteitscontroles geïmplementeerd is. Door een automatische methode van het opmeten van zone diameters te gebruiken kan het aantal fouten gereduceerd worden, waardoor de betrouwbaarheid van de resultaten van de bepalingen verder zal toenemen.

Hoofdstuk 5 beschrijft hoe vaak antimicrobiële resistentie voorkomt bij commensale bacteriën van mensen in Indonesië, zowel in het ziekenhuis als in de open bevolking. Bij in totaal 3995 mensen werd dragerschap van (resistente) *S. aureus* door middel van neuskweken en van (resistente) *Escherichia coli* door middel van rectumkweken onderzocht. Vier groepen personen werden in het onderzoek opgenomen: patiënten die werden opgenomen in een ziekenhuis (Dr. Kariadi ziekenhuis in Semarang en Dr. Soetomo ziekenhuis in Surabaya), patiënten die werden ontslagen uit het ziekenhuis na een opname van ten minste vijf dagen, patiënten die een algemeen wijkgezondheidscentrum bezochten en gezonde familieleden of huisgenoten van patiënten die werden opgenomen in het ziekenhuis. Negen procent van de onderzochte personen bleek drager van *S. aureus* in de neus, een lager percentage dan gewoonlijk wordt gevonden in Westerse, economisch ontwikkelde landen. Bij twee personen werd een meticilline-resistente *S. aureus* (MRSA) gevonden. Bij beide personen waren de kweken afgenomen op de dag van hun ontslag uit het Dr. Soetomo ziekenhuis in Surabaya. Resistentie tegen tetracycline bleek in alle vier groepen bij 20% of meer van de *S. aureus* stammen voor te komen. *E. coli* werd bij 3284 personen gevonden als dominante aëroob groeiende darmflora. De percentages van resistentie tegen gentamicine, cefotaxim en ciprofloxacine waren laag bij *E. coli* van patiënten gekweekt op de dag van opname in een ziekenhuis (respectievelijk 4%, 2% en 6%), maar hoog bij *E. coli* van patiënten gekweekt op de dag van ontslag uit het ziekenhuis (respectievelijk 18%, 13% en 22%). Resistentie tegen ampicilline, trimethoprim-sulfamethoxazol en chlooramfenicol kwam in alle groepen veel voor (respectievelijk 43%, 35% en 21%).

Deel III bestaat uit drie onderzoeken gericht op stafylokokken. In **hoofdstuk 6** wordt een analyse beschreven van de relatie tussen recent antibioticagebruik, sociaaleconomische, demografische, ziekte- en gezondheidszorg-gerelateerde factoren en neusdragerschap van een resistente *S. aureus*. Voor de analyse werden de mensen in twee groepen verdeeld: patiënten die opgenomen waren geweest in het ziekenhuis en bij wie de kweek was afgenomen op de dag van ontslag en mensen van buiten het ziekenhuis. In de ziekenhuisgroep bleek het gebruik van aminopenicillines geassocieerd te zijn met dragerschap van een resistente *S. aureus*, maar bij mensen van buiten het ziekenhuis die drager waren van een *S. aureus* bestond geen verband

tussen antibioticagebruik en resistentie. Bij mensen van buiten het ziekenhuis waren het wonen in een huis samen met negen mensen of meer en een laag inkomen risicofactoren voor het hebben van een multiresistente *S. aureus* in de neus.

De moleculaire epidemiologie en populatiestructuur van de *S. aureus* stammen wordt beschreven in **hoofdstuk 7**. Hoewel de prevalentie van MRSA onder de dragerschapsstammen laag was, bleek de prevalentie van "Panton-Valentine leukocidin" (PVL)-positieve *S. aureus* wel hoog (10.6%), hoger dan gewoonlijk gevonden wordt in collecties *S. aureus* uit Westerse landen. Dit is een zorgelijke bevinding, omdat PVL een exotoxine is dat geassocieerd wordt met invasieve huid- en weke delen infecties en zeer ernstige longontstekingen. De populatiestructuur van de *S. aureus* stammen hebben wij in kaart gebracht met behulp van een "high-throughput amplified fragment length polymorphism (AFLP)" analyse. De Indonesische *S. aureus* dragerschapsstammen bleken genetisch te behoren tot de reeds bekende AFLP clusters, zoals die gevonden zijn bij Nederlandse en Amerikaanse dragersschapsstammen. Opvallend was dat het in Nederland en Amerika veel voorkomende klonale cluster 30 nagenoeg afwezig was in Indonesië. PVL-positieve stammen bleken in alle vier grote AFLP clusters voor te komen, hetgeen suggereert dat de voor PVL coderende genen in verschillende subpopulaties van *S. aureus* in Indonesië geïntroduceerd zijn.

Tijdens onze surveillance studie naar dragers van (resistente) *S. aureus* werd bij een significant aantal personen onverwachts *Staphylococcus sciuri* aangetroffen in de kweek van de neusuitstrijk (**hoofdstuk 8**). *S. sciuri* is een coagulase-negatieve *Staphylococcus* soort, die op *S. aureus* lijkt, en vooral beschreven wordt als een commensaal bij knaagdieren. Vooral patiënten die opgenomen lagen in het Dr. Kariadi ziekenhuis in Semarang en recent antibiotica hadden gekregen liepen risico gekoloniseerd te raken met een multiresistente *S. sciuri*. De klinische betekenis van deze stafylokokkensoort is vooralsnog echter onduidelijk.

Deel IV bevat vijf studies die geheel gewijd zijn aan bacteriesoorten uit de familie Enterobacteriaceae, in het bijzonder *E. coli*. Een analyse naar de mogelijke determinanten van dragerschap van resistente *E. coli* in de Indonesische bevolking wordt beschreven in **hoofdstuk 9**. Opnieuw werden de onderzochte mensen voor deze analyse in twee groepen verdeeld: patiënten die opgenomen waren geweest in het ziekenhuis en bij wie een kweek was afgenomen op de dag van ontslag en mensen van buiten het ziekenhuis. Recent antibioticagebruik bleek in beide groepen de belangrijkste risicofactor voor dragerschap van een (multi-)resistente *E. coli*. Bij mensen van buiten het ziekenhuis werd een direct verband gevonden tussen het gebruik van β-lactam antibiotica en ampicilline resistentie en tussen het gebruik van sulfonamides en trimethoprim-sulfamethoxazol resistentie. Het opgenomen worden in een ziekenhuis, diarree en leeftijd onder de 16 jaar bleken ook factoren die te associëren

waren met dragerschap van een resistente *E. coli*; in de ziekenhuisgroep was dat het hebben van een ziektekostenverzekering. Alhoewel de percentages van resistentie van *E. coli* tegen ampicilline, trimethoprim-sulfamethoxazol, en chlooramfenicol het hoogst waren, waren de resistentiepercentages tegen ciprofloxacine en cefotaxim het meest verontrustend, met name van de *E. coli* waarmee in het ziekenhuis opgenomen patiënten gekoloniseerd waren. Dit omdat deze laatste middelen dikwijls ingezet worden als blinde therapie bij infecties verkregen in het ziekenhuis. Daarom werd besloten *E. coli* stammen die resistent waren tegen deze antibiotica verder te analyseren. In **hoofdstuk 10** en **hoofdstuk 11** wordt dieper ingegaan op de resistentie tegen ciprofloxacine. Wij hebben aangetoond dat het hoge percentage ciprofloxacine-resistente *E. coli* in de ziekenhuisgroep het gevolg was van enige klonale verspreiding, spontaan opgetreden puntmutaties leidend tot aminozuurveranderingen in het aangrijpingspunt en de aanwezigheid van plasmide-gemedieerde resistentiemechanismen. Op basis van een aanvullende analyse van virulentiefactoren kon bepaald worden dat de ciprofloxacine-resistente *E. coli* vooral behoorden tot de minder virulente types binnen de soort.

Hoofdstuk 12 beschrijft het onderzoek naar commensale *E. coli* en andere Enterobacteriaceae met een verminderde gevoeligheid voor cefotaxim. Op het moment van ontslag uit het ziekenhuis na een opname van ten minste vijf dagen bleek bijna 10% van de patiënten een extended-spectrum β-lactamse (ESBL)-producerende bacterie uit de Enterobacteriaceae familie bij zich te dragen als dominante aëroob groeiende darmflora. Op het moment van opname was dit slechts bij 1% van de patiënten het geval. ESBL-producerende bacteriën werden niet gedetecteerd bij gezonde personen of personen die een algemeen wijkgezondheidscentrum bezochten. De ESBL-positieve stammen werden geïdentificeerd als *E. coli* (n=68), *K. pneumoniae* (n=35), *Enterobacter cloacae* (n=3) en *Citrobacter freundii* (n=1). Het gen dat voor de ESBL CTX-M-15 codeert werd veel gevonden bij *E. coli* (47%) en *K. pneumoniae* (46%).

Vervolgens werden klinische ESBL-producerende *E. coli* en *K. pneumoniae* stammen prospectief verzameld in het Dr. Soetomo ziekenhuis in Surabaya gedurende een periode van 4 maanden in 2005 (**hoofdstuk 13**). Het gen dat voor CTX-M-15 codeert werd in bijna alle *E. coli* stammen aangetroffen (69/73, 95%) en in iets meer dan de helft van de *K. pneumoniae* (40/72, 56%). Met behulp van verschillende typeringsmethodes werd wel enige klonaliteit aangetroffen, wijzend op overdracht van patiënt naar patiënt, maar dit kon niet volledig de epidemiologie van de ESBL-producerende bacteriën verklaren.

In Deel V, **hoofdstuk 14**, worden de belangrijkste resultaten besproken en in bredere context geplaatst. Tevens worden suggesties gedaan voor toekomstig onderzoek.

Ringkasan dalam bahasa Indonesia
Indonesian summary

Pada tesis ini kami paparkan studi prevalensi, faktor - faktor risiko, epidemiologi molekuler dan mekanisme resistensi terhadap antibiotika pada bakteri komensal dari 4,000 penderita dan anggota keluarga yang sehat dari penderita tersebut, di Semarang dan Surabaya, di dua area perkotaan di pulau Jawa, Indonesia. Studi yang telah dikerjakan ini merupakan bagian dari studi *Antimicrobial Resistance in Indonesia: Prevalence and Prevention (AMRIN)*.

Bagian I memaparkan informasi beberapa latar belakang dari masalah resistensi bakteri terhadap antibiotika di Asia Tenggara dan aktifitas kelompok studi AMRIN yang ditujukan untuk mengetahui masalah resistensi di pulau Jawa, Indonesia.

Bab 2 memaparkan gambaran dari hasil telaah pustaka tentang resistensi bakteri penyebab infeksi terhadap antibiotika, di Asia Tenggara dari bulan Januari 1995 sampai dengan bulan Januari 2007. Simpulan umum adalah bahwa resistensi bakteri terhadap antibiotika di Asia Tenggara cukup tinggi, khususnya *Streptococcus pneumoniae*, *Neisseria gonorrhoeae* dan bakteri penyebab penyakit diare. Untuk beberapa bakteri yang berperan penting sebagai penyebab penyakit infeksi, seperti *Staphylococcus aureus*, sistem surveilans untuk resistensi terhadap antibiotika masih kurang dan data yang cukup mewakili dalam tatanan geografis dan waktu masih belum cukup untuk bisa dipakai sebagai pedoman.

Pada **bab 3**, uraian secara lengkap dari rancangan dan kerangka pikir dari studi AMRIN dipaparkan. Ini adalah studi yang pertama kali dikerjakan di Indonesia, tentang resistensi bakteri terhadap antibiotika, pada bakteri flora komensal pada beberapa kelompok individu di Indonesia.

Pada **bab 4** dari bagian II kami menyelidiki kemungkinan penggunaan metode difusi dengan disk antibiotika untuk uji kepekaan terhadap antibiotika di Indonesia dengan membandingkan akurasi pengukuran diameter daerah hambatan pada metode difusi dengan cara pengukuran secara manual di Indonesia dengan pengukuran secara otomatis (Oxoid *aura image system*) di Belanda. Perbedaan dianalisis dengan metode mikrodilusi cair. Hasil dari studi ini menunjukkan bahwa metode difusi cakram dengan pengukuran daerah hambatan secara manual, jika dikerjakan dengan kontrol kualitas yang baik, dapat digunakan sebagai data yang akurat sebagai uji kepekaan pada tempat dengan sarana-prasarana terbatas, seperti di Indonesia. Pengunaan metode

pengukuran daerah hambatan secara otomatis akan menurunkan tingkat kesalahan, dan ini dapat meningkatkan akurasi dari hasil uji kepekaan. **Bab 5** melaporkan prevalensi resistensi bakteri terhadap antibiotika pada isolat bakteri komensal yang ada di populasi Indonesia baik di dalam maupun diluar rumah sakit. Sejumlah 3,995 individu ditapis, apakah mengandung *S. aureus* di hidung dan *Escherichia coli* di rektum.

