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Antimicrobial resistance in bacterial infections in urban and rural Tanzania

Doctoral thesis by

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Le fléau n'est pas à la mesure de l'homme, on se dit donc que le fléau est irréel, c'est un mauvais rêve qui va passer. Mais il ne passe pas toujours et, de mauvais rêve en mauvais rêve, ce sont les hommes qui passent, et les humanistes en premier lieu, parce qu'ils n'ont pas pris leurs précautions.

(A pestilence isn't a thing made to man's measure; therefore we tell ourselves that pestilence is a mere bogey of the mind, a bad dream that will pass away. But it doesn't always pass away, and from one bad dream to another, it is men who pass away, and the humanists first of all, because they haven't taken their precautions.)

Albert Camus, La Peste, 1947

Preface

Paul Ehrlich described the concept of antimicrobial agents as “magic bullets” for killing microbes. This impression of antimicrobial agents as magic bullets was thoroughly reinforced when penicillin and other antibiotics came into clinical use in the 1940s. However, shortly after the introduction of these magic bullets in clinical practice, it was discovered that the bacteria were capable of developing resistance to the antimicrobials. The full magnitude of the resistance problem was not appreciated during the first decades of chemotherapy. However, bacteria became more resistant, new types of bacteria developed resistance, resistance genes spread among different bacteria, and resistant organisms spread to new geographical areas. Particularly serious resistance problems such as multidrug-resistant tuberculosis, methicillin-resistant *Staphylococcus aureus* and extended-spectrum beta-lactamase (ESBL) producing Gram-negative bacteria emerged and spread to most parts of the world.

Inappropriate use of antibiotics, use of broad-spectrum antibiotics, insufficient hygiene, immunosuppression and prolonged hospitalization may promote antimicrobial resistance. Use of antimicrobials of poor quality may contribute to emerging resistance and is a huge problem in countries with poor regulatory capacities. While antimicrobial resistance affects all countries, it has potential for doing more harm in developing countries since second-line antimicrobial drugs are often neither available nor affordable to those who need it. Diseases we have thought of as curable, such as pneumonia, bloodstream infections, typhoid fever and tuberculosis, may again become killers of people of all ages. If this scenario becomes real, developing countries may be where the harm will be felt first.

Summary

Infectious diseases cause one in every six deaths worldwide. Antimicrobial drugs have helped dramatically in curing patients suffering from bacterial infections. However, emerging antimicrobial resistance in bacteria threatens to undermine the management of bacterial infections. Developing countries have greater burden of infectious diseases. A number of factors, which may promote antimicrobial resistance such as availability of antimicrobials without prescription, use of counterfeit or substandard antimicrobial drugs, suboptimal hygiene, immunosuppression due to malnutrition or HIV, may be more frequent in developing countries. At the same time, consequences of antimicrobial resistance may be felt harder in resource-poor settings, since second-line antimicrobial drugs for resistant bacteria may be unavailable or unaffordable. There are many unresolved questions regarding antimicrobial resistance in general, including regarding its impact on patient outcome. In Sub-Saharan, some studies on antimicrobial resistance have been done, but, by and large, the issue has received far too little attention.

We set out to improve available antimicrobial susceptibility data in Tanzania. We implemented a free-of-charge computerized software (WHONET) for resistance surveillance in the University Teaching Hospital in Dar es Salaam. This exercise showed that resistance surveillance is feasible in the setting and provided useful data on antimicrobial resistance. The surveillance data indicated high rates of resistance to common antibiotics in Gram-negative bacteria. We performed a prospective, observational cohort study of bloodstream infections in 1828 admissions of children with fever or suspected serious infection at the hospital. We performed blood culture, malaria testing and HIV testing and collected clinical data from the study subjects. The study showed that a disturbingly high proportion of Gram-negative bacteria produced extended-spectrum beta-lactamases (ESBL), with prevalent genotypes being TEM-63, SHV-12 and CTX-M 15. The ESBL-producing bacteria had a high rate of resistance to almost all other available drugs, except for ciprofloxacin, and bloodstream infection caused by these multiresistant bacteria were associated with

extremely high case-fatality rates. The study showed that inappropriate treatment due to antimicrobial resistance, as well as malnutrition and HIV-infection, were risk factors for death in children admitted with bloodstream infections.

We investigated an outbreak of pediatric / neonatal meningitis at Haydom Lutheran Hospital, finding that *Salmonella* serovar Enteritidis, resistant to ampicillin and susceptible to gentamicin, was the cause of the outbreak. Although the numbers were small, the case-fatality rate for meningitis caused by these organisms was 100% (5/5).

Antimicrobial resistance varies greatly from one geographical area to another. Thus, data obtained at major hospitals in urban centers may not be representative for the whole country. We analyzed the antimicrobial susceptibilities of isolates of uropathogenic bacterial obtained from the urine of pregnant women in a rural area in Northern Tanzania. This study indicated that there is less antimicrobial resistance in *E. coli* isolates from this rural area than in isolates from the commercial capital, Dar es Salaam. In formulating guidelines for antimicrobial use this possible rural-urban difference should be taken into account.

For some of the bacteria carrying resistance traits for multiple antimicrobials, there are actually no good alternative drugs available. Based on the findings of these studies, we recommend sober, rational use of antimicrobial drugs, restrictions on sale and use of antimicrobials, and attention to hygiene.

List of publications

This thesis is based on the following papers, which will be referred to in the text by their numerals:

1. Blomberg B, Mwakagile DSM, Urassa WK, Maselle SY, Mashurano M, Digranes A, Harthug S, Langeland N. Surveillance of antimicrobial resistance at a tertiary hospital in Tanzania. BMC Public Health 2004, 4:45. (http://www.biomedcentral.com/1471-2458/4/45)

2. Blomberg B, Manji KP, Urassa WK, Tamim BS, Mwakagile DSM, Jureen R, Msangi V, Tellevik MG, Holberg-Petersen M, Harthug S, Maselle SY, Langeland N. Antimicrobial resistance predicts death in Tanzanian children with bloodstream infections: a prospective cohort study. (Accepted in principle for puplication in BMC Infectious Diseases)

3. Blomberg B, Jureen R, Manji KP, Tamim BS, Mwakagile DSM, Urassa WK, Fataki M, Msangi V, Tellevik MG, Maselle SY, Langeland N. High rate of fatal cases of pediatric septicemia caused by gram-negative bacteria with extended-spectrum beta-lactamases in Dar es Salaam, Tanzania. J Clin Microbiol 2005;43(2):745-749. (http://jcm.asm.org/cgi/content/abstract/43/2/745)

4. Vaagland H, Blomberg B, Krüger C, Naman N, Jureen R, Langeland N. Nosocomial outbreak of neonatal *Salmonella enterica* serotype Enteritidis meningitis in a rural hospital in northern Tanzania. BMC Infectious Diseases 2004; 4:35. (http://www.biomedcentral.com/1471-2334/4/35)

5. Blomberg B, Olsen BE, Hinderaker SG, Langeland N, Gasheka P, Jureen R, Kvåle G, Midtvedt T. Antimicrobial resistance in urinary bacterial isolates from pregnant women in rural Tanzania: implications for public health. Scand J Infect Dis 2005;37(4):262-268.

Contents

1. Background

1.1 List of Abbreviations

Bla	Beta-lactamase gene
CLSI	Clinical and Laboratory Standards Institute (formerly NCCLS)
DNA	Deoxyribonucleic acid
ESBL	Extended-spectrum beta-lactamase
HIV	Human immunodeficiency virus
IMCI	Integrated management of childhood illness
MecA	Methicillin resistance structural gene
MIC	Minimum inhibitory concentration
MRSA	Methicillin resistant *Staphylococcus aureus*
MUCHS	Muhimbili University College of Health Sciences
NCCLS	National Committee for Clinical Laboratory Standards (Now CLSI)
PBP	Penicillin-binding protein
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
UNICEF	United Nations Children's Fund
Van A/B	Vancomycin resistance genes A and B
VRE	Vancomycin-resistant enterococci
VRSA	Vancomycin-resistant Staphylococcus aureus
WHO	World Health Organization
WHONET	Antimicrobial resistance surveillance software from WHO

1.2 Definitions

1.2.1 Antibiotics, antimicrobial agents and chemotherapy

The word *antibiotic* is derived from the Greek words anti (against) and bios (life) and means, in principle, a substance, which kills any living organism. However, in medical practice it is taken to mean a substance, which is produced by a living organism and which kills or inhibits a bacterium. The classical example is penicillin which kills bacteria and which is produced naturally by the molds *Penicillium chrysogenum* and *Penicillium notatum*. The word *antimicrobial* comes from the Greek words anti (against), micros (small) and bios (life) and means a substance, which kills or inhibits microbes. It is mostly applied to substances working on bacteria (antibacterials), but can, in principal, also be applied to agents working on viruses (antivirals), fungi (antifungals) and parasites (antiparasitic agents). Antimicrobials include antibiotics produced by other organisms (e.g. penicillin, tetracycline, erythromycin), chemically modified antibiotics (e.g. doxycycline) as well as chemically produced substances (e.g. fluoroquinolones). The word cytostatic comes from the Greek words kytos (bag or cell) and statikos (causing to stop). Cytostatics are related to antibiotics in the sense that it is a substance, which kills living cells. However, in medical practice it is mostly applied to agents used to inhibit or kill cancer cells and some drugs used to inhibit immune processes involved in autoimmune diseases and in the rejection of transplanted organs. The word *chemotherapy* originates from Greek therapeia (curing, healing) and Arabic al-kimya (alchemy), which is believed to originate from either the Greek word khymos (sap, juice) or Khemia, an ancient name for Egypt. The word chemotherapy was first used by Paul Ehrlich to mean the treatment of infectious diseases with chemical substances.

1.2.2 Resistance, susceptibility and sensitivity

Resistance comes from the Latin words re (against) and sistere (to withstand). In microbiology, the term antimicrobial resistance is used to describe the phenomenon when a microbe can grow and multiply despite the presence of an antimicrobial agent. Depending on the microbe involved we use the terms antibacterial, antiviral, antifungal or antiparasitic resistance. Both *susceptibility* and *sensitivity* are commonly taken to mean the opposite of resistance, however, they have slightly different meanings. The word *susceptibility* comes from the Latin words sub (up from under) and capere (to take) while the word *sensitivity* comes from the Latin word sentire (to feel). In microbiology *susceptibility* is understood as a continuous variable, i.e. it can be used not only to describe whether a microbe is susceptible or resistant to a an antimicrobial, but also to quantify the degree to which it is resistant or susceptible as expressed by for instance the MIC result of antimicrobial dilution test or the zone diameters recorded from disk diffusion tests. *Sensitivity*, on the other hand, is a categorical variable commonly used to describe the interpretation of the susceptibility test into main groups such as sensitive (S), intermediate sensitive (I) and resistant (R).

1.2.3 Virulence and pathogenicity

Virulence derives from the Latin word *virus*, and may be related to the Sanskrit word *visha*, both of which mean poison or venom. *Pathogenicity* derives from the Greek words *pathos* (suffering) and *genesis* (creation), and means the ability to produce diseases (suffering). In microbiology, both *virulence* and *pathogenicity* means the ability to cause disease, but *pathogenicity* generally refers to the binary aspect, i.e. can the microbe cause disease or not, while *virulence* is a measure of the degree to which a microbe causes disease as indicated by case fatality rates for instance. Virulence factors are particular molecules that are responsible for the disease-causing ability of the microbe, such as toxins, adherence factors, proteins that mediate invasion of host cells. Genes coding for virulence factors may be located in gene regions called *pathogenicity islands*, which can be transferred horizontally between bacteria.

1.2.4 Mortality, lethality and case-fatality rates

Mortality is a measure of the proportion of the entire population that dies. Lethality is the proportion that dies due to a specific condition. In infectious diseases, the expression case-fatality rate is commonly used to describe the proportion that dies among those who contract a certain infectious disease. Thus, case-fatality rate is largely equivalent to lethality. Case-fatality rate is dependent not only of the proportion of observed deaths, but also on the rate of detection of the disease. This is particularly relevant in areas with poor coverage of health systems; for instance, in some areas of Central Africa, the described high case-fatality rates of hemorrhagic fevers is thought partly to be due to a low detection rate, i.e. a number of less severe cases go undetected by the local health system. The term 'attributable mortality' is an expression used in for instance case control studies, and refers to the excess mortality in those who have the case characteristic as compared to the controls.

