Antimicrobial Resistance Spread and the Role of Mobile Genetic Elements

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Antimicrobial Resistance Spread and the Role of Mobile Genetic Elements

Antimicrobiële resistentie verspreiding en de rol van mobiele genetische elementen

THESIS

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“Somewhere, something incredible is waiting to be known.”

Dr. Carl Sagan (1934-1996)

To family, friends and relatives

Contents

Chapter 1

General Introduction

A brief history of antimicrobial resistance

Alexander Fleming discovered the first antimicrobial agent, penicillin (a β-lactam), in 1928 in the mold *Penicillium notatum*. Penicillin was initially found to be active against staphylococcal strains, which at that time were a major source of infectious diseases. Indeed, the mortality rate of individuals with severe *Staphylococcus aureus* infection in the early 1940's was about 80% [1]. The discovery of penicillin gave clinicians an effective means to combat fatal microbial infections for the first time. Since that time, many different types of antimicrobial agents have been discovered, including aminoglycosides, macrolides and cephalosporins to name but a few. Further, current advances in biochemistry and engineering now mean that novel antimicrobial agents can be produced by 1) synthetically altering the structure of known antimicrobial agents, or by 2) high throughput screening strategies. However, even as new antibiotics are being discovered, bacteria are acquiring mechanisms to neutralise their effect.

Even at the time that penicillin was being commercially made available in 1945, reports of enzymes possessing β-lactamase activity had already been published by Abraham and Chain [2], and we now know that antimicrobial resistance mechanisms have been around for many millions of years, even before the large-scale use of antibiotic therapy in human populations began. For example, ampicillin (a derivative of penicillin) resistant bacteria estimated to be approximately 2000 years old have been recovered from glacial samples obtained from the Canadian Arctic Archipelago, while a more recent study detected TEM-type β-lactamases from a metagenomic library of cold seep deep-sea segments estimated to be approximately 10,000 years old [3, 4]. In fact, it is now known that many environmental organisms produce antimicrobial compounds in order to limit competition for nutrients or chemically alternative niches in defined niches. However, in response, bacteria have evolved (and are continuing to evolve) mechanisms to counteract these lethal antimicrobial compounds, in order to protect themselves from attack. It is these mechanisms that eventually create problems with respect to antimicrobial resistance and human health.

During the period after Alexander Fleming's discovery of penicillin, academia and the pharmaceutical industry discovered and developed many new antimicrobial agents. However, after each new class of antimicrobial agent was brought to market, new resistance mechanisms appeared that reduced the efficacy of the new antibiotics, seriously limiting patient treatment options. For example, one new class of antimicrobial agents discovered were broad-spectrum β-lactam antibiotics, the cephalosporins. These antibiotics have an extended-spectrum of action against both Gram-positive and Gram-negative bacteria and were originally extensively used for the treatment of serious infections caused by Gram-negative bacteria in the 1980s [5]. However, resistance to cephalosporins rapidly developed, creating new problems for the treatment of infectious diseases. This race between the introduction of new antimicrobial agents and the appearance of associated resistance mechanisms in microorganisms is still a major problem globally, limiting the successful treatment of infectious diseases by health care professionals. It is essential therefore to monitor the spread of existing, new, and emerging antimicrobial mechanisms in the world. The goal of this thesis is to provide further data that will help monitor existing antimicrobial resistances in order to add information to global antimicrobial resistance monitoring campaigns, whilst also helping to predict future trends.

Overview of antimicrobial resistance in microorganisms of major clinical importance

Both Gram-negative and Gram-positive microorganisms are associated with antimicrobial resistance in the clinical (and nosocomial) environments.

A. Gram-negative microorganisms:

Currently, the most important clinically relevant antimicrobial resistances among Gram-negative bacteria are associated with the enterobacteriaceae, namely:

1. Extended-spectrum β-lactam resistance in *Klebseilla pneumoniae*, *Escherichia coli* and *Salmonella* species.
2. Fluoroquinolone resistance in *Salmonella* species.