Individu – individu ini adalah penderita yang masuk rumah sakit, penderita yang keluar dari rumah sakit setelah 5 hari dirawat, penderita yang berkunjung di Puskesmas dan keluarga dekat penderita dari kelompok penderita yang masuk rumah sakit. Rata–rata *nasal carriage S. aureus* adalah 9%. Ditemukan 2 isolat *S. aureus* adalah metisilin resisten *S. aureus* (MRSA), yang keduanya berasal dari penderita yang keluar dari rumah sakit di Surabaya.

Sebanyak 25% isolat *S. aureus* resisten terhadap tetrasiklin, tetapi tidak ada hubungannya dengan perawatan di rumah sakit. *E. coli* sebagai flora usus yang dominan diperoleh dari 3,284 individu. Tingkat resistensi terhadap gentamisin, sefotaksim, dan siprofloksasin adalah rendah diantara *E. coli* yang dikultur dari penderita masuk rumah sakit (berturut-turut 4%, 2% dan 56%) tetapi tinggi pada penderita yang keluar dari rumah sakit (berturut turut 18%, 13% dan 22%). Resistensi terhadap ampisilin, trimethoprim-sulfametoksasol (Ko-trimoksasol) dan kloramfenikol ada pada semua grup (berturut-turut 43%, 35% dan 21%).

Bagian III terdiri dari tiga studi yang difokuskan pada *Staphylococcus* spp.

Bab 6 mendiskripsikan tentang analisis kemungkinan hubungan antara penggunaan antibiotik faktor - faktor seperti demografi, sosial ekonomi, penyakit dan perawatan kesehatan, terhadap pengidap nasal (*nasal carriage) S. aureus* resisten di populasi di dalam dan diluar rumah sakit di Indonesia.

Pada penderita yang dirawat di rumah sakit, penggunaan aminopenisilin berhubungan dengan pengidap bakteri resisten terhadap beberapa antibiotika yang diuji, tetapi pada komunitas tidak ada hubungan antara penggunaan antibiotik dengan resistensi. Di komunitas, kepadatan jumlah anggota yang tinggal serumah dan pendapatan yang rendah berhubungan dengan kejadian bakteri multi-resisten. Epidemiologi molekuler dan *population structure* dari isolat *S. aureus* dideskripsikan pada **bab 7**. Meskipun prevalensi MRSA rendah, kami menemukan tingginya prevalensi dari *Panton-Valentine Leukocidin (PVL)* yang positif pada metisilin sensitif *S. aureus* (MSSA). Hal ini perlu perhatian khusus, karena PVL adalah eksotoksin yang berhubungan dengan kejadian infeksi yang berat. Seperti telah diperiksa *high-throughput amplified fragment length polymorphism (AFLP)*, klaster isolat *S. aureus* orang –orang Indonesia terletak di dalam kluster kluster ALFP yang telah didefinisikan sebelumnya, namun demikian satu dari lima klaster mayor, *clonal complex* 30, hampir tidak ada sama

sekali di Indonesia. Isolat dengan PVL positif ditemukan pada masing masing klaster *genome* staphylococcus, hal ini menunjukkan bahwa PVL telah ada pada *phylogenic subpopulations S. aureus* tertentu, di Indonesia. Pada studi surveilans kami, tidak memperkirakan adanya hasil biakan *Staphylococcus sciuri*, yang merupakan stafilokokus koagulase negatif yang menyerupai *S. aureus*, dari sejumlah individu (**bab 8**). *S sciuri* pada umumnya ditemukan sebagai komensal pada hewan. Kami paparkan bahwa penderita yang dirawat di rumah sakit di Semarang dengan penggunaan antibiotika sebelumnya merupakan faktor resiko untuk terjadinya kolonisasi *S sciuri*. Peran khusus di klinik dari spesies stafilokokus ini belum diketahui.

Bagian IV berisi tentang lima studi tentang *Enterobacteriaceae*, khususnya *E. coli*. Analisis faktor - faktor resiko pengidap *E. coli* resisten pada populasi Indonesia di dalam dan di luar rumah sakit depaparkan pada **bab 9**. Penggunaan antibiotika merupakan faktor resiko terpenting terjadinya kebal ganda (*multiple drug resistance)* pada *E. coli*. Pada populasi yang tidak dirawat di rumah sakit, terdapat hubungan langsung antara penggunaan antibiotika golongan beta-laktam dengan resistensi terhadap ampisilin dan antara penggunaan sulfonamid dengan resistensi terhadap trimethoprim–sulfametoksasol. Namun demikian, penderita yang masuk rumah sakit, gejala diare, dan umur dibawah 16 tahun berhubungan dengan kejadian pengidap *E. coli* yang resisten terhadap beberapa antibiotika. Untuk penderita yang dirawat di rumah sakit, tidak berhubungan dengan status asuransi. Meskipun batas tertinggi dari resistensi diantara *E. coli* ditemukan terhadap ampisilin, trimethoprim-sulfametoksasol, dan kloramfenikol, tingkat resistensi terhadap golongan fluorokuinolon dan generasi ke 3 sefalosporin, menjadi perhatian utama, khususnya di dalam rumah sakit, karena hal ini bisa membatasi terapi empirik, khususnya pada pengananan infeksi nosokomial. Namun demikian, analisis mendalam dilakukan pada bakteri yang resisten terhadap antibiotika tersebut.

Bab 10 dan **bab 11** memaparkan studi fluorokuinolon resisten pada *E. coli*. Dari bukti klinik ditemukan bahwa tingkat resistensi yang tinggi terhadap kuinolon pada *E. coli* di rumah sakit di Indonesia adalah hasil dari penyebaran klonal terbatas, secara spontan terjadi mutasi titik (point mutation) yang berakibat perubahan asam amino pada ensim gyrase A, dan juga akibat resistensi yang diperankan oleh plasmid. *E. coli* yang resisten kuinolon ini menunjukkan bahwa sifat virulennya menurun namun sifat kemampuan adaptasinya meningkat.

Bab 12 melaporkan pada *E. coli* komensal dan *Enterobacteriaceae* lain dengan penurunan sensitivitas terhadap sefotaksim. Penderita yang keluar rumah sakit hampir 10% menjadi pengidap *Enterobacteriaceae* penghasil *extended-spectrum* β-*lactamase* (ESBL) sebagai flora fecal yang dominan. Pada penderita masuk rumah sakit, hanya 1% dari pasien yang dikolonisasi. Tidak ditemukan pengidap Enterobac-

teriaceae penghasil ESBL pada orang sehat dan pengunjung Puskesmas. Isolat dengan ESBL-positif termasuk 68 *E. coli*, 35 *K. pneumoniae*, 3 *Enterobacter cloacae*, and 1 *Citrobacter freundii*. $bla_{\text{CTX-M-15}}$. Dua tertinggi adalah *E. coli* (47%) dan *K. pneumoniae* (46%). Enterobacteriaceae penghasil ESBL dari spesimen klinik dikoleksi secara prospektif di RSUD Dr. Soetomo Surabaya selama periode 4 bulan, pada tahun 2005 (**bab 13**). Diantara 73 *E. coli* dengan ESBL-positif, gen pengkode CTX-M-15 adalah tinggi prevalensinya (95%). Diantara 72 *K. pneumoniae* yang memproduksi ESBL, $bla_{\text{CTX-M-15}}$ ditemukan pada 56% dari jumlah isolat. Kami menemukan beberapa klon diantara isolat yang positif ESBL, yang mengindikasikan penyebaran dari penderita ke penderita., tetapi tidak dapat dijelaskan secara penuh dari epidemiologi ESBL rumah sakit.

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Dankwoord
Juliëtte Astrid Severin

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Dr. Endang Sri Lestari, dear Endang. You have sacrificed a lot to do this PhD project. When you are in Rotterdam for the PhD ceremony, it will be your 8th visit to the Netherlands (or have I lost count?). In between, we kept in touch with hundreds of e-mails. Thank you for the pleasant cooperation.

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Stagiaires zijn onmisbaar voor ieder promotieonderzoek. Bij dit proefschrift zijn 3 stagiaires betrokken geweest. Allereerst Martijn Pastink. Jij hebt een groot deel van het moleculaire stafylokokken-werk uitgevoerd. Wendy Kloezen heeft zich ontfermd over de commensale cefotaxim-resistente Enterobacteriaceae. Tsz Shan Chan heeft een deel van het onderzoek van de ciprofloxacin-resistente *E. coli* stammen uitgevoerd. Allemaal bedankt voor de prachtige resultaten.

Alle analisten van het diagnostisch lab wil ik bedanken. Als ik vroeg om even een stammetje mee te nemen in de AccuProbe of latex, of om een E-test zus of zo, dan was iedereen altijd bereid dit te doen. Speciale dank aan de modi-analisten: Liesbeth van der Zwaan, Liset Dullaart, Willemien Zandijk en Jolanda Maaskant. Jullie hebben Diversilab en *mecA* PCRs tussen de dagelijkse berg werk door gedaan, met altijd fantastisch mooie resultaten.

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Curriculum vitae
Endang Sri Lestari

Endang Sri Lestari was born on October 16, 1966 in Purwodadi – Grobogan, Central Java, Indonesia. She graduated from elementary school (SD Negeri IV Purwodadi - Grobogan) in 1979 and junior high school (SMP Negeri 1 Purwodadi - Grobogan) in 1982. She moved to Semarang to continue her study at senior high school (SMA Negeri 1 Semarang) from 1982 to 1985. After completing her education from Medical Faculty, Diponegoro University Semarang as medical doctor in 1993, she worked at private clinics for one year until 1994. From 1994 to 1997 she worked at a primary health center (puskesmas) in Ngaliyan, Semarang. Since February 1997 until now, she works at Department of Clinical Microbiology, Medical Faculty, Diponegoro University, Semarang. She has been involved in the AMRIN study since January 2001. During the AMRIN study period, she was trained at Department of Infectious Diseases, Leiden University Medical Center (LUMC) (Prof. P.J. van den Broek, MD, PhD), Department of Medical Microbiology and Infectious Diseases, Erasmus University Medical Center, Rotterdam (Prof. H.A. Verbrugh, MD, PhD, and I.C. Gyssens, MD, PhD), and Department of Infectious Diseases, Radboud University Medical Center, Nijmegen (M. Keuter, MD, PhD). Endang is married to Achmad Fuadi and they have two sons, Qorri (1997) and Vaza (2000).

Curriculum vitae
Juliëtte Astrid Severin

Juliëtte Astrid Severin was born in Amsterdam, The Netherlands on July 8th 1975.

After completing her secondary education (Atheneum) at the Twents Carmellyceum in Oldenzaal in 1993, she went to Brazil for one year as an exchange student. At the Universidade Estadual de São Paulo in Rio Claro, Brazil, she attended courses in Anatomy and Ecology. In 1994 she began her medical training at the Faculty of Medicine at the Leiden University Medical Center (LUMC). As an undergraduate student she participated in research projects at the Department of Medical Microbiology at the LUMC on the molecular epidemiology of *Acinetobacter* spp. (L. Dijkshoorn, PhD) and at the Universidade Federal de São Paulo in São Paulo, Brazil, on the interaction of AIDS and tropical diseases (Prof. A.C. Pignatari, MD, PhD).

After receiving her Medical Doctor degree in 2001 she was a resident in Internal Medicine at the Reinier de Graaf Gasthuis Hospital in Delft (E. Maartense, MD, PhD). In November 2001 she started her residency training in Medical Microbiology in the Erasmus MC in Rotterdam (Prof. H.A. Verbrugh, MD, PhD) and Reinier de Graaf Gasthuis in Delft (R.W. Vreede, MD, PhD). Since 2002 she has been involved in the AMRIN study, but the official PhD period started in 2005. The results are presented in this dissertation.

Since November 2006 she has been working as a clinical microbiologist in the Department of Medical Microbiology and Infectious Diseases, Erasmus MC Rotterdam. Juliëtte is married to Marcelo Goossens and together they have a son, Sibolt (2008).