1.2.5 Bloodstream infections, bacteremia, septicemia and sepsis

Bacterial infections of the bloodstream are recognized as important causes of morbidity and mortality [1]. However, there has been much debate regarding the understanding of the common terminology related to these infections. Traditionally, the term bacteremia has been used as a microbiological diagnosis meaning the presence of viable bacteria in circulating blood. The term septicemia is a combined clinical and microbiological diagnosis and is commonly used to mean cases that have both bacteremia and a clinical signs indicating severe infection. Commonly, septicemia includes also cases of candidaemia with clinical signs of severe infection. Sepsis is a clinical diagnosis, which means that there are both signs of clinical infection and signs of systemic response. An additional frequently used term is bloodstream infection. Bloodstream infection is largely equivalent to the meaning of septicemia described above, meaning clinical signs of infection and bacteremia or candidaemia. However, there has been some ambiguity regarding this term as well, and it is now commonplace to use the extended term 'laboratory-confirmed bloodstream infection" to ensure a correct understanding, i.e. clinical infection plus a

verified bacteremia or candidaemia. In the articles presented in this thesis we have used both the term septicemia (paper 3 & 4) and laboratory-confirmed bloodstream infection (paper 2, thesis) as interchangeable terms, meaning clinical signs of infection and verified bacteremia or candidaemia. The current understanding of the terminology is based on the description by Bone et al [2] with modifications applicable for infants and children as described by Jafari and McCracken [3] as summarized in Table 1 (the table was not presented in the articles).

Table 1. Definitions

Bacteremia	Presence of viable bacteria in the circulating blood confirmed by blood culture
Sepsis	Clinical suspicion of infection accompanied by evidence of a systemic response manifested by at least two of the following conditions a) High (>38'C) or low (<36'C) body temperature, b) Elevated heart rate (adults >90, children >150 and infants >160 beats/minute) c) High respiratory rate (adults >20/min, children >50/min and infants >60/min) d) Elevated (>12,000/mm^3) or low (<4,000/mm^3) white blood cell count
Sepsis syndrome/ severe sepsis	Sepsis plus evidence of altered organ perfusion manifested by at least one of the following acute changes: a) Acute changes in mental status (reduction by 3 in Glasgow coma scale or Simpson and Reilly or Jacobi modification for children) b) Oliguria c) Elevated blood lactate d) Hypoxemia
Septic shock	Severe sepsis with hypotension (systolic blood pressure (mmHg: Adults <90 or 40 below baseline, children <75 and infants <65, or <5th percentile for age), which is responsive to therapy with i.v. fluids.
Refractory septic shock	Septic shock with hypotension, which lasts for more than one hour and is not responsive to i.v. fluid and pharmacological therapy, and requires vasopressor therapy
Multiorgan failure	Any combination of disseminated intravascular coagulation (DIC), respiratory distress syndrome, acute renal failure, hepatobiliary dysfunction and central nervous system (CNS) dysfunction

1.2.6 Asymptomatic bacteriuria and urinary tract infection

Asymptomatic bacteriuria is commonly defined as the finding of >100,000 bacteria per ml urine in a single midstream urine in a person with no symptoms of urinary tract infection. Urinary tract infection is the finding of >100,000 bacteria per ml urine

in a person with symptoms suggestive of urinary tract infection such as dysuria and frequent micturation.

1.3 Bacterial infections

Infectious diseases are responsible for an estimated 17.8% of all deaths world-wide, amounting to almost 10 million deaths per year [4]. While the majority of incidents of cardiovascular and neoplastic diseases affect the older part of populations and are important causes of death in developed countries, infectious diseases have an important impact on children and young adults, particularly in countries with scarce economical resources. In Africa, one in every six children dies before reaching the age of five years [5]. Malnutrition and infectious diseases are the main killers. However, it is notoriously difficult to assess which diseases contribute most to the suffering and death in the world because proper diagnostic tools are not available in large areas of the world and because many patients suffer from more than one condition at the time. Particularly, many children dying with infectious diseases are also suffering from severe malnutrition and it may be difficult to say which is contributing most to the suffering and death of the patients. Similarly, people dying with HIV infection almost invariably have one or more other conditions such as tuberculosis, other bacterial and parasitic infections and cancers. Thus, poverty, malnutrition and immunosuppression by HIV or other causes, all contribute to the complex picture of infectious diseases in the developing world. The World Health Organization (WHO) rank the major causes of mortality in children younger than five years in Africa as neonatal causes (26%, among which the entity “sepsis or pneumonia” contributes a quarter), pneumonia (21%), malaria (18%) diarrhea (16%) and HIV-infection (6%) [6].

1.3.1 Bloodstream infections

Bloodstream infection is an important contributor to morbidity and associated case-fatality rates exceed 25% [7, 8]. However, as bloodstream infection often occurs as

part of localized infections with defined foci, the significance of bloodstream infection as a death cause is often not reflected in published figures. Based on clinical examination alone, bacterial/fungal bloodstream infection and malaria are practically impossible to differentiate [9]. The WHO's IMCI guidelines have been reported to fail to identify up to half of the cases of bacterial bloodstream infections [10]. A recent study from Kenya [8] showed that bloodstream infection is the cause of death in approximately one quarter of the children who died in the hospital, outnumbering even malaria deaths. Bloodstream infection is associated with both malnutrition and HIV [8, 11]. The causative agents in bloodstream infections differ among various settings, and nontyphoid salmonellae are the predominant cause of bloodstream infections in children in Africa [12].

1.3.2 Meningitis

Bacterial meningitis is a serious infection associated with high case-fatality rates. Pneumococci, meningococci are common causes of meningitis. *Haemophilus influenzae* is decreasing dramatically as a cause of meningitis after the implementation of vaccine against *H. influenzae* type B. At the extremes of age, in neonates and old people, *E. coli*, other Gram-negatives and *Listeria monocytogenes* are important causes of meningitis, while Group B streptococci are particularly important in neonates. Nontyphoid salmonellae are an uncommon cause of meningitis in economically developed countries [13], but more common in tropical countries, particularly in children younger than six months, and often associated with higher case-fatality rates than meningitis caused by other bacteria [12, 14-16].

1.3.3 Asymptomatic bacteriuria and urinary tract infection in pregnant women

In non-pregnant women, asymptomatic bacteriuria is considered a harmless condition and is usually not treated with antimicrobials. However, asymptomatic bacteriuria affects five to ten percent of all pregnant women [17-22], among whom untreated asymptomatic bacteriuria may progress to pyelonephritis in as much as 20-30 percent

of cases. The reason for the much more serious implications of asymptomatic bacteriuria in pregnancy is thought to be mechanical obstruction of urinary flow from the enlarged uterus combined with hormonally (progesterone) mediated dilatation of the ureteres and renal pelvis, which favors ascending infection to the kidney [23]. Approximately 30-40% of all preterm deliveries are estimated to be caused by various infections, including urinary tract infections [24]. Pyelonephritis in pregnancy is associated with increased morbidity and mortality for mother and child, and if left untreated causes preterm birth in 20-50%. Studies have shown an association between asymptomatic bacteriuria in pregnancy and preterm delivery/low birth weight (<2500g) [19], but it has not been established whether asymptomatic bacteriuria is a separate risk factor or merely an indicator for low socioeconomic status, which is known to be associated with low birth weight [20].

1.3.4 Clinical assessment of sick children and IMCI

During the 1990s, the World Health Organization (WHO), UNICEF and other agencies developed the strategy known as Integrated Management of Childhood Illness (IMCI) in an attempt to integrate the many proven strategies for prevention and treatment of disease in children, and thereby increasing the number of lives saved. The diagnostic and treatment practices at MUCHS are generally rooted in the IMCI guidelines. In the study of bloodstream-infections in children (paper 2 and 3) we used the IMCI guidelines as a base for development of the questionnaire, classification of the patients and analysis of the data. It is beyond the scope of this thesis to review the whole IMCI strategy, but I will mention some of the most important decision tools used for classifying patients.

The following four signs were considered general danger signs, and patients were classified according to the number of danger signs present: 1) convulsions (may indicate cerebral malaria, meningitis or other serious illness), 2) lowered level of consciousness, 3) inability to drink/eat and 4) vomiting. The following three signs were considered suggestive of pneumonia: 1) high respiratory rate, 2) lower chest wall indrawing and 3) stridor. The normal respiratory rate varies by age, and thus we

consider different cut-off points for fast respiration as follows: 60 pr minute for young infant up to 2 months age, 50 pr minute for children aged 2 -12 months and 40 pr minute for children from 12 months to 5 years. We used the cut-off of 40 pr minute also for children aged 6 and 7 years, although the IMCI framework does not cover this age group.

1.4 Resistance to antimicrobial agents

1.4.1 Historical background

Penicillin was first discovered in 1928 [25], but it was not until the 14th of March 1942 that the first patient was successfully cured from infection with penicillin by Drs Bumstead and Hess. The drug went on to have a significant impact on saving lives during World War 2. The success with penicillin, anti-tuberculosis drugs and other antimicrobials had dramatic effect on the treatment of infectious diseases and led to a great deal of optimism. In 1969, the US Surgeon General summarized this enthusiasm with the following historical words to the Congress "The time has come to close the book on infectious disease." While many praised this vision, the realities of infectious diseases were to take an unexpected and completely different course in the following period.

Even before penicillin was used clinically, Abraham and colleagues had discovered an enzyme capable of destroying penicillin [26, 27]. By 1950, half of the *S. aureus* isolates were resistant to penicillin [28]. Penicillin-resistance first became prevalent among hospital-acquired staphylococci [29], but by the late 1960s also in community-acquired infections [30]. However, the implications of antimicrobial resistance were seriously underestimated and there was widespread confidence that science would find new solutions to this problem. Methicillin, introduced in 1959, offered a solution for treating penicillin-resistant staphylococci, however, already in 1961 Jevons described the first methicillin-resistant *S. aureus* (MRSA) [31]. Vancomycin was approved for clinical use in 1958 and was suitable to treat MRSA, and later on other

problem-organisms such as enterococci, various streptococci and *Clostridium difficile* [32]. By 1986, vancomycin-resistance started to emerge in enterococci in Europe [33]. The infectious disease experts feared that the much more virulent *S. aureus* would acquire resistance to vancomycin too. In 1997, vancomycin-intermediate resistant *S. aureus* (VISA) was discovered in Japan [34], and as recently as in 2002, the first clinical isolate of fully vancomycin-resistant *S. aureus* (VRSA) was isolated from a patient in Michigan, USA [35].

Similar developments of emerging resistance went on in other organisms, including Gram-negative bacteria. In 1948, Guiseppe Brotzu discovered that a substance produced by *Cephaloporium acremonium* effectively killed *Salmonella typhii*, laying the foundation for a whole new group of beta-lactam antibiotics, the cephalosporins. Starting with the use of cefalotin in 1964, the first-generation cephalosporins were succeeded by second-generation cephalosporins such as cefuroxime, and later on the third-generation oximino-cephalosporins, such as cefotaxime and ceftriaxone, which became fundamental in the treatment of Gram-negative bacteria, and ceftazidime, which had additional anti-pseudomonas effect. Ampicillin, the first penicillin with a broad-spectrum and activity against Gram-negative bacteria, was introduced in the early 1960s. Shortly after, Datta and colleagues in Greece described in a strain of *E. coli* a plasmid-mediated ampicillin-hydrolyzing beta-lactamases, which was named TEM-1 after the patient, whose name was Temoniera [36]. Another beta-lactamase, SHV-1, which is chromosomal in many strains of *Klebsiella* spp., spread via plasmids to *E. coli*, and other *Enterobacteriaceae*. The use of ampicillin selected for the spread of TEM-1 and other beta-lactamases. In 1985, Kliebe and colleagues discovered, SHV-2, the first extended-spectrum beta-lactamase (ESBL) capable of hydrolyzing third-generation cephalosporins in an isolate of *Klebsiella ozaenae* [37]. In the coming years, mutations have lead to the emergence of a large number of ESBL enzymes [38], currently counting over 100 in the TEM-family and over 50 in the SHV-family. Another type of ESBL, the CTX-M group, has probably evolved from chromosomal beta-lactamases in *Kluyvera* spp., and is particularly effective in hydrolyzing cefotaxime. The CTX-M group of ESBL now counts more than 40

variants, divided into 5 sub-classes, and is spreading fast, including in community-acquired isolates. Other beta-lactamases which are counted among the ESBLs are VEB, PER, GES and OXA.