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Endang Sri Lestari

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PhD Portfolio
Endang Sri Lestari

PhD Student	Endang Sri Lestari
Department	Medical Microbiology and Infectious Diseases
PhD period	2001-2009
Promoters	Prof.dr. H.A. Verbrugh, Prof.dr. K. Kuntaman
Supervisor	M. Keuter, MD, PhD

1. PhD training	Year
General courses	
- Didactic skills	2001, 2006, 2008
- Research Methodology	2001
- Data Analysis with SPSS Program	2001
Medical Training	
- Clinical Microbiology course, Semarang, Indonesia	2004
- Clinical Microbiology course, Surabaya, Indonesia	2004
- Course on Infectious Diseases, LUMC, Leiden	2001
- Course on Infectious Diseases, Radboud University Medical Center, Nijmegen	2001
- Course on Medical Microbiology and Infectious Diseases, Erasmus MC, Rotterdam	2001
In-depth courses	
- The 2nd National Workshop "Strategy to Combat the Emergence and Spread of Antimicrobial Resistance Bacteria in Indonesia". Jakarta, Indonesia.	2006
- The 1st National Workshop "Strategy to Combat the Emergence and Spread of Antimicrobial Resistance Bacteria in Indonesia". Bandung, Indonesia.	2005
- Training course on molecular analysis, Erasmus MC, Rotterdam	2003, 2004
- Training course on bacteriology, Erasmus MC, Rotterdam	2001, 2002
Presentations	
- The 5th Symposium of Indonesia Antimicrobial Resistance Watch (IARW), Update the Battle between Human and Pathogenic Microbial Intelligence. Jakarta, Indonesia (oral presentation).	2009
- Annual scientific meeting of the Indonesian Association of Clinical Microbiology (PAMKI). Surabaya, Indonesia (oral presentation).	2007
- The 1st National Workshop "Strategy to Combat the Emergence and Spread of Antimicrobial Resistance Bacteria in Indonesia". Bandung, Indonesia (oral presentation).	2005
- The 14th European Congress of Clinical Microbiology and Infectious Diseases. Prague, Czech Republic (poster presentation).	2004
(Inter)national conferences	
- The 5th Symposium of Indonesia Antimicrobial Resistance Watch (IARW), Update the Battle between Human and Pathogenic Microbial Intelligence. Jakarta, Indonesia.	2009
- Open Science Meeting Indonesia Netherlands: Towards a Sustainable World. Denpasar, Bali, Indonesia.	2007

- Annual scientific meeting of the Indonesian Association of Clinical Microbiology (PAMKI). Surabaya, Indonesia.	2007
- The 7th National Congress of the Indonesian Association of Clinical Microbiology (PAMKI). Malang, Indonesia.	2006
- The 3rd Symposium of Indonesia Antimicrobial Resistance Watch (IARW): Challenges in the use of antimicrobials in emerging and re-emerging infectious diseases. Jakarta, Indonesia.	2006
- The 2nd Symposium of Indonesia Antimicrobial Resistance Watch (IARW): Evidence Based Use of Antimicrobials in the Era of Alarming Resistance. Jakarta, Indonesia.	2005
- Symposium on Infectious Diseases and the problems of Antimicrobial Resistance, "Management of Infectious Diseases, from basic Microbiology to Clinical Applied. Surabaya, Indonesia.	2004
- The 14th European Congress of Clinical Microbiology and Infectious Diseases. Prague, Czech Republic.	2004
- Wetenschappelijke vergadering van de Vereniging voor Infectieziekten. Den Haag, The Netherlands.	2001
2. Teaching	
Lecturing	
- Lectures on various topics in microbiology (2nd and 3rd year medical students)	2001-present
- Training in basic microbiology (2nd and 3rd year medical students)	2001-present
Supervising thesis	
- Supervising Bachelor's theses (5 students)	2009
- Supervising Bachelor's theses (2 students)	2004
- Reviewer Bachelor's theses (11 students)	2006
3. Other	
Reviewer of Saudi Arabia Medical Journal	2008

PhD Portfolio
Juliëtte Astrid Severin

PhD Student	Julëtte Astrid Severin
Department	Medical Microbiology and Infectious Diseases
PhD period	2005-2009
Promoters	Prof.dr. H.A. Verbrugh, Prof.dr. K. Kuntaman
Supervisor	Dr. W.H.F. Goessens

1. PhD training	Year
General courses	
- Didactic skills	2007
Medical Training	
- Residency in Medical Microbiology, Erasmus MC	2001-2006
In-depth courses	
- Boerhaave course on Infectious Diseases. Noordwijkerhout.	2007
- ESCMID-SHEA Training Course in Hospital Epidemiology. Basic infection control. Baden, Austria.	2006
- Strategy to combat the emergence and spread of antimicrobial resistant bacteria in Indonesia (workshop). Bandung, Indonesia.	2005
Presentations	
- Open Science Meeting 2007. Towards a Sustainable World. Bali, Indonesia (oral presentation and laptop presentation).	2007
- Interscience Conference on Antimicrobial Agents and Chemotherapy. Chicago, USA (poster presentation).	2007
- European Congress of Clinical Microbiology and Infectious Diseases. Nice, France (poster presentation).	2006
- Vitek® 2 meeting on ESBL (oral presentation).	2006
- European Congress of Clinical Microbiology and Infectious Diseases. Prague, Czech Republic (poster presentation).	2004
(Inter)national conferences	
- European Congress of Clinical Microbiology and Infectious Diseases. Helsinki, Finland.	2009
- European Congress on Tropical Medicine and International Health. Amsterdam.	2007
- Scientific Spring Meeting NVMM & NVvM. Papendal.	2006, 2009
- International Conference of the Hospital Infection Society. Amsterdam.	2006
2. Teaching	
Lecturing	
- Lectures on various topics in microbiology (second year medical students)	2006-present
- Training in basic microbiology (second and fourth year medical students)	2002-present
Supervising thesis	
- Supervising Bachelor's theses (3 students)	2003-2007

Appendix

Antimicrobial resistance, antibiotic usage and infection control. A self-assessment program for Indonesian hospitals

TABLE OF CONTENTS

INTRODUCTION

Antimicrobial resistance of bacteria is a worldwide and ever growing problem, directly linked to the use of antimicrobial drugs. Resistant bacteria emerge under the selective pressure of antibiotics. In hospitals, where large-scale usage of antibiotics is common, bacteria resistant to several antibiotics frequently occur and generate serious problems for the treatment of patients with infections by these microorganisms. Well known (multi)-resistant bacteria causing problems in many countries all over the world are methicillin-resistant *Staphylococcus aureus*, vancomycin-resistent enterococci, penicillin-resistant pneumococci, extended-spectrum beta-lactamase-producing *Klebsiella pneumoniae*, carbapenem-resistant *Acinetobacter baumannii*, and multiresistant *Mycobacterium tuberculosis*.

To reduce the problem of antimicrobial resistance action should be taken along two tracks: promotion of the prudent use of antibiotics and prevention of the spread of resistant bacteria. With this the prevention of antimicrobial resistance becomes the responsibility of every healthcare workers. Doctors when it comes to rational use of antibiotics, doctors and every other healthcare worker who has contact with patients, when it comes to carefully applying the rules for infection control and hospital hygiene.

Between September 2000 and 2004 the AMRIN study was performed in Surabaya and Semarang to address the problem of antimicrobial resistance in hospitals.

The AMRIN study was a collaborative study of the University of Airlangga, Dr Soetomo Hospital in Surabaya, the Diponegoro University, Dr Kariadi Hospital in Semarang and three Dutch university centres, Leiden University Medical Centre, Erasmus University Medical Centre Rotterdam and Radboud University Medical Centre Nijmegen. The study was financially supported by a SPIN grant from the Dutch Royal Academy of Arts and Sciences.

One of the aims of the AMRIN study was to develop an efficient, standardized program for the assessment of antimicrobial resistance, the quantity and quality of antibiotic usage, and infection control measures in Indonesian hospitals. This program should be applicable in every Indonesian hospital. Hospitals, which evaluate their situation according to this validated program, can pool and compare their data. By doing so a nationwide surveillance of antibiotic resistance, antibiotic use, and infection control measures can be initiated.

This document presents the validated package for self-assessment, which is developed on the basis of the results of the AMRIN study.

PRE-REQUISITES

To be effective as a hospital in the fight against antimicrobial resistance the hospital needs a number of facilities:

- an antibiotic policy committee
- an infection control committee
- a clinical microbiology service
- a clinical pharmacy service
- an infectious diseases service

The professionals, who man these services and committees, should work together closely and their activities should be supported fully by the management of the hospital. They can use the self-assessment programme presented in this document as a guide for planned actions in their hospital.

ANTIMICROBIAL RESISTANCE

Resistance to antimicrobial agents is a common trait of microorganisms, especially of bacterial pathogens. Resistance to a given antimicrobial agent may be acquired through spontaneous mutations in the microbial genome or through the uptake of resistance genes from another microbe. Antimicrobial resistance emerges as a problem when such resistant mutants are given the opportunity to multiply and spread.

Surveillance of resistant microorganisms is crucially important in any effort to control the emergence of antimicrobial resistance. Surveillance of antimicrobial resistance is the systematic collection of resistance data on clinically relevant species of bacteria, the analysis of such data and the regular reporting of the information on resistance trends to those that need to know (including medical doctors, administrators). When considering surveillance of antimicrobial resistance several important issues arise.

1] Which bacterial species should we include in our surveillance?

Not all bacterial species that can cause disease in man need to be included in a surveillance system for antimicrobial resistance. For practical purposes a selection has to be made. The two major selection criteria are the societal relevance of the bacterial species (i.e. its prevalence and impact on health of the population), and the probability of yielding data with predictive value for similar resistance in other, related, species (sentinel function). For Gram-positive species the shortlist includes in decreasing order of importance: *Staphylococcus aureus, Streptococcus pneumoniae,*

Mycobacterium tuberculosis complex, *Streptococcus pyogenes, Staphylococcus epidermidis, Enterococcus faecalis, Enterococcus faecium, Streptococcus agalactiae*. For Gram-negative species the shortlist prioritizes: *Escherichia coli,* other species of *Enterobacteriaceae* (Klebsiella, Enterobacter, Salmonella, Shigella, Serratia, Citrobacter)*, Campylobacter jejuni, Neisseria gonorrhoeae, Pseudomonas aeruginosa, Stenotrophomonas maltophilia, Burkholderia cepacia, Haemophilus influenzae, Helicobacter pylori*.

In the AMRIN study only *Staphylococcus aureus* and *Escherichia coli* were included.

2] From what source(s) and materials should the bacteria come from or be isolated?

The sources of bacterial isolates included in the surveillance should be clearly defined in advance. Resistance data are often obtained from routine susceptibility testing of strains isolated from clinical cultures since these data are readily available at little additional costs. In this case it is crucial to register the type of facility (home, primary healthcare center, long term care facility, hospital etc), the ward (in- or outpatient, nursing unit) and medical specialty the patients was cared for at the time of sampling as well as the type of specimen from which the bacterial strain was isolated. However, information about resistance based on routine clinical culture is heavily biased and has limited predictability beyond the setting from which the strains originated. E.g. antimicrobial resistance patterns observed among isolates from ICU patients do not predict the level of resistance encountered elsewhere in the same hospital, let alone in populations in the community outside the hospital.

Resistance levels among bacteria present in the community at large can be monitored by screening individuals from the community. In this case sampling of the commensal microflora in nose, throat, skin, or faeces is needed, preferably taken from a random sample of the population in a well-defined geographical area. Such a population-based study was done in the AMRIN project by taking nasal (for isolating *Staphylococcus aureus*) and rectal cultures (for isolating *Escherichia coli* and other *Enterobacteriaceae*) of patients and their relatives presenting to primary health care centers and to hospitals regardless of their underlying diseases. However, resistance information thus obtained can, vice versa, not be extrapolated to clinical settings, e.g. the ICU. It is, therefore, important to a priori focus and define the questions that surveillance needs to address.

5] How many isolates of a bacterial species do we need to include in our surveillance?

The number of isolates needed is primarily determined by the accuracy required of the resistance information. When resistance rates are reported (see below), the accuracy

can be expressed as the confidence interval around each rate. Usually the accuracy or confidence interval needed is 5% or less. This translates into approximately 300 isolates per species that need to be tested minimally. A much greater accuracy (e.g. confidence intervals <1%) is rarely needed since it does not add much to the clinical consequences of the information provided to those that need to know.

One pitfall to avoid is to include repeat or multiple isolates of a species from the same patient or individual into the surveillance set. For each bacterial species only a single, usually the first one or the dominant one (in case of multiple strains in a sample), should be included in a given timeframe (e.g. per hospital admission or within a month).

3] Which antimicrobial agents should we include in our surveillance?

For each bacterial species included in the surveillance an à priori well-defined selection of antimicrobial agents is tested. The agents to be included are selected on the basis of natural susceptibility of the species, the actual use of the agent in the hospital or area under surveillance, and the availability of a validated test method for that particular combination of 'bug & drug'. A cross table of these combinations is given in attachment 2. This example is taken from reference 1. Reference 2 specifies several, more detailed, cross tables that can be used as a guide in selecting the appropriate combinations.

Not all antibiotics need to be tested, even when they are frequently prescribed in the population under study. Within each class of antibiotics (beta-lactams, aminoglycosides, macrolides etc) one can select one or a few agents for surveillance purposes as being predictive for the whole class of antibiotics. Thus, oxacillin resistance predicts resistance to all beta-lactam antibiotics in *Staphylococcus aureus,* and erythromycin may be used to monitor macrolide resistance in this pathogen. Likewise, cefotaxime may be used as the surveillance antibiotic for beta-lactamase mediated resistance against 3rd and 4th generation cephalosporins among strains of *Escherichia coli* and other *Enterobacteriaceae.* In the AMRIN study *Staphylococus aureus* isolates were tested for their susceptibility to: oxacillin, chloramphenicol, tetracycline, gentamicin, trimethoprim-sulfamethoxazole, and erythromycin. *Escherichia coli* and other *Enterobacteriaceae* were tested against: ampicillin, cefotaxime, chloramphenicol, tetracycline, gentamicin and ciprofloxacin. In this manner, much is learned by testing only a carefully selected range of antibiotics for surveillance (see also reference 2).

4] Which microbiological methods should we use to measure resistance?

There two major requirements for the microbiological methods that can be used for resistance surveillance. First and foremost, the test system must be validated and quality controlled. This can easily be achieved by adhering to published standards for susceptibility testing (e.g. by NCCLS [see www.nccls.org]), and by including reference strains with known resistance patterns in parallel during the susceptibility tests (see reference 1 for lists of appropriate strains). The test results of quality control strains should also be recorded and checked for stability over time. There are several methods that can be used for susceptibility testing, but in the AMRIN study the NCCLS based disk diffusion test on Mueller Hinton agar was employed, since it is less costly compared to other methods, and has a long history of reliable use throughout the world.

A second issue is the type of data collected. Quantitative resistance data, e.g. the minimal inhibitory concentrations (MIC's), or the sizes of the inhibition zones around antibiotic containing disks on agar, are preferably recorded; this is more informative than recording only qualitative interpretative data, i.e. whether the isolates are considered S(usceptible), I(ntermediately susceptible), or R(esistant). Quantitative data are directly related to the microbe's susceptibility and more sensitive in detecting changes in susceptibility over time. In contrast, interpretative resistance data (S,I,R) are heavily influenced by clinical and pharmacological parameters and are much less sensitive to emerging resistances among microbial pathogens. Since qualitative categorization into SIR classes remains possible when quantitative data are recorded, but not vice versa, it is recommended that the microbiological findings are recorded quantitatively, i.e. by zone sizes or MIC's.

6] Should we continuously run surveillance or only for limited periods at regular intervals?

Surveillance of antimicrobial resistance does not need to be a continuous process. Alternatively, carefully planned systematic data can be collected during certain periods of time each year. Planning should account for possible seasonal variation in the population under study, and should, of course, meet the minimum number of strains needed for accuracy (see above). In the AMRIN study a period of 3-4 months was sufficient to collect and examine nasal and rectal specimens from 2,000 persons in each of two cities on Java. Whatever surveillance system is chosen, however, it is important that surveillance is repeated at regular intervals so that trends in resistance can be detected. When resistance data are continuously collected over time, the analysis may reveal significant trends in resistance at an earlier stage. On the other hand, such continuous surveillance systems are more difficult and costly to maintain.

7] How can we best analyze resistance data and provide information on resistance to those that need to know?

The data from susceptibility assays should be analyzed and collated into presentations that are informative to those that need to know. Since the target audience is usually not trained in medical microbiology it is important to tailor such reports well. Simple tables showing rates of resistance may suffice. The resistant rate (%) is calculated as the number of resistant isolates divided by the total number of strains tested for that particular 'bug-drug' combination times 100. An example taken from the AMRIN study is given in attachment 3.

Apart from these simple rates of resistance more complex and more informative calculations can be made. Resistance rates can be specified per bacterial species and by type of specimen or by type of infection. More informative still may be the reporting of incidence rates, e.g. number of cases of MRSA bacteremia per 1,000 hospital days. Again, reports should be kept as simple as possible and directly tailored to the needs of those to whom the reports are presented. Remember, they will only act appropriately when given relevant information in an appropriate format!

ANTIBIOTIC USAGE

Two aspects of the usage of antibiotics are important to evaluate: the amount of antibiotics used, i.e. the quantity, and the appropriateness of the choice and dosage of antibiotics and the duration of therapy, i.e. the quality of use.

Quantity

The quantity of antibiotic usage in a hospital can be measured retrospectively or prospectively. In the AMRIN study a retrospective method was used because this method requires less time of the investigators than a prospective method. Validation of the method in one of the hospitals learned that the retrospective method leads to underestimation of the amount of antibiotics usage due to incompleteness of data in the medical records (Hadi U et al. Audit of antibiotic prescribing in two governmental teaching hospitals in Indonesia. Clin Microbiol Infect 2008; 14:698-707). The level of underestimation in a given setting should be determined. For comparisons between hospitals this is essential, for comparisons through time within one hospital this is optional.

Two outcome measures are used to quantify antibiotic consumption:

1. the percentage of admitted patients that are treated with antibiotics during stay in the hospital.

2. the amount of antibiotics used expressed as Defined Daily Doses (DDD) per 100 patient days.

The DDD of a drug is the assumed average maintenance dose per day for a drug used for its main indication in adults.

DDDs provide a fixed unit of measurement independent of price and formulation enabling the researcher to assess trends in drug consumption and to perform comparisons between population groups. DDDs are assigned by the World Health Organization Collaborating Centre for Drugs Statistics Methodology http://www.whocc.no/atcddd/(accessed 15 July 2004).

Calculation antibiotic DDDs is facilitated by the Antibiotic Consumption Calculator (ABCcalc) tool of the European Study Group on Antibiotic Policies (ESGAP), downloadable from the internet free of charge http://www.escmid.org/sites/index_f.asp?par=2.5

Retrospective determination of quantity of antibiotic use

Medical record viewing at discharge:
On the day of discharge of a patient the medical record is screened for prescriptions of antibiotics. Form 'Antibiotic usage in hospitals' (see attachments) is used to record the findings. The procedure is described in detail in a standard operational procedure (SOP 'Antibiotic usage in hospitals', see attachments). Of each prescription the name of the antibiotic, dose, frequency, duration, and whether the antibiotic was given for therapy, prophylaxis or unknown reasons are recorded (for definitions, see SOP). From these data the maximal amount of antibiotic prescribed can be calculated by multiplying dose, frequency and duration.

The medical records, containing the medical follow up notes and the nursing record are inspected to determine which doses of the prescribed antibiotics actually have been administered to the patient. From these data the minimal amount of antibiotic usage is calculated by adding up all the administered dosages. This is the amount one can assume that it has been administered to patients.

Prospective determination of quantity of antibiotic use

Daily interviews of patients and nurses:
For prospective determination of antibiotic usage patients should be followed up daily from the day of admission until discharge. Each day the patient is visited by the investigator and inquired about the medication taken during the last 24 hours.

The investigator checks all available sources of antibiotic prescribing and dispensing and, if indicated, asks the nurses whether an antibiotic has been administered. Each administered dose of antibiotic is recorded. The same form can be used as for the retrospective data collection (attachment 5). The amount of used antibiotics is obtained by adding up all the administered dosages.

Validation of retrospective determination

To find out to which amount antibiotic usage is underestimated by the retrospective method, the results are compared with those of the prospective method (see attachment "Validation of retrospective method to measure antibiotic use'). Validation should only be done in a subset of patients. It is not necessary to collect data on all hospitalization days of the patients. In that case the comparison between the two methods is limited to those days that are documented in the prospective study. Investigators should perform the retrospective and prospective collection of data independently. The prospective investigator includes patients. On the day of discharge he informs the retrospective investigator. Data collection is done as described above for the prospective and retrospective method.

Quality

The quality of antibiotic use is measured by inspection of the medical records by independent reviewers who use a standardized format to assess antibiotic prescriptions (Gyssens IC, Van den Broek PJ, Kulberg BJ, Hekster YA, Van der Meer JWM. Optimizing antimicrobial therapy. A method for antimicrobial drug evaluation. J Antimicrob Chemother 1992; 30:724-727; for flow chart see attachment 8).

Five outcome measures are used to qualify antibiotic use:

1. Percentage of prescriptions without justification.
2. Percentage of prescriptions with inappropriate choice.
3. Percentage of prescriptions inappropriate duration of therapy.
4. Percentage of prescriptions with inappropriate dosage.
5. Percentage of prescriptions appropriate in all respects.

Several of these outcome measures are subdivided in more detailed assessments. The choice of an antibiotic can be inappropriate because there is a more effective, less toxic, less expensive alternative or an alternative with less broad spectrum. Duration of therapy can either be too short or too long. The dosage can be inappropriate because the dose, the interval or the route of administration is not optimal.

The reviewers follow the flow chart for quality evaluation (see attachment 8) and report their findings using the report form for quality evaluation (see attachment 7). Quality evaluation is a time-consuming activity, therefore it should be done in a random selection of patients. The experience is that a selection of 40 to 50 patients gives a reliable picture of the quality of antibiotic use in a department.

At least two persons should review the same records independently. Differences can be minimalised by organizing focus discussion groups for reviewers. The reviewers should evaluate the cases independently and should be experienced in the treatment of infectious diseases, preferably have a specialist training in infectious diseases.

INFECTION CONTROL

Infection control in the hospital is assessed by surveillance of healthcare-associated infections, an inquiry into the knowledge, attitude, and behavior of healthcare workers concerning infection control, and evaluation of the infrastructure of infection control.

Outcome measures for infection control are:

1. the prevalence (or incidence) of healthcare-associated infections
2. level of knowledge, attitude and behavior regarding infection control
3. the infrastructure score

Surveillance

Surveillance of infections can be realized through cross-sectional studies resulting in prevalence rates or prospective follow-up studies resulting in incidence rates. The AMRIN study group explored the possibility for surveillance by retrospective inspection of the medical records. However, the medical records contained insufficient information to diagnose healthcare-associated infections in retrospect. In the framework of the AMRIN study a method for repeated cross-sectional studies was developed, which is presented here (attachments 9 and 10).

Knowledge, attitude and behavior

Information about knowledge, attitude, and behavior of the healthcare workers regarding infection control is very relevant for infection control committee members. Valuable information can be obtained by a questionnaire among healthcare workers in the hospital. An example of a questionnaire that was used in the AMRIN study is given in attachment 11.

Infrastructure of infection control

The infrastructure of infection control is measured by using a score system that was developed in Japan (Ota H et al. Standards for the evaluation of hospital infection control policies and procedures. J Nippon Med Sch 2000; 67: 396-399). The standard assesses whether a hospital-wide program for infection control is established and functions properly, whether adequate surveillance of healthcare-associated infections is performed, and whether infection control programs are executed in the various hospital departments.

FROM SELF-ASSESSMENT TO IMPROVEMENT

Assessment of the quantity and quality of antibiotic use in a hospital, the prevalence of antimicrobial resistance, the prevalance of nosocomial infections, and the quality of infection control is not a goal by itself. It becomes meaningful only when the results are the starting point for improvement, the beginning of a full quality circle. The self-assessment is the first step that makes it possible to identify problems in the hospital regarding antimicrobial resistance, antibiotic use, and infection control.

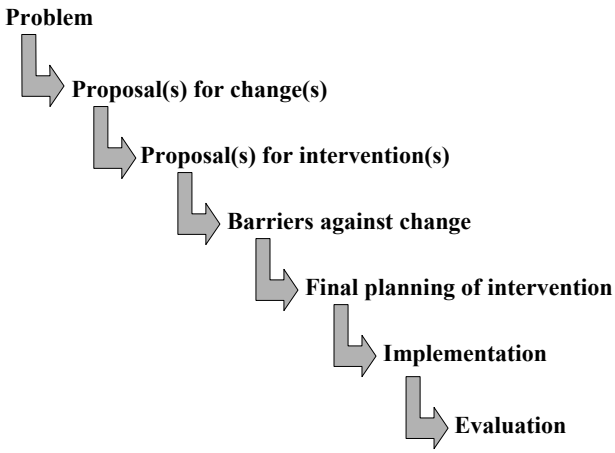

Figure. Planning interventions.

When the problems are defined, proposals for change can be formulated and an inventory of the actions needed to achieve these changes can be made. There will always be barriers against change. It is very important to analyze these barriers and propose ways to counter act these barriers. Barriers that cannot be overcome, can force one to abandon the plans for intervention. If this is not the case the final planning for intervention is made and the intervention is actually implemented. The

last step is to evaluate the effects of the intervention, which brings one back at the beginning of the quality circle, the continuous process of improvement.

Examples of interventions

The AMRIN study revealed several problems regarding antibiotic use and infection control. Following the decision process as described above the study group selected three topics for intervention:

- surgical prophylaxis,
- management of patients admitted with fever,
- adherence to standard precautions.