Resistance to oximino-cephalosporins also emerged in *Enterobacter* spp. and other Gram-negatives by mutations in inducible chromosomal class C (AmpC) beta-lactamases, resulting in "derepressed" mutants, which produce these beta-lactamases in abundance. AmpC beta-lactamases have also migrated from chromosomal locations to plasmids and are spreading into *E. coli* and *Klebsiella* spp.

As the medical community started to realize the magnitude of the resistance problem, another catastrophe struck with the dawn of the HIV epidemic in the early 1980s. According to UNAIDS, there are currently almost 40 million people infected with HIV and 3 million people dying from this disease every year [39]. As HIV infection progresses, the individual becomes susceptible to bacterial and other infections which needs treatment with antimicrobials. Persons infected with HIV also experience fever episodes for other reasons than bacterial infections, and may thus consume more antimicrobials than others. Use of antibacterial agents, particularly broad-spectrum agents such as cephalosporins, is a known risk factor for infection with resistant bacteria. There is evidence of an association between HIV infection and bacterial resistance, and this has been linked to co-trimoxazole prophylaxis against *Pneumocystis jirovecii* pneumonia [40-42]. Thus, the HIV epidemic may contribute substantially to the resistance problem.

The optimism of the "golden age" of antibiotics, has given way to a reserved feeling, as bacteria have generated resistance against virtually any antimicrobial agent that humans have developed. While the pharmaceutical industry has largely been passive in developing new antimicrobials the last few decades, the emergence of VRSA [35, 43], plasmid-mediated AmpC and carbapenemases [44], seem to herald that we may be entering what Cohen called the post-antibiotic area [45].

1.4.2 Consequences of antimicrobial resistance

Intuitively, antimicrobial resistance leads to ineffective chemotherapy, which subsequently leads to treatment failure, increased morbidity, increased cost and, ultimately, increased risk of death [45-47]. According to WHO, infections with resistant organisms are more often fatal and lead to prolonged illness [48]. Due to the prolonged illness, there is greater risk of spread of the infection to other people. Costs are increased, not only because of the use of more expensive antimicrobials, but also because of longer duration of care and hospitalization. Prompt treatment with appropriate antibiotics is essential to prevent serious complications and death, particularly in serious infections such as bloodstream infections. While this reasoning seems obvious, there is not extensive scientific proof of this association, and studies assessing the association between resistance and adverse outcome are challenging for a number of reasons [49]. Firstly, confounding factors may influence the outcome. In particular, various underlying conditions are important to consider. It can sometimes be difficult to tell whether the adverse outcome is the result of an underlying disease or a consequence of antimicrobial resistance in the bacteria causing the infection. Furthermore, the design of the study, particularly, the choice of reference group, has great impact on the conclusions. Whether comparing patients with infections caused by resistant organisms to patients with infections with similar, but non-resistant bacteria, or comparing to those without infection, makes a big difference. The first design would evaluate the effect of having a resistant bug as compared to having a susceptible bug, while the last design would measure the combined effect of having an infection and having an infection with resistant bacteria. Thus, the choice of reference group becomes increasingly important the more virulent the bacteria in question are [50]. Many published studies are retrospective, while more reliable information would be obtained from prospective studies. Furthermore, the size of studies also limits the ability to detect associations, if the number of observations is too small, a biological difference might not be detected. Thus, meta-analysis of the data obtained from several studies is sometimes used to increase the data set and thus increase the chance of detecting differences. The challenge with meta-analyses is that

the studies on which it is based may not be designed in the same way so that they may not be directly comparable. Finally, the type of study outcome will influence the chance of actually measuring a difference. In economically developed countries, cost and morbidity are more sensitive measures of resistance than its impact on mortality [49]. In many low-income countries, the surge in antimicrobial resistance is seen as potentially disastrous because of the lack of resources for purchasing expensive second-line drugs [51-53]. However, while this notion appears plausible, again, there is lack of evidence of an association between antimicrobial resistance and adverse outcome in developing countries.

Impact antimicrobial resistance on morbidity

Frequently, duration of hospital stay is used as a proxy for morbidity. It is intuitive that inappropriate chemotherapy would lead to more suffering for the patient. Several studies have documented an association between increased duration of hospital stay and infections with resistant bacteria [49]. The duration of hospital stay significantly ($p<0.001$) increases if *S. aureus* surgical site infections are caused by methicillin-resistant strains [54]. Likewise, the patients with infections caused by penicillin-resistant pneumococci stay longer in hospital than those with penicillin-susceptible pneumococci [55]. Infection with ESBL-producing *E. coli* and *Klebsiella* spp. is also associated with increased duration of hospital stay [56].

Cost implications of antimicrobial resistance

The cost of treating patients with infections caused by resistant bacteria increases due to the higher cost of second-line drugs and the longer duration of hospital stay. Significant association between infection with resistant causative microbe and higher cost has been shown for penicillin-resistant pneumococci [55], methicillin-resistant *S. aureus* bacteremia [57] and ESBL-producing *E. coli* and *Klebsiella* spp. [56].

Impact antimicrobial resistance on mortality

Many highly resistant bacteria, such as enterococci, have relatively little virulence and foremost cause disease in hospitalized patients with serious underlying diseases

and/or immunosuppression. In these cases, it may be difficult to determine whether the adverse outcome is related to the resistance or to the underlying conditions. Still, both prospective [58, 59] and retrospective studies [60-63] have shown an increased risk of death from enterococcal infections if it is caused by VRE, although two of these studies used patients without enterococcal infections as controls [60, 63].

Meta-analyses of available studies on bacteremia caused by *S. aureus* found that patients with MRSA had increased risk of fatal outcome compared to those with methicillin-sensitive S. aureus [64, 65]. A prospective study of hemodialysis patients with *S. aureus* bacteremia showed increased risk of death in patients with MRSA infection compared to those with MSSA. Similarly, surgical site infections with MRSA have been associated with increased risk of fatal outcome in prospective studies [54].

In infections caused by pneumococci, there has not been established any significant association between penicillin resistance and increased case-fatality rates [66-68]. Possible explanations for this observation could be that penicillin-resistant pneumococci may be less virulent, that patients acquire pneumococcal infections in the community and thus may have less underlying disease, or that empirical use of appropriate antimicrobial agents, such as vancomycin, is high in areas where penicillin-resistant pneumococci are prevalent.

A number of studies have assessed the effect resistance on outcome of infections caused by Gram-negative bacteria. Both for *Pseudomonas aeruginosa* [69] and for *Enterobacter* spp., the emergence of resistance in the causative isolate during treatment has been linked to increased case-fatality rates. A recent review by Cosgrove and colleagues in a leading journal [49] reported no studies showing significantly increase in case-fatality rates associated with infections with ESBL-producing Gram-negative bacteria, referring to a retrospective matched cohort study by Lautenbach and colleagues [56]. A previous retrospective study from South Korea does however report increased case-fatality rates in pediatric cases of bacteremia caused by ESBL-producing *E. coli* and *Klebsiella* spp. compared to those caused by

non-ESBL-producing isolates [70], although the reported figure was obtained by univariate analysis (patients dying from underlying diseases had been removed from the analysis). A microbiologic case-control study from Chicago [71] revealed that patients with bacteremia caused by ceftazidime-resistant *E. coli* and *Klebsiella* spp. were more likely to survive (p=0.02) if they received appropriate treatment within 3 days of start of the bacteremic episode.

1.4.3 Resistance mechanisms

The mechanisms for antimicrobial resistance in bacteria can be divided into three broad categories 1) enzymatic inactivation of the antimicrobial agent, 2) substitutions, amplifications or modifications of the drug target reducing the affinity of the drug to the target or 3) Reduced access of the antimicrobial agents to the target by means of permeability barriers or efflux pumps [72, 73].

Enzymativ inactivation of the antimicrobial agent

The typical example of enzymes, which inactivate the antimicrobial agent, is the beta-lactamases. The beta-lactamases are enzymes, which destroy beta-lactams. They may be chromosomal or plasmid-mediated and are involved in resistance in *S. aureus*, Gram-negative rods, gonococci and *Haemophilus influenzae*. They differ in antimicrobial spectrum from the simple penicillinases capable of hydrolyzing benzyl-penicillin, to more broad-spectrum beta-lactamases, such as TEM-1, which hydrolyzes ampicillin, to extended-spectrum-beta-lactamases, which hydrolyze oximino-cephalosporins, AmpC beta-lactamases, which are also inhibitor-resistant, and carbapenemases which neutralizes even carbapenems.

Furthermore, enzymatic modification of a variety of antimicrobials can occur by means of cytoplasmic modifying enzymes. Enzymatic degradation by aminoglycoside modifying enzymes (aminoglycoside phosphotransferases APH, acetyltransferases AAC and nucleotidyltransferases ANT) is an important mechanism for resistance to aminoglycosides in Gram-negative rods and enterococci. Enzymatic modification or inactivation can cause resistance to chloramphenicol

(chloramphenicol acetyl transferase), macrolide resistance in *Enterobacteriaceae* and staphylococci (*EreA*, *EreB*) and resistance to streptogramin A (acetyltransferase) and streptogramin b (hydrolyzing enzymes, *vgb*, *vgbB*). The *tetX* gene encodes a tetracycline-inactivating enzyme, but its clinical importance is not well known [73].

Altered target of the antimicrobial agent

Beta-lactams exert their antimicrobial action by inhibiting the transpeptidase and carboxypetidase activities of the cell-wall synthesizing enzymes, the so-called penicillin binding proteins (PBP). The mechanism for resistance to methicillin in *S. aureus* is alteration of the PBP. The *mecA* gene encodes an altered PBP, called PBP2a or PBP2', which has reduced affinity for beta-lactams, thus methicillin-like drugs such as cloxacillin will not be able to interfere with cell-wall synthesis. Penicillin-resistance in pneumococci is also caused by altered PBP. Pneumococci commonly have 6 PBPs, PBP1a, PBP1b, PBP2a, PBP2b, PBP2x, and PBP3. Resistance is the result of altered pbp1a, pbp2b, and pbp2x low-affinity, which are encoded by mosaic genes believed to contain gene material acquired from other species such as *Streptococcus mitis* [73]. The glycopeptides, vancomycin and teicoplanin, exert their action by binding to the D-alanyl–D-alanine side chains of peptidoglycan, thus preventing the cross-linking of the pedtidoglycan chain and thereby disrupting cell wall synthesis. Resistance to vancomycin and teicoplanin is the result of the production of a different ligase, VanA, encoded by the *vanA* gene, which produces peptidoglycan side chains with less affinity for glycopetide antimicrobials. The vanB1-3 genes only confer resistance to vancomycin, not teicoplanin. While vancomycin-resistant enterococci are important pathogens in nosocomial infections and immunocompromised hosts, unfortunately, vancomycin-resistance has recently become a reality also in the much more virulent *S. aureus* (VRSA) [35, 43].

Protein synthesis in the ribosomes is the main target of a number of antimicrobials, including aminoglycosides (gentamicin, tobramicin, streptomycin), tetracyclines and the MLS group of antimicrobials macrolides, lincosamins and streptogramins, and alterations of ribosomal targets can result in resistance to these drugs. MLS

antimicrobials have a wide selection of resistance mechanisms. In Gram-positive bacteria, alteration in 23S rRNA mediated by *erm* genes leads to resistance to macrolides, lincosamines and streptogramin B, but does not affect streptogramin A [73]. While aminoglycoside modifying enzymes are quantitatively more important, altered rRNA can also lead to resistance to aminoglycosides. Altered ribosomal target (TetM) causes tetracycline resistance in gonococci and *S. aureus*.