These topics were chosen because the results of the first half of the AMRIN study showed that a large proportion of antibiotics used inside hospitals to treat patients with fever and to prevent surgical site infections was not justified or inappropriate. Standard precautions were chosen because they are the basis of the prevention of spread of microorganism in hospitals and adherence appeared to be unsatisfactory.

Surgical prophylaxis

The intervention studies on surgical prophylaxis were performed in the departments of Surgery, and Gynaecology and Obstetrics in Semarang and Surabaya using a staggered entry design. The departments were asked to develop an evidence-based consensus guideline on antibiotic prophylaxis. In each department the guideline was introduced by interactive educational sessions followed by feedback after a fixed time period. Surgical departments received the training six weeks after the departments of Gynaecology and Obstetrics. Continuous recording of outcome parameters such as the percentage of patients receiving justified or unjustified antibiotic prophylaxis, the amount of antibiotic used for surgical prophylaxis, was performed during the entire study. In connection to this intervention an improved protocol for the surveillance of surgical wound infection was tested including post-discharge surveillance.

Management of patients admitted with fever

The intervention studies on the management of patients with fever were performed in Semarang and Surabaya in the departments of Internal medicine and Paediatrics. The departments were asked to reach consensus on the management of patients with fever on admission and to lay down this consensus in a guideline. The guideline consisted of a flowchart with decision steps of giving antibiotics or no antibiotics, the introduction of routine blood cultures and a formularium which antibiotics to use for which suspected diseases. This was introduced by teaching activities followed by feedback after some time. The introduction, training, and feedback were not given at the same time in the different departments. Continuous recording of

outcome parameters such as the percentage of patients receiving justified or unjustified antibiotics according to the local guideline, the amount of antibiotic used, was performed during the entire study. Concurrent to these activities the management of patients with fever was followed using outcome measures such as the quality of antibiotic therapy as judged by independent reviewers. By dividing the introduction of guidelines in several periods (in the separate departments), which did not coincide with each other, allowed us to make comparisons between these 4 periods (pre intervention, post guideline announcement, post training period and post feedback period) in these separate departments and cities. Herewith one can identify the most important determinants of adherence to guidelines.

Adherence to standard precautions

The intervention on standard precautions was performed in the departments of Paediatrics and Internal medicine in Semarang, the department of Gynaecology served as control. The departments reached consensus on the application of standard precautions in their departments, wash basins were installed, alcohol hand rub was made available, and on the wards instruction was given about hand hygiene, the use of gowns, gloves and masks and safe handling of needles. Outcome measures were the adherence to the guidelines measured by observations on the wards before, during and after the intervention activities. Feedback on the adherence was given to healthcare-workers on both departments a month after the study. On Internal Medicine, feedback was given twice more, several moths after the intervention.

On both departments, adherence to guidelines, especially on hand hygiene, improved drastically after the intervention. There was a 50% increase in the compliance with handwashing in Internal Medicine and a more than twofold increase in Paediatrics. However, in Paediatrics compliance decreased slightly after four months, while in Internal Medicine it remained stable. Overall, in both departments, the adherence to guidelines on hand hygiene was more than 60%, which is very good.

The intervention on standard precautions showed that it is possible to improve adherence to general infection control guidelines in an Indonesian hospital through a multifaceted intervention.

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ATTACHMENTS

Attachment 1. Antimicrobial susceptibility testing
Attachment 2. 'Bug-drug' combinations
Attachment 3. Antimicrobial resistance from AMRIN study
Attachment 4. SOP 'Antibiotic usage in hospitals'
Attachment 5. Form 'Antibiotic usage in hospitals'
Attachment 6. Form 'Quality assessment of antibiotic use'
Attachment 7. Flowchart assessment of quality of antibiotic use
Attachment 8. Form 'Surveillance of healthcare-associated infections'
Attachment 9. SOP 'Surveillance of healthcare-associated infections'
Attachment 10. Example of inquiry to measure knowledge, attitude and behavior

ATTACHMENT 1. ANTIMICROBIAL SUSCEPTIBILITY TESTING

The susceptibility test is performed by DISK DIFFUSION METHOD recommended by NCCLS. The principle of this method is as follows: the specified bacterial suspension turbidity is spread on the surface of Mueller Hinton agar. The disks with specified content of antimicrobial agent are placed on the surface of the test agar. After overnight incubation at 37°C, the appearence of inhibition zones around the disks is noticed, and compared to the zone standard to specify the categories of 'SENSITIVE' and 'RESISTANCE' (Baron et al, 1994). For fastidious microorganisms (for instance *Streptococcus spp*) or if the microorganism is unable to grow satisfactorily after overnight incubation on Mueller Hinton agar, 5% defibrinated sheep blood may be added to the agar.

The performance of the test should be monitored by quality control strains as follows: 1) *Staphylococcus aureus* ATCC 25923; 2) *Escherichia coli* ATCC 25922; 3) *Pseudomonas aeruginosa* ATCC 27853 (Table 1). The reference strain will be tested 2 times a week in the first 4 weeks; and once a week thereafter if the results are correct. The slight haze of growth of Staphylococci around the oxacillin or methicillin disk should be carefully examined by transmitted light. Such a haze indicates a resistant subpopulation and the organism should be reported as resistant (Baron et al, 1994).

Table 1. Quality control for inhibition zone of antimicrobial susceptibility test.

Antimicrobial	Inhibition Zone in millimetre		
	E. coli ATCC 25922	*S. aureus* ATCC 25923	*P. aeruginosa* ATCC 27853
Ampicillin (AMP10)	16-22	27-35	-
Chloramphenicol (C30)	21-27	19-26	-
Gentamicin (CN10)	19-26	19-27	16-21
Cefotaxime (CTX30)	29-35	25-31	18-22
Ciprofloxacin (CIP5)	30-40	22-30	25-33
Co-trimoxazole (SXT1.25/23.75)	24-32	24-32	-
Ceftazidime (CAZ30)	25-32	16-20	22-9
Piperacillin (PIP100)	24-30	-	25-33
Meropenem (MEM10)	28-34	29-37	27-33
Tetracycline (TET30)	18-25	24-30	-
Oxacillin (OXA1)	-	18-24	-
Erythromycin (ERY15)	-	22-30	-

Method

1. Mueller Hinton (MH) Agar Preparation (Ferraro et al, 2000)

    1) MH agar is prepared from the commercially available dehydrated base according to the manufacturer's instruction.

2) Immediately after autoclaving, allow cooling at 45 – 50°C.
3) Pour the freshly prepared and cooled medium into glass or plastic flat-bottomed Petri dishes on a level horizontal surface to give a uniform depth of approximately 4 mm. This corresponds to 60 to 70 ml for plates with diameter of 150 mm and 25 to 30 ml for plates with a diameter of 100 mm.
4) The agar medium should be allowed to cool to room temperature and, unless is used the same day, stored in a refrigerator (2 to 8°C). The plates should be used within seven days.
5) The pH of MH agar should be between 7.2 to 7.4 at room temperature.
6) If excess surface moisture is present, incubate the plate just before use, until excess surface moisture is evaporated (usually 20 to 30 minutes).

2. Storage of Antimicrobial disks (Ferraro et al, 2000)

1) In AMRIN the disks were commercially acquired through Oxoid Ltd.
2) Refrigerate the disk container at 8°C or below, or freeze at minus 14°C or lower. Beta-lactam containing disks should be stored frozen
3) The disk container should be removed from refrigerator one to two hours before use.
4) Once opened cartridges should be placed in tightly sealed containers after use.

3. Turbidity standard for inoculum (Ferraro et al, 2000)

1) The inoculum should be standardized by the turbidity standard of BaSO4 that is equivalent to MacFarland 0.5.
2) The MacFarland standard is replaced monthly.

4. Inoculum preparation (Ferraro et al, 2000)

1) Bacteria are first grown to pure culture on MacConkey agar (for rectal isolate) and nutrient agar (for nasal isolate).
2) Subsequently bacteria are grown in 4 to 5 ml of a suitable broth medium (trypticase soy broth).
3) The broth culture is incubated 35°C until it achieves or exceeds the turbidity of the MacFarland 0.5. Usually 2 to 6 hours is enough)
4) The turbidity is adjusted with sterile saline or broth to obtain turbidity equivalent to MacFarland 0.5. This suspension contains approximately 1 to 2 x 10^8 CFU/ml.

5. Inoculum Test Plates (Ferraro et al, 2000)

    1) Within 15 minutes after turbidity adjusting, a sterile cotton swab is dipped into the adjusted bacterial suspension. The swab should be rotated several times and pressed firmly on the inside wall of the tube above the fluid level to remove the excess fluid.
    2) Inoculate agar plates by streaking the swab over the entire agar surface, three times at different angles.
    3) Leave for 3 to 5 minutes, but no more than 15 minutes, to allow for any excess surface moisture to be absorbed into the agar before applying the disks.

6. Application of Disks to Inoculated Agar Plates (Ferraro et al, 2000)

    1) Place the disks on the predetermine location on the agar surface.
    2) Press down the disks to ensure complete contact with the agar surface. The disks must be spaced evenly so that they are no closer than 24 mm from center to center. Ordinarily no more than 12 disks can fit on 150 mm plate or 5 disks on 100 mm plate.
    3) The plates are inverted and placed in a incubator set to 35°C within 15 minute after the disks are applied.

7. Reading Plates and Interpreting Results (Ferraro et al, 2000)

    1) Examination is done after 16 to 18 hour incubation.
    2) A good result is shown by uniformly circular zones of inhibition and a confluent lawn of growth.
    3) The diameter of the inhibition zones are measured by using a sliding calliper which is held to the back of the inverted petri plate.
    4) The sizes of the zone of inhibition are recorded and may subsequently be interpreted by referring to the zone standards.

8. Antimicrobial disks used for the test in AMRIN (Ferraro et al, 2000)

Enterobacteriaceae	***Pseudomonas/Acinetobacter***	***Staphylococcus* spp**
1) Ampicillin	1) Ceftazidime	1) Tetracycline
2) Chloramphenicol	2) Piperacillin	2) Oxacillin
3) Gentamicin	3) Gentamicin	3) Erythromycin
4) Cefotaxime	4) Ciprofloxacine	4) Co-trimoxazole
5) Ciprofloxacine	5) Aztreonam	5) Chloramphenicol
6) Co-trimoxazole	6) Meropenem	6) Gentamicin
		And
		7) Beta-lactamase test (Nitrocefin test)

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1. Baron, E.J., Peterson, L.R. and Finegold, S.M. 1994. Bailey and Scott's Diagnostic Microbiology, Ninth Ed. CV. Mosby, Toronto.
2. Ferraro, MJ., Craig, WA., Dudley MN. Et al. 2000. Performance standards for antimicrobial Disk Susceptibility Test; Approved Standard-Seventh Edition. M2-A7, Vol. 20 Number 1; ISBN 1-56238-393-0. ISSN 0273-3099. NCCLS.

ATTACHMENT 2. 'BUG-DRUG' COMBINATIONS

Antimicrobial agent	*S. epidermidis*	*S. aureus*	Haemolytic strep. A & B	*S. pneumoniae*	*E. faecalis / E. faecium*	*Enterobacteria-ceae*	*P. aeruginosa / Acinetobacter*	*C. jejuni*	*H. influenzae / Neisseria / Moraxella*	*H. pylori*
Penicillin										
Amoxicillin					+	+			+	+
Amoxicillin+clavulate					+	+			+	
Penicillin	+	+	+	+						
Cloxacillin	+	+								
Piperacillin						+	+			
Piperacillin+tazobactam							+			
Cephalosporin										
Cefuroxime (2nd)				+		+			+	
Cefotaxime (3rd)			+	+		+				
Ceftazidime (3rd)						+	+			
Other beta-lactams										
Imipenem				+		+	+			
Aminoglycoside										
Streptomycin					+					
Gentamicin	+	+			+	+	+			
Amikacin							+			
Chloramphenicol			+	+	+				+	
Tetracycline										
Doxycycline	+	+		+	+	+			+	+
Macrolide										
Erythromycin	+	+	+	+	+			+	+	
Clarithromycin										+
Lincosamid										
Clindamycin	+	+	+	+						
Glycopeptide										
Vancomycin	+	+	+		+					
Teicoplanin	+									
Sulfonamide										
Trimethoprim						+				
Co-trimoxazole	+	+	+			+	+	+		
Quinolone										
Ciprofloxacin	+	+	+		+	+	+	+	+	
Nitrofurantoin					+	+				
Metronidazole										+
Rifampicin	+	+		+						

ATTACHMENT 3. ANTIMICROBIAL RESISTANCE FROM AMRIN STUDY

Table 1. Antimicrobial resistance rates (n [%]) of *E. coli* and *S. aureus* from different cohorts in Indonesia.