Quinolones exert their antimicrobial action by inhibiting the DNA gyrase, which is pivotal in the coiling of DNA. The primary mechanism responsible for resistance to fluoroquinolones in Gram-negative rods is alteration of the DNA gyrase, particularly the GyrA subunit encoded by the *gyrA* gene. In Gram-positive organisms, alteration of the topoisomerase IV confers resistance.

The folate inhibitors, trimethoprim and the sulfonamides, exert their antimicrobial action by inhibiting folic acid synthesis in the target organism. Resistance to folate antagonists is caused by altered target enzymes, DHFR for trimethoprim and DHPS for sulfonamides.

Impaired access of the antimicrobial agent

Bacteria can reduce the access of antimicrobials by two principle ways, reduced permeability for the drug or by efflux pumps that remove the drugs from the cell. Reduced permeability makes Gram-negative bacteria inherently resistant to macrolides, lincosamines and streptogramines, and causes resistance to beta-lactam antimicrobials and aminoglycosides in *Pseudomonas aeruginosa* and *E. cloacae* [73]. Macrolide efflux pumps cause resistance to macrolides in staphylococci, and tetracycline efflux pumps cause resistance to tetracyclines in both Gram-negatives and Gram-positives (TetA-E and TetG-H). Efflux pumps (NorA) also cause resistance to fluoroquinolones in Gram-negative rods and *S. Aureus*.

1.4.4 How does resistance emerge and spread

Selection pressure and risk factors

The mold growing and inhibiting bacteria on Fleming's agar plates [25] probably developed the bactericidal substance, penicillin, as a means to survive in a natural environment in competition with numerous other organisms. In a similar manner, bacteria develop antimicrobial resistance mechanisms as a defense against any, for them, toxic substance, which nature or humans throw at them. Thus, antimicrobial resistance is a natural phenomenon, which helps microbes survive in an environment with toxic substances. In an environment free of the particular toxic substance or antimicrobial agent, the presence of antimicrobial resistance mechanisms may incur a cost for the bacterium. However, in an environment where antimicrobials are present, such as hospital settings, bacteria harboring resistance mechanisms get an advantage in surviving by Darwinian selection [74]. Consequently, any use of antimicrobial drugs, whether appropriate or not, has the potential to lead to the selection of resistant bacteria [75, 76]. In Europe, it is striking that both outpatient antimicrobial consumption and antimicrobial resistance rates are higher in Southern European countries than in Northern Europe [77].

While even appropriate antimicrobial use may select resistant bacteria, this problem is bound to be greater with exaggerated and irrational use of drugs. Using narrow-spectrum antimicrobials in a sufficient dose, for the correct duration, kills off the intended bacteria, while leaving the least possible effect on the natural flora of the host. Conversely, using unnecessarily broad-spectrum antimicrobials leads to a higher degree of "collateral damage" in terms of unwanted ecological effects, selection of resistant bacteria and colonization or overt infection with resistant bacteria [78]. Cephalosporin and fluoroquinolones have been embraced by clinicians for their combination of bactericidal properties towards a broad spectrum of relevant clinical pathogens and their relatively infrequent side effects. However, there is increasing evidence to link cephalosporin use to infection with ESBL–producing *Klebsiella pneumoniae*, vancomycin-resistant enterococci and "antibiotic-associated diarrhea"

caused by *Clostridium difficile*. Similarly, use of fluoroquinolones is associated with MRSA infections and increasing rates of resistance to fluoroquinolones in Gram-negative bacilli, including *Pseudomonas aeruginosa*.

Inappropriate use of antimicrobial therapy may further increase the risk of selecting resistant bacteria, since sub-therapeutic drug levels may only suppress bacteria, but not eradicate them, thus increasing the number of bacteria that are exposed to the drug and the time of exposure, and allowing for the survival of partially treated microbes. Inappropriate use of antimicrobials is common and may be propelled by erroneous prescription and availability of antimicrobials over-the-counter without prescription [79, 80]. In developing countries, the use of poor-quality and counterfeit pharmaceuticals is an extremely serious problem, which appears to be disturbingly widespread [81-85]. If available drugs are of poor-quality, even the best attempt at rational treatment will become *de facto* inappropriate.

Besides the use of antimicrobials, other factors have been identified as risk factors for acquiring infections with resistant bacteria. Risk factors for hospital-acquired infections with ESBL-producing bacteria are admission to intensive care units, receipt of parenteral nutrition, use of indwelling catheters, renal failure and burns [86]. Risk factors for acquiring infections with ESBL-producers outside hospitals are antimicrobial treatment during the last 3 months, particularly with cephalosporins, age over 60 years, underlying diabetes and a history of recent hospitalization [87]. Finally, the HIV epidemic may also contribute to the current worldwide surge in antimicrobial drug resistance [40-42].

Acquisition and spread of resistance traits

Some resistance traits are inherent to particular bacteria such as ampicillin-resistance in *Klebsiella pneumoniae*, cephalosporin-resistance in enterococci and erythromycin-resistance in many Gram-negative bacteria. Other resistant traits are acquired. Bacteria can acquire resistance traits by three principally different ways: 1) accumulation of mutations in the bacterial chromosome, 2) acquisition of a new gene and 3) intrageneic recombination of genes to form mosaic genes which encode

resistance traits [88]. Examples of mutations leading to antimicrobial resistance are the mutations in gyrA, gyrB, parC, parE genes leading to fluoroquinolone resistance and the mutation in the rpoB gene in *M. Tuberculosis* leading to rifampicin resistance. Acquisition of new genes encoding for resistance traits can occur by different mechanisms such as plasmid transfer and conjugation, which occurs in both Gram-positive and Gram-negative bacteria, and transformation and transduction in Gram-positive bacteria. Intrageneic recombination of genes is the cause of emergence of penicillin-resistance in pneumococci [88].

Resistance traits can spread by proliferation of the bacteria harboring these traits, so-called vertical transfer, which means that resistant bacteria multiply and get offspring with similar resistance traits. Poor hygiene allows resistant bacteria to spread more easily. In hospitals where there is a high consumption of antimicrobials, resistant bacteria get a competitive advantage over susceptible ones. In addition, resistance genes may spread horizontally among bacteria, e.g. via plasmids. There is evidence that coliform bacteria can exchange plasmids with resistance genes in the gut [89, 90].

2. Rationale for the study

Bacterial infections are a major cause of morbidity and mortality, particularly in low-income countries [4-6]. The global emergence of antimicrobial resistance undermines the management of infectious diseases [45, 46, 49]. Availability of antimicrobials without prescription, use of poor-quality antimicrobials and other factors, which promote the emergence of antimicrobial resistance, may be more frequent in developing countries [81-85]. At the same time, the consequences of antimicrobial resistance may be felt harder in a setting of scarce economical resources, because alternative antimicrobial drugs tend to be unavailable or unaffordable [51-53, 91]. The HIV epidemic may influence both the spectrum of bacteria causing infections [92, 93] and their antimicrobial resistance patterns [41, 94, 95]. Despite its obvious importance, there is little published information on antimicrobial resistance in the developing world. Available data from Tanzania [96-98] and neighboring countries [99-104] suggested there was significant rates of antimicrobial resistance particularly in Gram-negative bacteria. Since antimicrobial resistance varies greatly among geographical locations it is essential to base empiric therapy of serious infections such as bloodstream infections on sound knowledge of the prevalence and antimicrobial resistance patterns of local bacterial isolates [105]. The rationale for the study was to gain more insight into the epidemiology of certain bacterial infections and their resistance patterns in selected areas of Tanzania in order to increase the evidence available to make sensible decisions on antimicrobial therapy both at the level of the practicing clinician and at the level of authorities responsible for developing guidelines.

3. Aims of the study

The aims of the study were:

- To implement and evaluate a computerized system for surveillance of antimicrobial resistance at MUCHS.
- To determine the prevalence of various pathogenic bacteria, fungi and malaria parasites as etiological agents in bloodstream infections in infants and children presenting with fever at MUCHS.
- To describe the susceptibility patterns of the isolated pathogenic bacteria and the presence of specific resistance problems such as MRSA and ESBL.
- To assess any impact of HIV co-infection on the prevalence and antimicrobial susceptibilities of the causative agents.
- To assess the impact of resistant bacteria on the patient outcome.
- To evaluate the microbial etiology and resistance patterns in pediatric meningitis cases at Haydom Lutheran Hospital.
- To assess the microbial etiology and susceptibility patterns bacteriuria in pregnant women in Mbulu and Hanang district.
- To compare antimicrobial resistance data from urban and rural areas of Tanzania.

4. Study population and methods

4.1 Study settings

The studies were performed in two areas of Tanzania. Papers 1, 2 and 3 are based on work from Muhimbili University College of Health Sciences (MUCHS) in the commercial capital, Dar es Salaam, and papers 4 and 5 are based on work done in and around Haydom Lutheran Hospital in a rural area in Manyara Region, Northern Tanzania. Microbiological investigations were done at the Department of Microbiology and Immunology, MUCHS, at Institute of Medicine, University of Bergen, Norway and at Department of Microbiology, Ullevål University Hospital, Oslo.

4.1.1 Tanzania

The country comprises 945 090 square kilometer. It has an estimated population of 34.4 million and an annual population growth rate of 2.9%. The official sizes of the populations of the two study areas are 2.5 million in the region of Dar es Salaam and 1.0 million in Manyara region (www.tanzania.go.tz/census/). Eighty percent of the population is employed in agricultural activities. Small-size (0.9-3.0 hectare) farms dominate the agricultural sector. Seventy percent of farmland is cultivated by hoe, 20% by ox-plough and only 10% by tractor. The agricultural production suffers from poor farming tools and a combination of unstable weather conditions and lack of irrigation facilities. The main staple crops are maize, sorghum, millet, rice, wheat, beans, cassava, bananas and potatoes. The main export crops are coffee, cotton, cashew nuts, tobacco, sisal, pyrethrum, tea, cloves, other spices and flowers. Tanzania is considered one of the economically poorest countries in the world with an estimated per capita income at only 330 USD in 2005 (www.worldbank.org). Tanzania spends 12 USD per capita on health annually, or an estimated 4.1% of the GDP [4]. The country has prioritized primary health care and has excellent coverage

of the childhood immunization program. Lately, the net enrollment in primary school has increased considerably from 59% in 2000 to 95% in 2005. Although still high, there has been a significant decrease in infant mortality (from 100 to 68 per 1000 live births) and child mortality (from 156 to 112) from 2000 to 2004. Maternal mortality remains extremely high at 1.5% of all births (compared to 0.006% in Norway), and the country has a high burden of the major infectious diseases such as malaria, tuberculosis (estimated incidence 371 pr 100,000) [106] and HIV infection (estimated prevalence of 7% of the population) [107]. Currently the estimated life expectancy at birth is only 46 years. The country has 8 consultant/specialized hospitals, of which 4 are government run. There are 17 regional hospitals (all government) and 68 district hospitals (55 government). 479 health centers (409 government) and more than 3955 dispensaries (2450 government).

4.1.2 Dar es Salaam, MUCHS

With more than 1000 beds Muhimbili National Hospital / MUCHS is the largest hospital in the country, serving as a national referral and university teaching hospital, as well as a primary and referral hospital for the population in the Dar es Salaam area. Dar es Salaam is expanding rapidly under the influx of people from other parts of the country and abroad. The Department of Microbiology and Immunology at MUCHS analyses specimens from inpatients and outpatients at the hospital, as well as specimens from a number of nearby situated hospitals. The Department of Pediatrics has a neonatal section (Ward 36), two wards for general pediatrics (ward A and B), one ward mainly for gastroenteritis (ward 17) and a ward for malnutrition (Makuti). Only patients aged 0 to 7 years are admitted to the pediatric wards. The most commonly used antimicrobial treatment regimens for common infections are presented in Table 2, and the dosages used in Table 3 (these tables were not presented in the articles).

Table 2. Treatment regimens for bacterial infections in infants* and children† at MUCHS

Diagnosis	Drugs (administration route ‡)	Duration (days)
Septicemia	* Ampicillin (iv) + cloxacillin (iv) + gentamicin (iv)	5 – 10 depending on the severity
	† Benzyl penicillin (im/iv) + gentamicin (iv)	
	† Benzyl penicillin (im/iv) + chloramphenicol (iv)	
Meningitis	* Ampicillin (iv) + cloxacillin (iv) + gentamicin (iv)	14 - 21
	† Benzyl penicillin (im/iv) + chloramphenicol (iv)	
Pneumonia	* Ampicillin (iv) + cloxacillin (iv) + gentamicin (iv)	7 - 10
	† Benzyl penicillin (im/iv) + gentamicin (iv)	
Upper respiratory tract infections, sore throat, ear infections	Amoxicillin (po), erythromycin (po) or cephalexin (po)	5 - 7
Skin infections	Phenoxymethyl-penicillin (po), cloxacillin (po), erythromycin (po) or cephalexin (po)	5 - 7
Osteomyelitis	Ampicillin (iv) + cloxacillin (iv) + gentamicin (iv)	6 weeks
Urinary tract infection	Co-trimoxazole (po), ampicillin (po), amoxicillin-clavulanate (Augmentin) (po)	
Severe urinary tract infection	Ampicillin (iv) + gentamicin (iv)	7 - 14
Necrotizing enterocolitis	Ampicillin (iv) + gentamicin (iv)	7 - 14
	Ampicillin (iv) + chloramphenicol (iv)	
	Gentamicin (iv) + metronidazol (iv)	
Suspected staphylococcal infection (skin, surgery, late onset sepsis)	Cloxacillin (iv/po) + gentamicin (iv)	14

* Infants. † Children ≥ 1 month. ‡ po = oral, im = intra-muscular, iv = intra-venous. In addition, there is occasional use of cefuroxime, cefotaxime, ceftriaxone, and amikacin

Table 3. Dosage schedules for commonly used antimicrobial agents in children at MUCHS

Drug (administration route*)	Total daily dose	Number of doses per 24 hours	
	(mg/kg)	Age <= 1 week	Age > 1 week
Benzyl-penicillin (iv)	100 - 200	2	3
Phenoxymethyl-penicillin (po)	25 - 50	4	4
Ampicillin (po, im, iv)	50 - 200	2	3
Amoxicillin (po)	20 – 40	3	3
Amoxicillin clavulanate (po)	20 – 40	3	3
Cloxacillin (po, iv)	100	2	3
Cephalexin (po)	25 – 100	4	4
Cefuroxime (iv)	50 – 240	2	2
Cefotaxime (im, iv)	50 – 180	4	4
Ceftriaxone (im, iv)	50 – 100	2	2
Erythromycin (po)	20 - 40	3	3
Co-trimoxazole (po)	8 / 40	2	2
Gentamicin (iv)	5	1	1
Gentamicin (iv) †	2.5	1	2
Amikacin (iv)	15	1	1
Amikacin (iv) ‡	20	1	1
Chloramphenicol (iv)	50	2	3
Chloramphenicol (iv) †	25	1	1
Metronidazole (po, iv)	15	2	3

* po = oral, im = intra-muscular, iv = intra-venous. † Low birth weight. ‡ Below 1 year of age

During the year 1997-98 the neonatal ward admitted 7,236 patients and ward A + B together admitted 7,241 patients. The department has a total of 120 beds and 70 cots. There are approximately 30-40 deliveries per day at the hospital, out of which

approximately 20% are done by cesarean section. The resources available in the pediatric department include nutrition via nasogastric tube, IV/IM administration of drugs, blood transfusions, phototherapy, oxygen treatment with mask and electrocardiography. A CPAP machine was available, but was out of order by the time of the study. Respirators and cardiopulmonary surveillance are not available.

4.1.3 Haydom Lutheran Hospital, Mbulu and Hanang

The study of urinary bacterial pathogens was undertaken at antenatal care visits through eleven outreach clinics run by Haydom Lutheran Hospital and one stationary clinic at the hospital [108]. Dongobesh and Basotu, in Mbulu and Hanang districts, respectively, are typical rural areas in Manyara region (previously part of Arusha region) in northern Tanzania. The major causes of stillbirths and perinatal mortality in the study area are infections (39%), particularly malaria and pneumonia, as well as asphyxia (24%) and immaturity (15%) [109]. The HIV-sero-prevalence in the study area was low, only 0.3% and 0.4%, respectively, in two studies from 1996 and 1998 [110]. Haydom Lutheran Hospital is situated 300 km from Arusha, which is the nearest major city. The outreach clinics, located five to one hundred kilometers from Haydom Lutheran Hospital were visited on a monthly basis.

4.2 Study populations

The WHONET surveillance study (paper 1) was a laboratory-based study, in which all bacterial isolates of clinical significance, a total of 7617 isolates, from specimens received at MUCHS during the period July 1st 1998 to December 31st 1999 were recorded and analyzed. The specimens examined included urine, pus/secretions (swabs from skin, surgical and traumatic wounds, burns, umbilical cords, throat, nose, eye and ear discharge and genital swabs), blood, cerebrospinal fluid, other body fluids, stools and other specimens. Mycobacteria and anaerobic bacteria were not included in the study.

The study population in the bloodstream infection study (paper 2) consisted of consecutively admitted patients who upon admission to the pediatric department at MUCHS had temperature instability or other signs or symptoms of serious systemic infection, such as sepsis, meningitis, pneumonia, typhoid etc. A total of 1787 patients were enrolled, corresponding to 1828 admissions.

The study population in the study on ESBL-producing bacteria (paper 3) was a subset of the study population in the bloodstream infection study (paper 2), and included all children who had *E. coli*, *Klebsiella* spp. or salmonella isolated from their blood cultures, corresponding to a total of 113 children.

The study population of the meningitis investigation (paper 4) included 24 children with suspected meningitis and/or septicemia, out of a total of 360 children, who were admitted at Haydom Lutheran Hospital from July to August 2000.

The study population in the bacteriuria study (paper 5) included 5153 pregnant women consecutively enrolled between mid-April 1995 and mid-March 1996 as they attended antenatal care visits through eleven outreach clinics and one stationary clinic run by Haydom Lutheran Hospital [108]. The majority of the study subjects (n=3715) were residents of two divisions, Dongobesh and Basotu, in Mbulu and Hanang districts, and the study covered an estimated 68% of the pregnant women in those two divisions [108].

4.3 Study designs

The WHONET surveillance study (paper 1) was a laboratory-based prospective, observational cohort study, and a qualitative evaluation of the intervention of introducing a computerized surveillance system at MUCHS.

The bloodstream infection study (study 2 & 3) was a prospective, observational cohort study with consecutive inclusion of study subjects suspect of having systemic infection. In both papers, nested case-control designs within the cohort of the main

study were used to assess risk factors for infection caused by bacteria with certain resistance traits, and risk factors for adverse outcome.

The investigation of the meningitis cases at Haydom Lutheran Hospital (paper 4) was an outbreak investigation as part of the quality assurance of medical services at the hospital. The design of a prospective, observational cohort study was used to assess the association between salmonella meningitis and fatal outcome.

The study of bacteriuria in pregnant women (paper 5) was a prospective, observational cohort study including consecutively pregnant women attending antenatal clinics. A nested case-control design within the main cohort was used to assess the impact of bacteriuria and antimicrobial resistance on outcome of the pregnancy.

4.4 Methods

4.4.1 Specimen collection, transport and bacterial isolation

In the WHONET surveillance study (paper 1), microbiological specimens were obtained as part of the routine diagnostic services in accordance with regular practice at the hospital.

In the bloodstream infection study (paper 2 and 3), blood specimens (1 ml from neonates, 5 ml from older children) were inoculated bedside in BACTEC Myco/F lytic blood culturing vials (Becton Dickinson, Franklin Lakes, NJ). Positive blood cultures were subcultured on Columbia II agar base (Oxoid Ltd, Basingstoke, UK) with five percent human blood, chocolate agar and MacConckey agar (Difco/BD Diagnostic Systems, Sparks, MI, USA). The culturing vials also support the growth of *M. tuberculosis* and other mycobacteria.

In the meningitis investigation (paper 4), blood and spinal fluid specimens were inoculated in BBL SeptiChek blood-culture bottles (Becton Dickinson, Sparks, MD USA) and on locally prepared non-selective Thayer-Martin medium in slanted tubes,

respectively. All cultures were incubated at 35˚C for 5 days and inspected daily for bacterial growth. Positive bacterial specimens were shipped to University of Bergen, Norway, for further study.

In the bacteriuria study (paper 5), 'clean-catch' midstream urine specimens were collected in pre-boiled and air-dried plastic containers. Part of the specimen was inoculated immediately using the Uricult ® dip slide (Uricult ®, Orion Diagnostica, Espoo, Finland). The dip slides were transported to the hospital within 2-9 hours and incubated at 37˚C for 18-24 hours. Significant bacteriuria was defined as growth of more than 100,000 colony-forming units per ml of one or two bacterial isolates [21, 22]. The remaining urine was examined for leukocyte esterase, nitrite, blood, albumin and glucose using a reagent strip (Nephur-Test ® + Leuco, Boehringer Mannheim Gmbh, Mannheim, Germany). Positive dip slides were sent to Norway for further microbiological investigations.

4.4.2 Identification

Bacterial isolates were identified using standard laboratory methods [111, 112], including the use of API20E, API20NE and API 20 AUX systems (bioMérieux SA, Marcy l'Etoile, France). The identify of isolates of enterococci and *S. aureus* were confirmed by PCR [113].

4.4.3 Susceptibility testing

The antimicrobial susceptibilities of the bacterial isolates were examined by disk diffusion methods. In the WHONET surveillance study (paper 1), disk diffusion testing was done according to the Stokes' method [114] on Iso-Sensitest (Oxoid Limited, Basingstoke, UK) agar plates. In the bloodstream infection study (paper 2 & 3), disk testing was done according to CLSI (NCCLS) guidelines [115]. In the meningitis investigation (paper 4) and the bacteriuria study (paper 5) disk testing was done according to Scandinavian guidelines on PDM medium (AB Biodisk, Solna, Sweden) [116]. In the bloodstream infection study (paper 2 & 3) Gram-negative

isolates from blood cultures with reduced susceptibilities to cefotaxime (zone diameter <27mm) and/or ceftazidime (zone diameter <22mm) according to guidelines for laboratory detection of ESBL from the Centers for Disease Control and Prevention (http://www.cdc.gov/ncidod/hip/Lab/FactSheet/esbl.htm) were tested for ESBL phenotype with three different Etest ESBL strips, ceftazidime / ceftazidime + clavulanate, cefotaxime / cefotaxime + clavulanate and cefepime / cefepime + clavulanate (AB Biodisk, Solna, Sweden).

4.4.4 Detection and characterization of resistance genes

In the bloodstream infection study (paper 2 & 3), we used a multiplex PCR to confirm the presence of the *mecA* gene conferring methicillin resistance and the *nuc* gene, which verifies that the isolate is a S. aureus [113, 117]. Isolates with ESBL phenotype were examined for the presence of bla_{TEM}, bla_{SHV} and bla_{CTX-M} by PCR [118-120]. The PCR products were sequenced with the ABI PRISM BigDye cycle sequencing ready reaction kit (PE Biosystems, Norwalk, CT) using the same primers. The products were analyzed on an ABI PRISM 3700 DNA sequencer (PE Biosystems). Sequences were aligned with known ESBL sequences (www.lahey.org/studies/) using Vector NTI version 6 (Informax, Frederick, Maryland, US).

4.4.5 Evaluation of relatedness of bacterial isolates

Gram-negative isolates from the bloodstream infection study (paper 2 & 3) and the meningitis investigation (paper 4) were explored with amplified fragment length polymorphism [121]. Salmonella isolates from meningitis investigation (paper 4) were also genotyped by pulsed-field gel electrophoresis.

4.4.6 Resistance surveillance

The WHONET software, available free-of-charge from WHO [122], was implemented and used for the surveillance of antimicrobial resistance at MUCHS (paper 1).