	Admission	Discharge	Relatives	Primary health center (Puskesmas)	Total
E. coli	(n=822)	(n=784)	(n=815)	(n=863)	(n=3,284)
Gentamicin	32 (3.9)[a]	141 (18.0)[d,e]	11 (1.3)[c]	18 (2.1)[b]	202 (6.2)
Chloramphenicol	210 (25.5)[a]	335 (42.7)[d,e]	64 (7.9)[c]	95 (11.0)[b,f]	704 (21.4)
Trimethoprim-sulfamethoxazole	342 (41.7)[a]	435 (55.5)[d,e]	164 (20.1)[c]	209 (24.2)[b,f]	1,150 (35.0)
Ampicillin	416 (50.6)[a]	571 (72.8)[d,e]	162 (19.9)[c]	271 (31.4)[b,f]	1,420 (43.2)
Cefotaxime	17 (2.1)[a]	98 (12.5)[d,e]	6 (0.7)[c]	8 (0.9)	129 (3.9)
Ciprofloxacine	48 (5.8)[a]	173 (22.1)[d,e]	17 (2.1)[c]	17 (2.0)[b]	255 (7.8)
S. aureus	(n=84)	(n=98)	(n=82)	(n=97)	(n=361)
Tetracycline	29 (34.5)	24 (24.5)	18 (22.0)	19 (19.6)[b]	90 (24.9)
Oxacillin	0 (0)	2 (0.6)	0 (0)	0 (0)	2 (0.6)
Gentamicin	2 (2.4)	1 (1.0)	0 (0)	1 (1.0)	4 (1.1)
Erythromycin	4 (4.8)	5 (5.1)	1 (1.2)	2 (2.1)	12 (3.3)
Chloramphenicol	12 (14.3)	9 (9.2)	7 (8.5)	6 (6.2)	34 (9.4)
Trimethoprim-sulfamethoxazole	10 (11.9)	7 (7.1)[d]	7 (8.5)	0 [b,f]	24 (6.6)

Resistance rates were compared in defined pairs (see a-f), using the Chi-square test or Fisher's exact test as applicable ($P<0.05$ was considered significant).

[a] significant difference for admission versus discharge

[b] significant difference for admission versus puskesmas

[c] significant difference for admission versus relatives

[d] significant difference for discharge versus puskesmas

[e] significant difference for discharge versus relatives

[f] significant difference for puskesmas versus relatives

ATTACHMENT 4. SOP 'ANTIBIOTIC USAGE IN HOSPITALS'

1. Name of antibiotic
   - according to the doctor's instruction
   - in case the antibiotic was not given according to the doctor's instruction, then it should be written down who provided the antibiotic, the nurse (N) or the Patient (Pat.)

     Example: Doctor in charge instructed to give Cefotaxime 3x1 gr, but the patient bought ampicillin (4 days), that was followed by penicillin procaine by the nurse.

Name of antibiotic	Daily dose	1	2	3	4	5	6	7	8	9
1.Cefotaxime	3X 1 gr	0	0	0	0	0	0	0	-	-
1.a. Ampicillin (Pat)	3X 1 gr	3	3	3	3	-	-	-	-	-
1.b. Penicillin procaine (N)	4x 600.000 iu	-	-	-	-	4	4	4	-	-

2. Daily dose: fill in the frequency and the dose given during one day
3. Route: oral, intravenous or suppositories
4. Type of therapy (T, P, U)

   It can be looked up at the problem list or progress report or diagnosis on admission

   Therapy (T) is filled in if:
   a. in the medical record the doctor has stated that the antibiotic is given for therapy
   b. the doctor wrote down the diagnosis of an infectious disease
   c. the doctor wrote down the clinical sign of infectious disease e.g. fever
   d. the doctor didn't mention anything specific regarding infection, but from the medical record it is clear that there are clinical signs of infection e.g. fever on the day the antibiotic was started

   Prophylaxis (P) is recorded only if in the medical record the doctor clearly has stated that the antibiotic is given for prophylaxis or the antibiotic is given for not more than one day in relation to an intervention.

   Unknown therapy (U) is filled in if there was no information about whether it was therapy or prophylaxis.
5. Indication

   It should be looked up at the doctor progress note or can be discussed with the doctor in charge if possible.
6. Days of antibiotic uses

The number of antibiotics should be filled in according to the days that patient took antibiotics by looking it up at the nurses book, injection book, or ask to the patient directly (if the patient can be met).

If we don't know exactly (the nurse only wrote that the drug was given) we should fill in a question mark.

No	Name of antibiotic	1	2	3	4	5	6	7	8
1	Ampicillin inj. 3x 1 gr	3	2	-	-	-	-	-	-
2	Amoxycillin 3x 500 mg	-	-	0	3	1 ?2	?3	2	-

ATTACHMENT 5. FORM 'ANTIBIOTIC USAGE IN HOSPITALS'

No	Name of antibiotic (generic/ brand)	Daily dose (mg)	Route Oral, iv	Type of therapy (T,P,U)	Indication (UTI, etc.)	Day of antibiotic usage (start from day on admission)											
						1	2	3	4	5	6	7	8	9	10	11	12
1																	
2																	
3																	
4																	
5																	

Note: T, treatment; P, prophylaxis; U, unknown
UTI: urinary tract infection

Day of antibiotic usage																	
Day	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29
Date																	
1																	
2																	
3																	
4																	
5																	

ATTACHMENT 6. VALIDATION OF RETROSPECTIVE METHOD TO MEASURE ANTIBIOTIC USE

For the validation study, patients are selected on the day of admission, approximately 10 per department, to obtain a total of 100 patient-antibiotic-days per department.

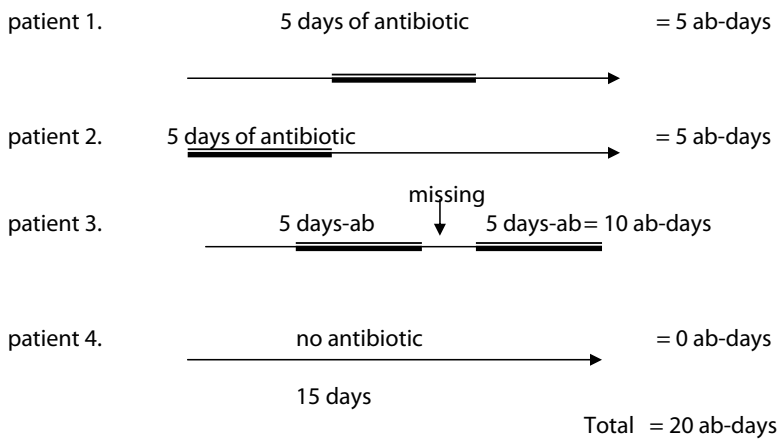
The validation study is done in a prospective and a retrospective way by different persons, for example a pharmacist and a medical doctor.

Each day the patients are asked directly about their antibiotic use of the day before, and also the nurses may be interviewed, and one may look in the injection book of the nurses. For validation, it is forbidden to look in the medical record or to ask the doctor in charge. The nurses in these wards shouldn't know the aim of the study.

Then the information on the antibiotic consumption of the same patient-days is also gathered by studying only the medical record and nursing report at discharge of these patients (see retrospective measurement of antibiotic usage).

Scheme of validation study

I. Prospective (concurrence-study): actual days that patient took antibiotic



II. After these patients are discharged from the hospital, the information from the medical record is looked up in the retrospective way as described above.

The analysis is done on a total of 100 patient-ab-days per department after the last 2 months of registration.

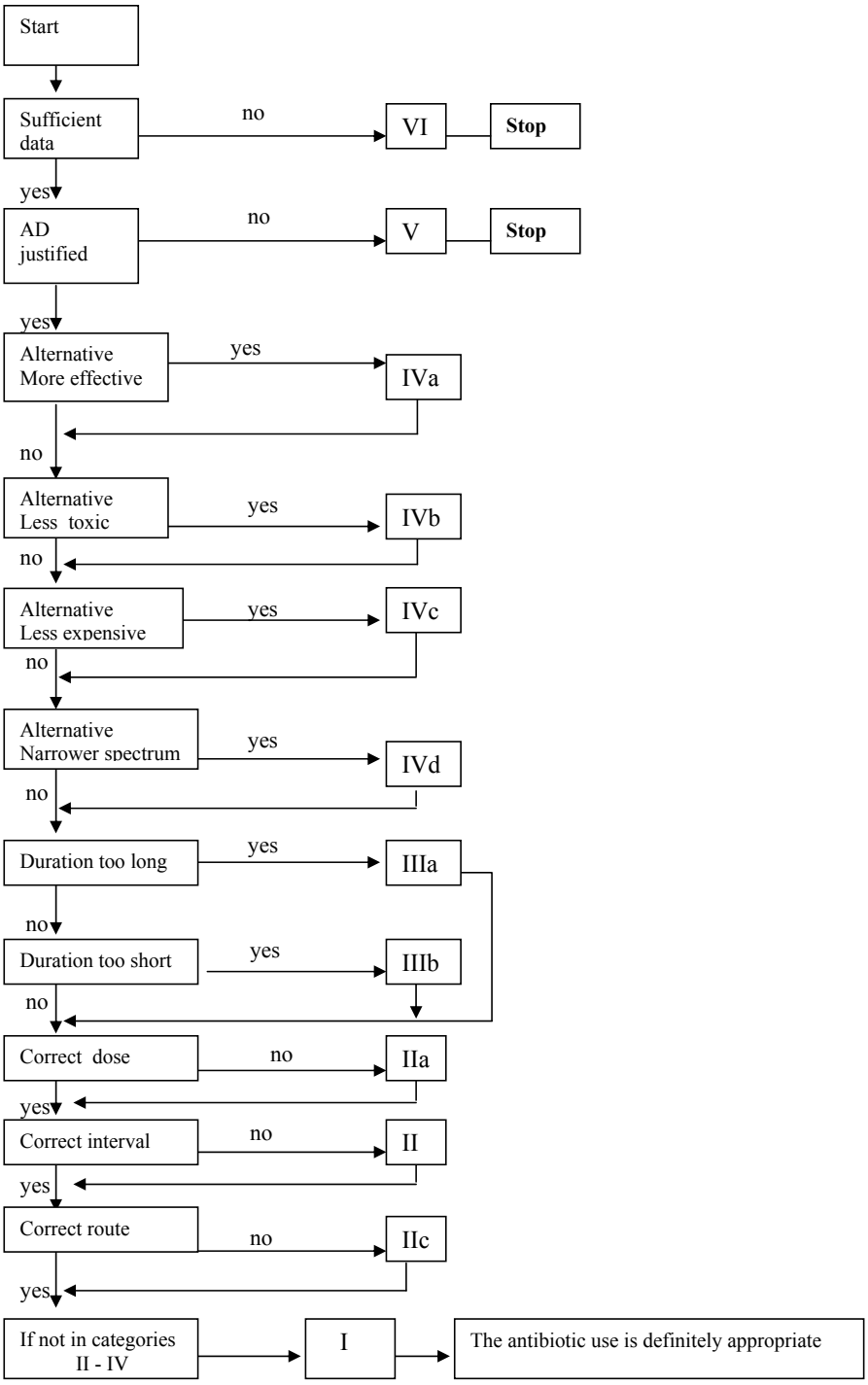
Missing periods of observation in the prospective study are not evaluated or compared with the retrospective approach.

ATTACHMENT 7. FORM 'QUALITY ASSESSMENT OF ANTIBIOTIC USE'

Type of therapy	Name of antibiotic (generic)	Unit dose (mg)	Freq (X)	Route	Start (d/m/y)	Stop (d/m/y)	Duration (days)	Total Doses	CATEGORY / GROUP											
									I	II			III		IV				V	VI
										A	B	C	A	B	A	B	C	D		
ADE																				
Aa																				
ADET																				
Aa																				
ADD																				
Aa																				
ADP																				
Aa																				
ADU																				
Aa																				

ADE: Antimicrobial Drug Empiric therapy; ADET: Antimicrobial Drug Extended Empiric therapy; ADD: Antimicrobial Drug Documented therapy;
ADP: Antimicrobial Drug Prophylaxis; ADU: Antimicrobial Drug Unkown therapy; Aa: Alternative agent

ATTACHMENT 8. FLOW CHART QUALITY ASSESSMENT ANTIBIOTIC USE



ATTACHMENT 9. FORM 'SURVEILLANCE HEALTHCARE-ASSOCIATED INFECTIONS'

SURVEYOR: …… **BAGIAN: ……..** **RUANG:……**

n	name	t	adm	med. rec.	a	s	diagnosis on adm	ab	cul	lab	exposure					nosocomial infection				
											v	k	ob	bt	ok	iv	isk	is	ip	o

Catatan: t, temp (suhu badan); adm, admission date; med. rec., medical record number; a, age (umur); s: sex; ab, antibiotic;cul, pemeriksaan kultur; lab, pemeriksaan; v, kateter intravena; k, kateter urine; ob, operasi bersih; bt, operasi bersih-terkontaminasi; ok, operasi kotor; iv, intravenous catheter infection; isk, infeksi saluran kemih; is, infeksi operasi incisi superfic.; ip, infeksi operasi incisi profunda; o, infeksi operasi organ.t

ATTACHMENT 10. SOP 'SURVEILLANCE HEALTHCARE-ASSOCIATED INFECTIONS

PP = point-prevalence
AB = antibiotics
NI = nosocomial infection
BC = blood culture

We look for nosocomial infections. The persons carrying out the PP will be provided with CDC definitions and criteria for NI.