4.4.7 Statistical methods

The WHONET software was used for entry and preliminary analysis of microbiology data (paper 1, 2 & 3). We used Stata 8.0 for Macintosh (Stata Corporation, College Station, Texas, USA) for further analysis of data. Assessment of differences of proportions and univariate assessment of risk factors for intra-hospital death was done by Fisher's exact test with a two-sided *P*-value and odds ratios, and 95% confidence intervals were obtained by the 'logistic' function in Stata. Multivariate analysis (papers 2 & 3) was performed by automated and manual backwards step-wise logistic regression where factors with $P>0.2$ were removed from the model. Comparisons of medians of time variables were done by Wilcoxon rank-sum (Mann-Whitney) test. Outcome data on intrahospital death was also evaluated by Kaplan-Meyer survival analysis.

4.5 Ethical considerations

The WHONET surveillance study (paper 1) was a laboratory-based exercise with an aspect of quality assurance and did not involve any intervention concerning the patients directly. It was not deemed necessary to seek ethical clearance for this study.

The study of pediatric bloodstream infections (paper 2 & 3) was performed as part of the regular laboratory support for the pediatric department. Informed consent from the patient's parents or responsible family member was obtained before taking blood for microbiological investigations when feasible. The Tanzanian national language, Kiswahili, was used for obtaining consent using consent forms. When patients were critically ill with suspected sepsis or meningitis, a blood specimen was taken based

on oral consent, since these investigations are strongly recommended as routine test in such situations, and since it may be unethical and inappropriate to waste time on paperwork in such situations. In such cases, written consent was obtained in retrospect. When patients' parents did not accept HIV testing, they were allowed to opt out for HIV testing and still be included for bacteriological investigations. The Muhimbili University College of Health Research Ethics Committee approved the study protocol. The protocol was also submitted to the Regional Committee for Ethics in Medical Research for Western Norway (previously REK III, now REK Vest), which gave a preliminary recommendation for the study.

The meningitis investigation (paper 4) was a case investigation requested by the hospital as part of the quality assurance of the medical services and, as such, did not need ethical clearance.

The bacteriuria study (paper 5) was approved by the Commission for Science and Technology (COSTECH) in Tanzania and the Regional Committee for Ethics in Medical Research for Western Norway (REK III/ REK Vest). Participation in the study was voluntary. Study subjects received free treatment with nitrofurantoin if they had asymptomatic bacteriuria or urinary tract infection.

5. Main results of the studies

5.1 Paper 1 – The resistance surveillance study

The paper describes the implementation of a computerized system for surveillance of antimicrobial resistance. The WHONET software was well suited to enter and analyze data on a large number of bacterial isolates. The study evaluated more than 7500 bacterial isolates, of which 10% were from blood cultures, and over 40% each from pus and urine cultures. Gram-negative bacteria showed relatively high rates of resistance to most antimicrobial drugs, except for fluoroquinolones, gentamicin and third-generation cephalosporins. The software was free-of-charge, thus, the direct cost of implementing the surveillance system was small, limited to the purchase of a basic computer, as well as some basic training activities. The running cost of the surveillance program was limited to human sources for operating the software, minimum 50% of a laboratory technician position. An important aspect of a surveillance system is its function as a quality assurance tool and its ability to attract focus on laboratory issues which need to be improved. Susceptibility data would give more information if results were recorded as inhibition zone diameters instead of interpreted values (“R”, “I” or “S”). Furthermore, it was highlighted that the surveillance system is dependent on susceptibility testing of acceptable quality. While quality susceptibility testing may incur extra costs, surveillance data may improve empiric therapy for infections and contribute to containing or reducing antimicrobial resistance, which in the long term may help reducing morbidity and mortality, and diminish the need for expensive second-line antimicrobial agents and thus save lives and reduce suffering.

5.2 Paper 2 – The study of bloodstream infections

This paper describes the prospective observational cohort study of bloodstream infections in 1828 admissions of children aged 0-7 years. As expected in a cohort of children with suspected systemic infection, almost all (94%) received antimicrobial therapy. Table 1 in Paper 2 (Annex) shows details on the antibiotic consumption in the study population. Table 4 (not presented in the article) shows the market shares of the most common antimicrobials as estimated from a survey of 15 randomly selected pharmacies in Dar es Salaam (not shown in Paper 2) [123]. The prices of commonly used antimicrobial formulations are shown in table 5 (not presented in the article). The survey was done in September 2000 by two Norwegian medical students attached to our project using a questionnaire in Kiswahili, which was completed and returned anonymously by the pharmacist.

Table 4. Sales of antimicrobial drugs (percentage of total defined daily doses (DDD) sold) from 15 randomly selected pharmacies in Dar es Salaam in September 2000.

Penicillin	2.8%
Ampicillin, amoxicillin	23.1%
Cloxacillin	8.8%
Cephalosporins	0.04%
Tetracycline	19.8%
Erythromycin, and other macrolides	9.1%
Co-trimoxazole	5.2%
Trimethoprim	4.6%
Quinolones	14.2%
Aminoglycosides	1.3%
Chloramphenicol	1.3%
Metronidazole	8.6%

Table 5. Cost of antimicrobials per defined daily dose (DDD

Drug sales at pharmacies	Form	DDD (mg)	TSH pr DDD	Euro* pr DDD
Nitrofurantoin	Tab	200	30	0.003
Tetracycline	Caps	1000	60	0.006
Co-trimoxazole	Tabs	2000	62.5	0.064
Metronidazole	Tabs	1500	75	0.075
Amoxicillin	Caps	1000	160	0.160
Erythromycin	Tabs	1000	200	0.200
Ampicillin	Caps	2000	280	0.280
Penicillin	Tabs	2000	280	0.280
Cloxacillin	Caps	2000	320	0.320
Ampicillin+cloxacillin	Caps	2000	400	0.400
Chloramphenicol	Caps	3000	420	0.420
Ciprofloxacin	Tabs	1000	600	0.600
Azithromycin	Caps	300	900	0.900
Nalidixic acid	Tabs	4000	1200	1.200
Amoxicillin-clavulanate	Tabs	1000	2000	2.000
Cefalexin	Caps	2000	2000	2.000
Cefaclor	Caps	1000	4000	4.000
Penicillin	Inj	3600	450	0.450
Gentamicin	Inj	240	750	0.750
Chloramphenicol	Inj	3000	1800	1.800
Ampicillin	Inj	2000	2000	2.000
Cefuroxime †	Inj	3000	31200	31.200

*The exchange rate for Euro to Tanzanian shillings at the time was roughly estimated 1:1000.

†Third-generation cephalosporins were not available from the pharmacies surveyed, but were generally more expensive than cefuroxime, which was the most costly drug among those available.

These figures from the market survey agree with the antibiotic consumption data in paper 2, if we take into account that the sales figures incorporate antimicrobial use in adults (tetracyclines and fluoroquinolones) as well as children.

The incidence of laboratory-confirmed bloodstream infection was 13.9% (255/1828) of admissions. The most frequent isolates were *Klebsiella* spp., salmonellae, *E. coli*, enterococci and *S. aureus*. Furthermore, 21.6% had malaria and 16.8% HIV infection. One third (34.9%) of the children with laboratory-confirmed bloodstream infection died. The case-fatality rate from Gram-negative bloodstream infection (43.5%) was more than double that of malaria (20.2%) and Gram-positive bloodstream infection (16.7%). Significant risk factors for death by logistic regression modeling were inappropriate treatment due to antimicrobial resistance, HIV infection, other underlying infectious diseases, malnutrition and bloodstream infection caused by Enterobacteriaceae, other Gram-negatives and candida. The study shows that bloodstream infection was less common than malaria, but caused more deaths. The finding that antimicrobial resistance, HIV-infection and malnutrition predict fatal outcome calls for renewed focus on stopping the further emergence of resistance, improving HIV care and nutrition for children.

5.3 Paper 3 – The ESBL study

This paper describes a nested case-control study within the cohort of the study of bloodstream infections (paper 2) examining patients with bloodstream infections caused by ESBL-producing strain of the three most common species of *Enterobacteriaceae*. ESBL was present in high proportions of *E. coli* (25% [9 of 36]), *Klebsiella pneumoniae* isolates (17% [9 of 52]) and one isolate of salmonella (*S.* Newport) causing pediatric septicemia at MUCHS. Patients with septicemia due to ESBL-producing organisms had a significantly higher fatality rate than those with non-ESBL isolates (71% versus 39%, P =0.039). This is the first report of the CTX-M-15 genotype of ESBLs on the African continent and the first observation of SHV-12 genotype in an isolate of *Salmonella enterica* serotype Newport. The study

demonstrates that the spread of ESBL-producing bacteria has extremely serious implications in a resource-constrained hospital in Sub-Saharan Africa.

5.4 Paper 4 – The meningitis investigation

This paper describes the microbiological investigation of an outbreak of pediatric meningitis with unusually high case-fatality rate at a rural hospital in northern Tanzania. We established a provisional microbiology laboratory, obtained blood and spinal fluid specimens, which were cultured. Among 24 children with suspected meningitis and/or septicemia, five neonates had meningitis caused by *Salmonella enterica* serotype Enteritidis, all of whom died. Two children had *S.* Enteritidis septicemia without meningitis and both survived. Genotyping with pulsed-field gel electrophoresis suggested a clonal outbreak. The salmonella strain was resistant to ampicillin and sensitive to gentamicin, the two drugs commonly used to treat neonatal meningitis at the hospital. The investigation reaffirms that nontyphoid salmonellae can cause meningitis associated with very high case-fatality rates. Resistance to multiple antimicrobial agents increases the risk of treatment failure and may have contributed to the fatal outcome in all of the five patients with salmonella meningitis. The investigation indicated that the outbreak was nosocomial and the outbreak subsided after hygienic measures were instituted. The study demonstrates that it is practical and valuable to establish provisional microbiological services to investigate and control disease outbreaks even in remote rural areas.

5.5 Paper 5 – The study of bacteriuria in pregnant women

This study describes the prevalence and antimicrobial susceptibility of bacteria causing bacteriuria in pregnant women in a rural area in Northern Tanzania. Urine specimens from 5153 pregnant women were inoculated on dip slides, and a total of 101 positive dip slides were identified and tested for susceptibility to antimicrobial agents by disc diffusion. The most frequent isolates were *E. coli* (n=27) and

enterococci (n=15). *E. coli* isolates showed low rates of resistance to ampicillin (17%), mecillinam (9%), cefalexin (0%), nitrofurantoin (4%), trimethoprim-sulfamethoxazole (0%), trimethoprim (13%) and sulfamethoxazole (0%), while other Gram-negative bacteria displayed higher rates of antimicrobial resistance. All enterococcal isolates were sensitive to ampicillin. Bacteriuria with *E. coli* was correlated with adverse outcome of pregnancy (relative risk 4.13, 95%CI: 1.50-11.38). This study shows that urinary isolates of *E. coli* and enterococci from rural areas of northern Tanzania are more frequently susceptible to antimicrobials than isolates from urban areas such as Dar es Salaam. The findings suggest that susceptibility data from both rural and urban areas should be taken into account when planning antibiotic policies.

6. Discussion

6.1 Surveillance of antimicrobial resistance

Surveillance of antimicrobial susceptibility of clinical bacterial isolates is important to guide empiric therapy of bacterial infections. The surveillance study showed that it is feasible and inexpensive to implement a computerized surveillance system at the level of a tertiary hospital in Sub-Saharan Africa. Appropriate software such as WHONET is available free-of-charge [122]. In a laboratory, which already performs susceptibility testing, only a minimal extra investment for a computer and running costs for a technician to operate the software can result in the accumulation of highly useful information on antimicrobial susceptibility.

While determination of MIC values would be more accurate, the higher cost and associated workload makes it an unfeasible option for routine surveillance activities. Thus, disk diffusion testing is generally used for surveillance, and is probably the only method that is feasible for routine use, at lest in a developing country setting. Unfortunately, disk diffusion testing is far from standardized internationally, and worldwide there are at least twelve different in vitro methods in use, and only in Europe the number is at least ten [124]. To further complicate the issue, there are ongoing changes in the interpretive criteria for susceptibility testing [125]. Yet, routine susceptibility testing data are regarded suitable for surveillance even if obtained with different methods [126].

One of the most important aspects of the surveillance system is to alert the professionals of particular emerging resistance-problems, and to kick off targeted research on these topics. Indeed, the WHONET surveillance study identified resistance in *Klebsiella* spp. and other Gram-negatives as a particular problem, and communication with the pediatricians strengthened the suspicion that these resistant Gram-negative organisms were of great clinical importance. On this background, we decided to do a prospective study on bloodstream-infections in children as described

in Papers 2 & 3. While routine surveillance is performed with the relatively inexpensive disk diffusion method, prospective studies targeting crucial problems such as bloodstream infections justifies the use of more accurate methodology, including MIC determination and molecular methods to describe the problem as detailed as necessary.

Other positive developments associated with the surveillance activities were: 1) opportunities to create awareness about antimicrobials resistance issues, 2) establishment of a chapter of APUA (Alliance for the prudent use of antibiotics, www.apua.org) in Tanzania, 3) identification of opportunities for further improvements in the surveillance testing methodology. The laboratory was using Stokes' method for disk diffusion testing [127], which relied on visual interpretation of the difference in inhibition zones between the clinical isolate and the control strain. This method is robust in the case of using non-standard, in-house made antibiotic discs of uncertain strength. However, provided quality reagents are available, a method such as the one recommended by the CLSI (NCCLS) and others [115] would have advantages and allowing for more sophisticated analysis of data, such as the detection of gradual shifts in antibiotic susceptibility and opportunities for early warning of emerging resistance problems.

6.2 Resistance patterns

The routine surveillance (paper 1) indicated that Gram-negative bacilli frequently were resistant to commonly used antibiotics, as reported in the region [99, 100, 128, 129] and elsewhere [130], and that a smaller proportion of *E. coli* (5%), *Klebsiella* spp. (6%) and *Enterobacter* spp. (10%), but no salmonella (0%) were resistant to third-generation cephalosporins. In the study of bloodstream infections (paper 2 & 3), more in debt investigation with Etest, PCR and DNA sequencing revealed a high proportion of ESBL-producers among common Gram-negative isolates, 18% of the *Enterobacteriaceae* isolates (*E. coli* 9/37, *Klebsiella* spp. 9/53, *Enterobacter* spp. 5/9, salmonella 1/39 and *Pantoea* spp 2/2) involving TEM-63, SHV-2a, SHV-12 and

CTX-M-15 genotypes, and in 3 isolates of non-*Enterobacteriaceae* (one *Acinetobacter* spp. and the 2 *Chryseobacterium* spp.).

Our study was the first report of TEM, SHV and CTX-M or other types of ESBL-producing bacteria in Tanzania, and one of few reports on ESBL-type resistance from Sub-Saharan Africa [118, 131-138], although others have followed [139-145].

The proportions of ESBL-producing *Enterobacteriaceae* in our study was higher than those reported from South Africa [133] and comparable to ESBL-affected institutions in US, Taiwan, mainland China and Japan [38]. CTX-M-15 had been found in India, Japan, Europe and elsewhere [146], however, our study was the first report of CTX-M-15 genotype on the African continent, although CTX-M-12 had previously been reported in *K. pneumoniae* isolates from Kenya [118]. Our study was also the first report of SHV-12 type ESBL in an isolate of *Salmonella* Newport. Recently, SHV-12-like ESBL was reported in isolates of S. Enteritidis and S. Babelsberg obtained in France from several children adopted from one particular orphanage in Mali [136]. However, apart from this, our report was the first account of SHV-12 genotype ESBL from Sub-Saharan Africa. Gentamicin-resistance is common in ESBL-producing Gram-negative bacteria, sometimes in as much as 96% of isolates [147]. In our study, ESBL-producers showed a high degree of resistance to gentamicin, chloramphenicol, doxycycline and trimethoprim-sulfamethoxazole.

The surveillance study indicated a very low prevalence of MRSA, consistent with previous data from the same hospital [148, 149], and this was confirmed with PCR for the *mecA* gene in the bloodstream infection study.

The surveillance study revealed relatively low prevalence of enterococci compared to studies from high-income countries [150], and suggested a low rate of ampicillin-resistant enterococci (ARE). However, the bloodstream infection study revealed high rates of combined ampicillin-resistant and high-level gentamicin-resistant (HLGRE) *E. faecium* and HLGRE *E. faecalis*.

While other countries in the region have been affected by penicillin-resistant pneumococci [151, 152], the surveillance study (paper 1) indicates that pneumococcal disease in Dar es Salaam can safely be treated with penicillin.

6.3 Trends of antimicrobial susceptibility

While resistance to ampicillin, tetracycline and sulfonamides in Gram-negative bacteria was frequent already in the seventies [96, 97], it is worrying, but not unexpected, that resistance to trimethoprim-sulfamethoxazole, chloramphenicol, and other drugs appear to have increased compared to previous studies [97, 98]. The extensive use of chloramphenicol for the treatment of presumed cases of typhoid fever and the use of trimethoprim-sulfamethoxazole for the ambulatory treatment of chest infections, malaria and, not least, for prophylaxis in people with HIV, may have contributed to the high prevalence of resistance to these two drugs.

The increasing rate of gentamicin-resistance in *Enterobacteriaceae* is worrying, considering the importance of this drug in the treatment of bloodstream infections. Gentamicin-resistance in *E. coli* has increased from zero in 1978-79 [97] to 2% in 1995 [98] , 8% in the surveillance study (paper 1), and 29% and 46% in community-acquired and hospital-acquired bloodstream infections, respectively (paper 2). Similar increases in gentamicin-resistance in *E. coli* has been noted in neighboring Kenya [99, 153]. In *Klebsiella* spp., which are inherently resistant to ampicillin, gentamicin-resistance is even more alarming and has reached almost 50% in both community- and hospital-acquired infections, which means that half of the cases of bloodstream-infection caused by *Klebsiella* spp. at the hospital will not have any effect of the commonly given combination of ampicillin and gentamicin.

While tetracyclines are not recommended for children, it is interesting to observe the decline in tetracycline resistance in *S. aureus* from in 57% in 1979 and 74% in 1982 [148] to 49% in 1998-99 (paper 1) and 38% in community-acquired infections in 2001-02 (paper 2), although hospital-acquired *S. aureus* showed 65% resistance. In the late seventies, huge quantities of tetracycline was used to prevent and treat

Cholera in Tanzania; as much as 1788 kilograms of tetracycline were used during a period of only 5 months [154]. As *Vibrio cholerae* developed tetracycline-resistance, the drug was much less used, which may have influenced resistance rates in other species, such as S. aureus.

6.4 Community-acquired and nosocomial infections

Among patients coming to the hospital, there may be of selection of patients with infections caused by resistant microbes, since many patients rely on health centers and pharmacies to cure simple ailments, and only come to the hospital when primary treatment fails. The study identified only a few resistance traits, which were more common in hospital-acquired infections (or inpatients), such as resistance to ampicillin (paper 1) and amoxy-clavulanate and cephalosporins (paper 2) in *E. coli* and resistance to gentamicin (paper 1) and co-trimoxazole (paper 1 & 2) in *Klebsiella* spp.. Kaplan-Meier survival graphs showed that deaths in patients with septicemia due to ESBL-producing bacteria occurred later than those caused by non-ESBL-producing isolates. Time from admission to blood culture was a significant risk factor for infection with ESBL. The majority (6/7) of TEM-63-producers were isolated from nosocomial infections, and the three TEM-63-producing isolates of *Klebsiella* spp. were virtually identical on genotyping with amplified fragment length polymorphism. These findings indicate nosocomial spread. However, half (3/6) of CTX-M-15-producers and almost two-thirds (9/15) of SHV-12 were from community-acquired infections, indicating that ESBL-producers are a problem in the community as well.

ESBL genes of the TEM, SHV and CTX-M families can reside in conjugative plasmids [38, 118, 119, 155, 156], and this has recently been demonstrated for CTX-M-15 [146, 157]. Previous reports have demonstrated that ESBL genes can spread via epidemic strains, but also by plasmid dissemination between unrelated strains [158]. One study found the same ESBL gene (TEM-24) in as many as 4 different species of *Enterobacteriaceae* in one single patient, indicating that horizontal transfer of ESBL-genes occurs *in vivo* at a considerable rate [89, 90]. The presence of identical ESBL

genotypes in multiple bacterial species in the current study, seems to support the notion that interspecies plasmid dissemination may contribute to the spread of ESBL in our setting also.

Genotyping with pulsed-field gel electrophoresis suggested there was a clonal outbreak of bacteremia and meningitis caused by *S.* Enteritidis at Haydom Lutheran Hospital (paper 4). The genotyping information, the susceptibility patterns and the clinical information that all children with *S.* Enteritidis infections were born at the hospital and that the majority never left the hospital before they became ill, suggests that the outbreak was nosocomial. Nosocomial outbreaks of nontyphoid salmonella in neonatal wards is known from the literature [159]. Neonates are at particular risk of infection because of relatively reduced gastric acidity and peristalsis [12]. While medications, diagnostics, blood products, human milk, eggs and contaminated suction tubes have been sources of previous outbreaks [159, 160], the source of the outbreak at Haydom Lutheran Hospital was not established. However, despite unaltered antimicrobial treatment for meningitis at the hospital, the swift interventions with reinforcement of hygiene were followed by a drop in case-fatality rates from pediatric meningitis from >60% before the intervention to 40% by 2001 hospital annual reports.

Further work on the *Enterobacteriaceae* isolates which produced SHV-12 [161], documented that resistance towards gentamicin (aac(3)-II gene), doxycycline, chloramphenicol and co-trimoxazole was transferred by the plasmid harboring ESBL-gene blaSHV-12. This finding implies several important notions. First, resistance traits mediated by the same plasmid makes both the empiric first-line treatment regimen (ampicillin/penicillin + gentamicin) and the reserve regimen (ceftriaxone) for treatment of septicemia ineffective. Second, treatment with gentamicin, which generally is accepted as ecologically sound, may indeed contribute to the selection of ESBL-producing strains since the genes encoding for these different resistance traits are located on the same plasmid. Perhaps even more worrying, considering the high rates of HIV infection, is that the cheap co-trimoxazole, which is widely used as prophylaxis against opportunistic infections in HIV-infected persons, may contribute

to the selection of ESBL-producing strains. Likewise, chloramphenicol, the long-standing drug of choice for the treatment of typhoid fever, is also frequently used and may contribute to selecting these ESBL-producers.

6.5 Antimicrobial resistance in urban and rural areas

It is reassuring, that *E. coli* isolates from the bacteriuria study in Northern Tanzania were highly susceptible to all tested drugs. Consequently, an important observation from this study is that antimicrobial resistance can vary considerably between rural and urban areas within a country. This should be taken into account when formulating antibiotic policies. In Tanzania, the great majority of the population lives in rural areas. Policies developed for urban areas may endorse the use of antibiotics, which are unaffordable for poor rural dwellers, including broad-spectrum antibiotics, which have the additional disadvantage of promoting further resistance. In countries with large rural populations, such as in Tanzania, resistance data from rural areas must play a significant role when deciding on antibiotic policies.

6.6 Incidence of septicemia

We found an incidence of septicemia of 13.9% (255/1828) of all admissions in the study. The incidence was higher in the youngest patients. For early-onset septicemia (within the first week of life) the incidence was 17.1%, for late-onset neonatal septicemia (week 2-4) 14.2% and for older children (>1month) 13.1%.

Table 6 (not presented in the articles) shows an overview of published bacteremia studies from Sub-Saharan Africa. It is evident that there is great variation in reported incidences of bacteremia, ranging from 5.8% to 46.0%. The incidence reported from our study lies slightly lower than the median these studies.

Table 6. Incidence of bacteremia per hospital admission among African children.