All fields in the form must be filled in. Fill in a + when a criterium is present. When a criterium is not present, always fill in -. Don't leave any fields blank.

Method:

1. The number of patients present in the ward on a certain day (with their names) are recorded from a small rectangular book which is found in nurse's office.
2. In the same book the temperature of each patient is found. Fill in the temperature.
3. In the same office, the headnurse keeps a large book with successively admitted patients. In this book you can find the admission date, medical record number, age, sex and diagnosis on admission.
   NB: If more people are carrying out the point-prevalence, they can also use the medical record to find this data. However, they should only look for the needed data and not spend time searching the medical record.

4. With the form at hand and accompanied by the nurse, the patients are visited. The patient and the nurse are asked about the use of medication. If yes, specify if antibiotics are used. If yes, record antibiotic use (+) on the form. First ask the nurse about the route of administration. A. Ask the patient or the nurse whether he or she can show the antibiotic or knows the name. B. Look on the chart which may hang on the bed. If not: C. (NB do this only after you have finished your round). If the antibiotic is taken orally: look at the oral patient medication, the nurse will know where to find this. If the antibiotic is administered parenterally, look in the injection book. If not everything is clear: look in the medical record. Write down the name.
5. Inquire about recently taken cultures and other recently taken radiological or laboratory tests. If yes, record this information on the form, with the specimen or test involved.
6. Now it is time to direct your attention to risk factors and nosocomial infections.

7. Look if the patient has an intravenous catheter (phlebitis yes/no, pus yes/no, lymphangitis yes/no). If pus is visible, be assured that a culture will be taken (WHO). In case of lymphangitis, blood cultures should be taken. In case pus is visible, both pus cultures and blood cultures should be taken.
8. Look if the patient has a urinary catheter (urine turbid yes/no, suprapubic pain yes/no). If there are signs of a urinary tract infection, be assured that a culture will be taken (WHO).
9. Ask for surgical wounds. If the patient has had an operation, ask which kind of operation (score: clean, clean-contaminated or dirty) and the operation day (which day after admission=day 0). If possible, look at the wound, or ask the nurse what it looks like. If it is -reported to be- dirty, be assured that cultures will be taken (WHO), both blood cultures and cultures of the site of the infection.
10. Repeat step 4 to 9 for every patient present in the ward.
11. Select the patients with a. antibiotics or b. fever or c. visible infections.
12. For patients who use antibiotics: if necessary, perform step 4C. Now it is known on which day antibiotics have started. Day 0 is admission day.
13. Of all selected patients, find the medical records.
14. If antibiotics are used: try to find the indication in the file (DMK 6 and 7 and the form called 'masalah') on the day antibiotics were started and on the day before this date. Has the resident written down the reason, were there any clinical signs or symptoms recorded on those specific days (blood pressure, pulse, shivering, chills, malaise). For fever on those specific days, look in the temperature graph. For laboratory results, look on the lab-forms. Use the above mentioned definitions and criteria to assess at this moment whether you can register a nosocomial infection. If an infection is likely, make sure that a blood culture is taken. If yes: score NI.
15. If the patient has fever on the PP day: Possibilities:
16. Only fever and no visible signs nor AB use: Look for reasons for fever (risk factors such as written down in step 7, 8, and 9) combined with signs of infection in the medical record on the PP day and the day before (blood pressure, pulse, shivering, chills, malaise) or /and results of diagnostic tests. Verify the fever on the temperature graph. For laboratory and radiological results, look on the lab-forms and in the radiology map. Make sure that a blood culture is taken. Use the above mentioned definitions and criteria to assess at this moment whether you can register a nosocomial infection. If yes: score NI.
17. Fever and antibiotic use see step14
18. Fever and visible signs of infection: If these are present, an infection is proven. Write down on what day the infection started. Make sure that a blood culture is taken. Use the above mentioned definitions and criteria to assess at this moment whether you can register a nosocomial infection. If yes: score NI.

19. Visible signs of infection but no fever nor antibiotic use: Look for reasons for infection (risk factors such as written down in step 7, 8, and 9) combined with signs of infection in the medical record on the PP day and the day before (bloodpressure, pulse, shivering, chills, malaise) or /and results of diagnostic tests. Verify the temperature on the temperature graph. For laboratory and radiological results, look on the lab-forms and in the radiology map. Use the above mentioned definitions and criteria to assess at this moment whether you can register a nosocomial infection.

Small nurse's book (buku suhu) or medical record

1. Number, Name
2. Temperature

Big nurse's book (buku register) or medical record

3. Admission date
   Medical record number
   Age
   Sex
   Diagnosis on admission

Go to patients (with nurse)

4. Antibiotics → if +
   → A. ask nurse / ask patient to show → name
   → B. look on chart (bed)
   → C. (after round) perform step 4C (of SOP)

5. Cultures taken? → MR
   Other diagnostic tests done? → MR

6. Risk factors

7. iv catheter → if + → pus → culture (+BC) NI
   lymphangitis ? if yes (BC) NI

8. urinary catheter → if + → turbid → if yes culture
   suprapubic pain → if yes culture
   → look MR

9. surgical wounds → if + → MR → kind operation
   day operation
   dirty → culture (+BC) NI

10. Select patients with fever → step 16 + BC

ATTACHMENT 11. EXAMPLE OF QUESTIONNAIRE TO MEASURE KNOWLEDGE, ATTITUDE AND BEHAVIOR

KUESIONER
PENGENDALIAN INFEKSI

Untuk:

Staf & Perawat
RSUD.Dr.Soetomo
Surabaya
2001

KUESIONER PENGENDALIAN INFEKSI

Pengukuran pengendalian infeksi rumah sakit merupakan sarana yang sangat penting dalam mengurangi jumlah infeksi nosokomial. Saat ini "The Indonesian – Dutch AMRIN Study Group" melakukan penelitian tentang pengendalian infeksi yang bekerjasama dengan "RSUD Dr Soetomo Infection Control Committee".

Kami ingin mengetahui tentang sikap dan perilaku sehari-hari dari para petugas yang berhubungan dengan pengendalian infeksi. Oleh karena itu kami meminta saudara untuk mengisi kuesioner ini. Tidak perlu mencantumkan nama dan kuestioner ini bukan merupakan test. Hal tersebut sangat penting bagi kami untuk mengetahui kebenaran, oleh karena itu sudi apalah kiranya sedapat mungkin saudara mengisi kuesioner ini dengan bijaksana.

Silakan menjawab semua pertanyaan-pertanyaan. Kalau tidak tahu jawabnya, silakan mengisi 'tidak tahu'. Silakan memekai V untuk mengisi jawab anda di (hokje). Kalau ada jawaban salah, silakan (doorkruisen).
Banyak terima kasih dan kami sangat menghargai kerjasama saudara.
Setelah selesai diisi silakan kuesioner ini dimasukkan ke dalam kotak yang telah kami sediakan.

1. Silakan menyatakan Departemen saudara saat ini

Bagian Ilmu Penyakit Dalam	
Bagian Ilmu Kesehatan Anak	
Bagian Ilmu Bedah	
Bagian Ilmu Kebidanan & Penyakit Kandungan	

2. Apakah profesi saudara?

Perawat	Dokter	Asisten perawat	Yang lain (sebutkan)

3. Sudah berapa lama saudara bertugas sebagai profesi tersebut?

0-4 tahun	5-9 tahun	10-14 tahun	15-19 tahun	20 tahun atau lebih

4. Pernahkan saudara divaksinasi terhadap hepatitis B? ya ☐ tidak ☐

A. INSTRUKSI UNTUK PETUGAS

Pernahkah rumah sakit memberikan petunjuk-petunjuk pada suadara berikut dibawah ini

	ya	tidak
Pernahkah saudara memperoleh petunjuk tentang pentingnya keber -sihan (hygiene) rumah sakit?		
Pernahkan saudara memperoleh petunjuk tentang pedoman pen-genda - lian infeksi rumah sakit?		
Dapatkah saudara kemukakan profesional manakah di rumah sakit yang mengkoordinir pengendalian infeksi?		
Pernahkah saudara diminta dengan segera untuk melaporkan tanda-tanda dan gejala-gejala suatu keadaan infeksi kepada supervisor atau petugas pengendalian infeksi?		
Pernahkah saudara memperoleh petunjuk tentang apa yang harus dila- kukan dalam kasus tertusuk jarum suntik?		

B. PENANGANAN YANG AMAN TERHADAP BENDA TAJAM

1a. Pernahkah saudara mengalami tertusuk jarum suntik? ya ☐ tidak ☐

1b. Apabila ya, apa yang saudara lakukan?

	ya	tidak	tidak ingat
Mencuci dengan air mengalir dan sabun atau mengusap dengan alkohol			
Melapor kepada supervisor			
Melapor kepada perawat bagian pengendalian infeksi			
Lain-lain			

2. Silakan menyatakan apakah pernyataan ini benar atau salah

	benar	salah	tidak tahu
Setelah tertusuk jarum suntik, kasus HIV berpindah 0.5%			
Setelah tertusuk jarum suntik, kasus HCV berpindah 3%			
HIV dapat dicegah dengan memberikan terapi antiretrovi-ral secepatnya setelah tertusuk jarum suntik			
Sebagian besar petugas rumah sakit pernah mengalami tertusuk jarum suntik, karena penanganan yang tidak aman terhadap benda tajam			

3. Silakan menyatakan apakah saudara setuju dengan pernyataan-pernyataan berikut ini

	ya	tidak	tidak tahu
Untuk menghindari tertusuk jarum suntik, jarum harus ditutup kembali			
Setelah tertusuk jarum suntik, petugas harus segera mela –por kepada supervisor atau perawat bagian pengendalian infeksi			
Untuk menghindari tertusuk jarum suntik, harus digunakan konteiner khusus untuk jarum			
Memakai sarung tangan dalam hal menangani peralatan yang tajam, melindungi dari tertusuk jarum suntik			

4. Silakan menyatakan apakah saudara telah melakukan hal seperti berikut ini

	ya	tidak
Untuk menghindari tertusuk jarum suntik, saya tidak pernah menutup kembali jarum suntik		
Untuk menghindari tertusuk jarum suntik, saya menggunakan kontei- ner khusus untuk jarum		
Untuk menghindari tertusuk jarum suntik, saya mengurus pada konteiner jarum tidak diisi terlalu penuh		
Dalam hal menangani jarum suntik, saya memakai sarung tangan		

5. Bekerja sesuai degan pedoman kadang-kadang dapat menjadi sukar, karena alasan –alasan yang berbeda. Kami ingin memperoleh informasi tentang problem-problem yang saudara alami

Bekerja sesuai dengan pedoman untuk keamanan dalam menangani darah sukar karena	Setuju	tidak setuju	tidak tahu
…tidak ada bukti bagi kepentingan penanganan darah yang aman			
…hal tersebut menyebabkan pekerjaan saya lebih berat			
… memerlukan banyak waktu			
…tidak cukup tersedia konteiner khusus			
… membuat perawatan pasien sangat teknis			

C. KEBERSIHAN TANGAN

1. Silakan menyatakan apakah pernyataan berikut ini benar atau salah

	benar	salah	tidak tahu
Penyebaran bakteri di rumah sakit timbul terutama melalui tangan petugas			
Infeksi nosokomial terutama disebabkan oleh bakteri yang dibawa masuk ke rumah sakit melalui petugas rumah sakit			
Memakai perhiasan tangan tidak memungkinkan membersihan tangan dengan sempurna			

2. Silakan menyatakan apakah saudara setuju dengan pernyataan-pernyataan berikut ini:

	ya	tidak	tidak tahu
Sebelum menyentuh penderita yang mengalami pe –nurunan kekebalan tubuh, tangan harus selalu dicuci dengan sabun dan air atau dibasuh dengan alkohol			
Membersihkan atau membasuh dengan alkohol tangan hanya perlu dikerjakan sebelum dilakukan operasi kecil atau perawatan luka pada penderita dengan sistem kekebalan yang normal			
Tangan harus dicuci sebelum memulai melakukan pe –kerjaan di ruangan			
Tangan yang kelihatan kotor harus dicuci dengan air dan sabun			
Setiap petugas rumah sakit berkewajiban agar sedapat mungkin menjaga tangannya bebas dari bakteri			
Sesudah menangani linen yang kotor, tangan harus dicuci dengan sabun dan air atau dibasuh dengan alkohol			
Kuku harus dipotong pendek, bersih dan dirawat dengan baik			
Petugas ruangan harus menggunakan tissue habis pakai untuk menghilangkan ingus dari hidungnya			
Petugas ruangan harus mencuci tangan mereka setelah menghilangkan ingus dari hidungnya			