Location, period	Incidence	Population	Ref.
South Africa	5.8% (315/5397)	All admitted children	[162]
Kilifi, Kenya	6.6% (1094/16570)	All admitted children	[8]
Addis Ababa, Ethiopia	7.7% (49/634)	Febrile children, 0-14years	[163]
Shongwe, South Africa	9.6% (31/323)	Malnourished children	[164]
Ilesa, Nigeria	9.9% (15/152)	Severely anemic children	[165]
Benin City, Nigeria	11.1% (71/642)	Children (1m-5y) with acute fever	[166]
Nairobi, Kenya	12.1% (32/264)	Febrile hospitalized children	[167]
Kigali, Rwanda	12.4% (112/900)	Children with fever (≥39')	[129]
Dar es Salaam, Tanzania	13.9% (255/1828)	Children with suspected BSI†	*
Jos, Nigeria	15.6 (139/891)	Children with suspected BSI†	[168]
Lwiro, D. R. Congo,	15.9% (124/779)	All children admitted	[169]
Kampala, Uganda	17.1% (76/445)	Malnourished children (<60 days)	[170]
Blantyre, Malawi	17.2% (365/2123)	Children with fever	[171]
Kumasi, Ghana	20.3% (51/251)	Children suspect of having malaria	[9]
Kigali, Rwanda	26.7% (36/135)	Children having blood cultured	[172]
Nairobi, Kenya	28.6% (26/91)	Malnourished children, 2-60 months	[173]
Harare, Zimbabwe	30.7 (95/309)	Age <8y, temp>38', suspect infection	[174]
Nairobi, Kenya	31.7% (19/60)	Children, clinical septicemia	[175]
Lagos, Nigeria	31.7% (19/60)	Sicklers (hz), 3m-13y, with acute illness	[176]
Ibadan, Nigeria	38.2% (39/102)	Febrile infants <1year	[177]
Bulawayo, Zimbabwe	43.4% (92/212)	Children (0-5y), dead <3h before adm.	[11]
Ile-Ife, Nigeria	44.6% (54/121)	Sick, young infants	[178]
Calabar, Nigeria	46.0% (552/1201)	Children (0-15y), suspected BSI†	[179]

* Paper 2, †BSI = bloodstream infection

The incidence of septicemia among children admitted to hospitals is dependent on several factors including the health-care seeking behavior in the population, the type of study performed, the criteria for inclusion into the studies, antimicrobial use before blood-culture and the blood-sampling technique, transport time and culturing techniques. For instance, in the recent study from a rural hospital in Kilifi in neighboring Kenya, the incidence of community-acquired bacteremia was lower than in our study, 12.8% in children younger than 2months and 5.9% in older children [8]. However, the studies were designed differently with different inclusion criteria, which may explain this difference. In our study only patients admitted with features suggestive of infection were investigated while the Kenyan study included all children admitted to the hospital, except for children admitted for elective procedures.

In resource-constrained settings, such as Sub-Saharan Africa, there are generally higher reported incidences of bacteremia in children than in the economically developed world. This may be due to a number of factors, including higher infection rates of organisms such as salmonella due to suboptimal hygiene. Furthermore, there may be a higher prevalence of immunosuppression due to malnutrition and the HIV epidemic. However, the high incidences of septicemia may partly reflect that admission is delayed because caretakers do not have sufficient funds to pay for transport, admission fees etcetera.

Amore accurate way of describing the incidence of bacteremia/septicemia is by estimating minimal annual incidences (MAI) expressed as the number of occurrences of the condition in the total population per year. The calculation of MAI requires population-based studies in the sense that there is knowledge about the size of the population, which would come to the study site if falling sick. Thus, such studies may be easier to perform in rural areas or small towns where there are a limited number of health facilities that handle cases. In the rural area in Kilifi, Kenya, the minimal annual incidence of community-acquired bacteremia was estimated at 1.5% of infants under one year of age, 1.1% among children under two years, and 0.5% of children under five years [8]. In The Gambias, an incidence of community-acquired bacteremia of 1.1% and 1.0% was found in children aged 2-29 months who had

received or not received pneumococcal conjugate vaccine [180]. The study site for our study was a university teaching hospital in a major city with several million inhabitants and, apart from the study hospital, there are several four public district hospitals, several private hospitals and a great number of health centers and pharmacies, all of which may treat children with systemic bacterial infections. Thus, since we do not have sufficient information on size of the population that actually uses MUCHS as a primary hospital we are not able to calculate any accurate estimate of minimal annual incidence of pediatric bacteremia from our study.

6.7 Prevalence of organisms causing septicemia

Bloodstream infections caused by *Klebsiella* spp. are much more common in developing countries, and particularly in hospital-acquired neonatal infections [91, 181]. *Klebsiella* spp. were the most common cause of neonatal septicemia in our study (paper 2) as well, particularly in early-onset septicemia, and *Klebsiella* spp. and *S. aureus* were the most frequent agents causing hospital-acquired infections. It has been estimated that 70% of infections caused by *Klebsiella* spp. in developing countries will not be covered by the widely used empirical treatment with ampicillin and gentamicin due to inherent ampicillin resistance and emerging acquired resistance to gentamicin [91]. Again, our study (paper 2) supports these findings, as half of the isolates of *Klebsiella* spp. were resistant to gentamicin.

Salmonella is one of the major causes of bacteremia in African children and has been linked to various risk factors, including malnutrition, recent malaria, HIV co-infection[169, 175, 182]. In line with other studies, our study showed that salmonella was the most common pathogen causing septicemia in children older 1month, and, along with *E. coli,* the most common cause of community-acquired septicemia.

Pneumococci are a major cause of invasive disease and child death [183, 184], but were not detected in our study of bloodstream infections (paper 2). Possible reasons for this may be antimicrobial therapy prior to blood culture and a selection bias, as people with pneumococcal disease may already have been treated and/or cured with

penicillin in other health facilities. Human blood is used in agar-production in the lab, and it has been speculated that this may be suboptimal, since there may be remnants of antimicrobials in the blood that may inhibit bacterial growth.

6.8 Septicemia versus malaria

The incidence of septicemia in the study (paper 2) was high (13.9%). While malaria was more frequent (21.6%), septicemia was involved in more deaths. In a study from Rwanda in the 1980s [129] the case-fatality rate of malaria was similar to that of bloodstream infection. However, in our study, bloodstream infection was associated with a much higher case-fatality rate than malaria. This is in line with the study from Kenya [8], where deaths from bloodstream infection also outnumbered malaria deaths. A reason for the trend of higher case-fatality rate in bloodstream infections than in malaria may be that antimicrobial resistance is seriously undermining the treatment strategies for bacterial bloodstream infections, while malaria still can be effectively treated with quinine. In view of this, it is an unfortunate and pressing dilemma that bloodstream infection and malaria are difficult to distinguish based on clinical presentation [9, 10, 185-187].

6.9 Septicemia and HIV infection

The HIV-prevalence (16.8%) in the study-population (paper 2) was higher than national average (7%), which may be explained by the selection of study population and refusal by some relatives to test their child. Contrary to others [40-42], we did not find any significant association between HIV co-infection and resistance to drugs such as co-trimoxazole, which is used prophylaxis against *Pneumocystis jirovecii* pneumonia. However, HIV-positive children did receive inappropriate antibacterial therapy more frequently than HIV-negative.

6.10 Clinical outcome

It has been shown that prompt antimicrobial treatment is important for the survival of patients with bacterial bloodstream infection [188]. An adverse impact of antimicrobial resistance on survival has been shown for certain problematic organisms such as MRSA [64, 65] and VRE [58, 59]. However, for other resistant microbes, such as ESBL-producers, there is a relative lack of scientific proof of an impact on mortality [49, 70]. Our bloodstream infection study (paper 2 & 3) confirms that inappropriate treatment of bloodstream infections due to antimicrobial resistance increases the risk of fatal outcome, and this association seems to be independent of underlying diseases as shown by logistic regression. The study also shows that both HIV-infection and malnutrition adversely affected the outcome of the patients as well.

Although the numbers are small, the case-fatality rate of meningitis caused by *S.* Enteritidis in Northern Tanzania was 100% (5/5). The strain responsible for the outbreak was resistant to two of the first-line drugs, ampicillin and chloramphenicol, but sensitive to gentamicin, which is in line with reports of multi-drug-resistant *S.* Enteritidis in the region [189]. We speculate that the high case-fatality rate in these patients was partly due to antimicrobial resistance. The children were given ampicillin + gentamicin. Since the strain was resistant to ampicillin, the clinical outcome demonstrates that effective monotherapy with gentamicin probably is suboptimal as treatment for *S.* Enteritidis meningitis.

In the bacteriuria study in Northern Tanzania (paper 5), growth of *E. coli* from a urinary specimen was associated with a significantly increased relative risk for negative outcome of the pregnancy. This finding is in line with previous studies by Kass and others [17-19], but unexpected in the sense that all women with positive dip slides received nitrofurantoin treatment and, thus, should have been cured from their bacteriuria. A plausible explanation for this may be that compliance with treatment is low, and particularly so in asymptomatic persons.

6.11 Strengths and limitations

Selection bias is a serious and easily overlooked potential source of error in studies assessing antimicrobial. When studies are performed at major hospitals in urban centers such as MUCHS, Dar es Salaam, there is a possibility that many patients receive antimicrobial therapy at primary or secondary health services prior to presenting at the tertiary hospital. Persons with infections with susceptible bacteria may well be cured at the primary health facility and never come to the major hospital, while people with infections with resistant bacteria will not be cured at the periphery, and may eventually end up coming to the major hospital. We have quantified the problem with antimicrobial treatment prior to blood-culture (paper 2), and speculate on its implication, e.g. for reduced detection of fastidious organisms. We have tried to address problem with the selection bias by also performing susceptibility studies in an unselected population in a rural part of Tanzania, and this exercise confirms that there are differences in antimicrobial susceptibility patterns between urban and rural areas.

7. Conclusions

Data from this thesis shows that:

- Computerized surveillance of antimicrobial resistance can be implemented at a tertiary hospital in Tanzania at low cost.

- The resistance surveillance system can 1) provide useful information on antimicrobial resistance patterns, 2) function as a quality assurance tool, 3) increase awareness of the resistance issues, and 4) pinpoint particular resistance-problems which needs to be targeted by dedicated research.

- Bacterial bloodstream infections are a frequent cause of morbidity in hospitalized children in Dar es Salaam and associated with higher case-fatality rates than malaria.

- There are high rates of antimicrobial resistance, particularly in Gram-negative bacteria causing bloodstream infections.

- Bacteria that produce ESBL (including SHV-12, TEM-63 and CTX-M-15) have been described for the first time in Tanzania.

- ESBL-type and other resistance mechanisms towards given antimicrobial therapy are significant risk factors for death from bloodstream infections.

- Meningitis caused by ampicillin-resistant *S.* Enteritidis was uniformly fatal in neonates receiving combination therapy with ampicillin and gentamicin, but the problem diminished upon reinforcement of hygiene.

- Growth of *E. coli* in urine culture from pregnant women was correlated with adverse outcome of pregnancy.

- The rates of resistance towards antimicrobials vary within a country, with lower rates in remote, rural areas than in populated urban centers.

8. Recommendations

Hospitals with bacteriology laboratories, such as MUCHS, should implement computerized systems for surveillance of antimicrobial resistance.

As far as possible, standardized methods should be used for susceptibility testing. Currently there is lack of standardization at international level, and efforts to harmonize resistance surveillance efforts across borders should continue [190, 191].

All measures must be taken to limit the further spread of antimicrobial resistance traits, particularly the ESBL-type resistance. This may include:

- Restrictions on antimicrobial use and prescriptions.
- Reemphasizing rational antimicrobial use, including the use of narrow-spectrum rather than broad-spectrum antimicrobials, when appropriate.
- Reinforcement of hygiene, particularly in hospitals.

Currently, multidrug-resistant, ESBL-producing Gram-negative bacteria, which cause bloodstream infections, are susceptible to few or none of the available antimicrobials in Tanzania. Despite previous concern regarding adverse effects of fluoroquinolones in children, drugs such as ciprofloxacin can be resorted to for treatment of life-threatening infections [192, 193].

The described nosocomial outbreak of nontyphoid salmonella-meningitis underlines the importance of stringent hygiene, particularly in neonatal wards.

The bacteriuria study supports the notion that asymptomatic bacteriuria and urinary tract infection in pregnant women should be treated.

Differences in antimicrobial resistance between rural and urban areas should be taken into account when formulating guidelines for use of antimicrobial agents.

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