3. Silakan menyatakan apakah saudara siap bekerja dengan cara-cara sebagai berikut:

	Ya	tidak	tidak tahu
Saya akan mencuci tangan saya yang kelihatan kotor dengan air dan sabun			
Cuci atau membasuh dengan alkohol tangan sebelum dan sesudah meyentuh setiap penderita			
Cuci atau membasuh dengan alkohol tangan sebelum melakukan pembedahan kecil dan merawat luka pada penderita dengan sistem kekebalan yang normal			

4. Bekerja sesuai dengan pedoman kebersihan tangan kadang-kadang dapat menjadi sukar, karena alasa-alasan yang berbeda. Kami ingin mendapatkan informasi tentang problem-problem yang saudara alami

Bekerja sesuai dengan pedoman dalam hal kebersihan tangan adalah sukar karena:	setuju	tidak setuju	tidak tahu
… tidak ada bukti untuk pentingnya kebersihan tangan			
… pekerjaan saya menjadi lebih berat			
….memerlukan banyak waktu			
….di ruangan tidak cukup tersedia tempat pencucian tangan (wastafel)			
… membuat perawatan pasien sangat teknis			
….kulit tangan saya menjadi teriritasi			
… orang lain tidak mengikuti pedoman			

D. KEBERSIHAN PETUGAS DAN PERALATAN PELINDUNG

1. Untuk pernyataan–pernyataan berikut ini, silakan menyatakan apakah hal tersebut benar atau salah

	benar	salah	tidak tahu
Ada bukti bahwa apron, baju panjang dan masker efektif untuk mencegah infeksi nososkomial			
Sarung tangan dapat mengurangi kontaminasi pada tangan tetapi tidak melindungi dengan sempurna			

2. Silakan menyatakan apakah saudara setuju dengan pernyataan-pernyataan berikut ini

	ya	tidak	tidak tahu
Untuk setiap pasien yang dirawat dengan sarung tangan, petugas harus mengganti dengan sarung tangan baru			
Sarung tangan yang tidak steril harus dipakai dalam ka- sus kontak dengan kulit yang luka			
Sarung tangan yang tidak steril harus dipakai ketika memasang infuse			
Sarung tangan yang tidak steril harus dipakai setiap kontak langsung dengan penderita			
Sarung tangan yang steril harus dipakai selama mema- sang kateter urin			
Sarung tangan yang steril harus dipakai dalam kasus kontak dengan selaput lendir			
Menangani linen yang kotor dan linen yang bersih harus dipisahkan			
Apron (plastik) yang sekali pakai dapat digunakan ketika ada resiko bahwa baju atau seragam dapat terkena darah, cairan tubuh, sekresi dan ekskresi kecuali keringat			
Petugas boleh makan atau minum ketika merawat pasien			

3. Silakan menyatakan apakah sdr.telah melakukan pekerjaan dengan cara ini:

	ya	tidak
Saya memakai sarung tangan yang tidak steril pada kasus melakukan kontak dengan kulit yang luka		
Saya hanya memakai apron (plastik) apabila ada resiko bahwa baju atau seragam saya dapat terkena darah, cairan tubuh, sekresi atau ekskresi kecuali keringat		
Sesudah menangani linen yang kotor, saya mencuci tangan saya atau membasuh dengan alcohol		

4. Bekerja sesuai dengan pedoman kadang-kadang dapat menjadi sukar, karena alasan-alasan yang berbeda. Kami ingin memperoleh informasi tentang problem-problem yang saudara alami.

Bekerja sesuai dengan pedoman tentang keber sihan bagi petugas dan peralatan pelindung adalah sukar karena:	setuju	tidak setuju	tidak tahu
… tidak ada bukti bahwa hal itu penting			
… pedoman tersebut tidak jelas			
… menjadikan pekerjaan saya lebih berat			
… memerlukan banyak waktu			
… tidak seorangpun perduli hal tersebut			
… di ruangan kami tidak cukup tersedia sarung tangan			
… di ruangan kami tidak cukup tersedia apron			

E. PENGGUNAAN KATETER URIN

1. Untuk pernyataan –pernyataan berikut ini, silakan menyatakan apakah menurut saudara bahwa hal itu benar atau salah

	benar	salah	tidak tahu
Gangguan pada saluran urin merupakan indikasi yang baik untuk dilakukan kateterisasi			
Pencegahan terhadap dikubitus merupakan indikasi yang baik untuk dilakukan kateterisasi			
Inkontinen urine merupakan indikasi yang baik untuk dilakukan kateterisasi			
Pemberian cairan yang cukup menurunkan resiko terhadap ISK pada penderita yang dikateterisasi			
Pada penderita yang dilakukan kateterisasi, pemberian salep antibiotik pada ujung urethra menurunkan resiko terhadap ISK			

2. Silakan saudara mengemukakan apakah saudara setuju dengan pernyataan-pernyataan berikut ini.

	ya	tidak	tidak tahu
Selalu bekerja dengan menggunakan tehnik aseptik			
Penderita yang menggunakan kateter harus minum paling sedikit sehari 3000 ml			
Krim antibiotik harus dioleskan pada lubang uretra penderita yang dikateterisasi			
Penderita yang dikateterisasi daerah genital dicuci setiap hari, seperti halnya terhadap penderita–penderita yang lain			

3. Silakan menyatakan apakah saudara sudah melakukan pekerjaan dengan cara seperti ini

	ya	tidak
Saya yakin penderita yang dikateterisasi minum paling sedikit 3000 ml sehari		
Saya kosongkan kantong urine paling sedikit 4 kali sehari atau bila mungkin lebih sering		
Untuk mengambil sampel urin saya menggunakan tehnik yang steril dan tertutup		
Saya mencuci daerah genital penderita yang memekai kateter sama halnya dengan penderita lain yang tidak memekai kateter		

4. Bekerja sesuai dengan pedoman pengendalian ISK kadang-kadang dapat menjadi sukar, karena alasan-alasan yang berbeda. Kami ingin memperoleh informasi tentang problem-problem yang saudara alami

Bekerja sesuai dengan pedoman untuk pencegahan infeksi saluran kandung kemih adalah sukar karena:	setuju	tidak setuju	tidak tahu
… tidak ada bukti bahwa hal itu penting			
… membuat pekerjaan saya menjadi lebih berat			
… memerlukan banyak waktu			
… tidak seorangpun peduli hal tersebut			
… membuat perawatan pasien sangat teknis			
… sistem pengumpulan tidak diikuti pengambilan sampel urin secara tertutup			
... orang lain tidak mengikuti pedoman tersebut			

F. PENANGANAN LUKA OPERASI

1. Untuk pernyataan–pernyataan berikut ini, silakan menyatakan apakah menurut saudara bahwa hal itu benar atau salah

	benar	salah	tidak tahu
Pencukuran sebelum operasi mengurangi kemungkinan infeksi pada luka operasi			
Mandi dengan sabun antibakteri sebelum operasi mengurangi kemungkinan infeksi pada luka operasi			

Pencukuran apabila diperlukan, harus dilakukan perioperatif memberikan risiko ILO lebih kecil dari pada waktu pencukuran yang lebih lama			

2. Silakan saudara mengemukakan apakah saudara setuju dengan pernyataan-pernyataan berikut ini.

	ya	tidak	tidak tahu
Rambut hanya boleh dicukur (dikerok) kalau rambut disekitar daerah yang dioperasi lebat			
Sebelum operasi apabila rambut perlu dihilangkan segera sebaiknya menggunakan alat cukur listrik			
Pada operasi terencana penderita perlu mandi (atau dimandikan) setidaknya pada malam sebelum operasi dengan sabun antiseptik			
Penderita dengan luka yang potensial menular harus diiso- lasi untuk tindakan pencegahan sesuai dengan pedoman yang ada			
Petugas harus mencuci tangannya sebelum dan setelah merawat luka operasi			
Melindungi luka operasi terutama harus ditutup dengan pembalut steril selama 24 sampai 48 jam paska operasi			
Bilamana pembalut luka harus diganti, harus menggunakan tehnik yang steril			
Kalau kasa steril menjadi basah harus diganti			

3. Silakan menyatakan apakah saudara sudah melakukan pekerjaan dengan cara seperti ini

	ya	tidak
Pada operasi terencana, saya minta penderita untuk mandi (atau dimandikan) setidaknya pada malam sebelum dioperasi dengan sabun antiseptik		
Kalau rambut didekat daerah yang dioperasi sangat lebat yang akan dapat mengganggu prosedure operasi, saya menghilangkannya		
Saya selalu mencuci tangan saya sebelum dan sesudah merawat luka operasi		
Apabila pembalut luka harus diganti, saya menggunakan tehnik yang steril		

4. Bekerja sesuai dengan pedoman penegndalian ILO kadang-kadang dapat menjadi sukar, karena alasan-alasan yang berbeda. Kami ingin memperoleh informasi tentang problem-problem yang saudara alami

Bekerja sesuai dengan pedoman sangat sukar karena:	setuju	tidak setuju	kurang lebih
… tidak ada bukti bahwa hal itu penting			
… membuat pekerjaan saya menjadi lebih berat			
… memerlukan banyak waktu			
… tidak seorangpun peduli hal tersebut			
… membuat perawatan pasien sangat teknis			
… kami tidak cukup memiliki pembalut yang steril			
… orang lain tidak mengikuti pedoman tersebut			

G. KATETER INTRAVENA PERIFER

1. Untuk pernyataan–pernyataan berikut ini, silakan menyatakan apakah menurut saudara bahwa hal itu benar atau salah

	benar	salah	tidak tahu
Pemberian krim antibiotik pada tempat tusukan mengurangi resiko plebitis infeksi atau bakteriemi			
Plebitis selalu disebabkan oleh infeksi			
Penggantian infus perifer dalam kurun waktu 3x24 jam mengurangi risiko plebitis dan bakteriemi			

2. Silakan saudara mengemukakan apakah saudara setuju dengan pernyataan-pernyataan berikut ini.

	ya	tidak	tidak tahu
Sebelum memassukkan obat lewat iv-kateter, penghubung harus disucihamakan			
Gunakan kasa steril atau pembalut transparan untuk menutupi bagian kateter			
Apabila digunakan kasa dan plester pada pembalut kateter, gantilah apabila pembalut tersebut basah			
Apabila digunakan kasa dan plester pada pembalut kateter, gantilah apabila pembalut diperlukan untuk pemeriksaan kateter			

Gantilah sistem infus yang digunakan untuk memasukkan darah pada saat selesai pemberian transfusi atau 24 jam dari awal pemberian transfusi			
Gunakan krim antimikroba pada tempat tusukkan sebagai bagian dari perawatan kateter secara rutin			

3. Silakan menyatakan apakah saudara sudah melakukan pekerjaan dengan cara seperti ini

	ya	tidak
Sebelum memassukkan obat lewat iv-kateter, permukaan luar tangkai kateter dan tempat penghubung disucihamakan		
Apabila digunakan kasa dan plester pada pembalut kateter, saya menggantinya ketika pembalut tersebut basah		
Apabila digunakan kasa dan plester pada pembalut kateter, saya menggantinya ketika diperlukan pemeriksaan letak kateter		
Saya memberikan krim antimikroba pada letak pemasukkan infus sebagai bagian dari perawatan kateter secara rutin		
Saya mengganti sistem infus yang digunakan untuk memasukkan darah pada saat selesai pemberian transfusi atau 24 jam dari awal pemberian transfusi		

4. Bekerja sesuai dengan pedoman pengendalian plebitis / bakteriemi kadang-kadang dapat menjadi sukar, karena alasan-alasan yang berbeda. Kami ingin memperoleh informasi tentang problem-problem yang saudara alami.

Bekerja sesuai dengan pedoman untuk pencegah- an infeksi terhadap kateter sukar sebab:	setuju	tidak setuju	tidak tahu
… tidak ada bukti bahwa hal itu penting			
… membuat pekerjaan saya menjadi lebih berat			
… kami tidak ada krim antibiotik di ruangan			
….tidak seorangpun peduli hal tersebut			
….sangat teknis dan tidak mudah diterima oleh penderita			
….orang lain tidak mengikuti pedoman tersebut			

Terima kasih banyak atas kerjasama saudara.

Silakan memasukkan kuesioner ini pada kotak yang tersedia.