1. β-lactam resistance in *E. coli* was first identified in the early 1920's. Subsequent studies showed that β-lactam resistance among enterobacteriaceae was caused by the so-called TEM-1 and SHV-1 β-lactamases, which possess an amino-acid similarity of 68%. As the discovery of new antibiotics quickened, a new class of antibiotics, the broad-spectrum β-lactam antibiotics (cephalosporins), were introduced due to their improved spectrum of activity against both Gram-negative and Gram-positive bacteria. Coincidently, β-lactam resistant strains of enterobacteriaceae were also found to be susceptible to these broad-spectrum antibiotics. However, soon after the introduction of the cephalosporins, resistant enterobacterial isolates began to emerge. The mechanism facilitating this particular resistance was found to be a mutation in either TEM or SHV β-lactamase genes, which lead to changes in the relevant β-lactamase amino-acid sequence, giving rise to different phenotypic variants that possessed "extended" β-lactamase activities. The fact that the new TEM and SHV type β-lactamases possessed "extended" activity against β-lactam antibiotics, meant that they were named "extended-spectrum β-lactamases" or ESBLs [6]. The first ESBL (SHV-type) reported to cause the inactivation of extended spectrum β-lactam antibiotics was reported in Germany in 1983 [7]. However, since 1995, the rate of dissemination and reporting of ESBLs among different bacterial isolates has increased rapidly in most parts of the world [8-10]. In fact, the increased frequency of microbial isolates carrying these mutation events most probably occurred via an increase in "selection pressure" after the introduction and large-scale use of cephalosporins, which removed cephalosporin sensitive bacterial isolates from the clinical environment, thereby allowing cephalosporin resistant isolates to take advantage of the newly available niches [11-14].

Untill the 1990's, ESBLs found in clinical isolates of bacteria were mostly identified as TEM and SHV-type enzymes. However, a different class of ESBL enzymes (called CTX-M) began to emerge after this date. CTX-M enzymes are a new class of β-lactamase that have become increasingly prevalent in the clinical and nosocomial environments, affecting all populated continents. Indeed, since first being reported in Germany in 1990 [15], several different classes of CTX-M enzyme groups have emerged, with CTX-M carrying enterobacterial isolates having quickly become the most widespread and clinically relevant group of ESBL enzymes. To illustrate this point, to date, more than 50 different CTX-M enzymes have been reported, which are grouped into five main subgroups (CTX-M-1, CTX-M-2, CTX-M-8, CTX-M-9 and CTX-M-25) based on their amino acid sequence similarities [10].

2. Fluoroquinolone resistance within the enterobacteriaceae (and in particular within the *Salmonella* species) is also of great economic and nosocomial importance. Fluoroquinolone antibiotics have been available since the 1960s, when nalidixic acid was introduced. Subsequent structural modifications to the quinolone molecule led to the development of newer compounds such as ciprofloxacin and norfloxacin, which provided better results in the treatment of severe (enterobacterial) infections. However, microorganisms including salmonellae rapidly developed resistance to these fluoroquinolone compounds, mainly due to binding-site mutations in the DNA gyrase (the enzyme affected by fluoroquinolones that is required for correct DNA synthesis) and due to active efflux system. Fluoroquinolone resistaance in enterobacteriaceae is currently one of the main concerns associated with the developing world.

The major aspect causing concern with respect to treatment failures in Gram-negative bacterial infections currently, is the in-vivo acquisition of both extended-spectrum β-lactamase (ESBL) AND fluoroquinolone resistance genes. This characteristic has now been well-established in isolates from several countries [16-18], especially in countries where "over-the-counter" antibiotics are available without a general practitioner's prescription. In India, this problem tends to be particularly associated with (non-typhoidal) salmonellae isolates [19].

B. Gram-positive microorganisms:

The most important clinically relevant antimicrobial resistances in Gram-positive bacteria are:

1. Methicillin resistance in *Staphylococcus aureus.*
2. Vancomycin resistance in *Staphylococcus aureus* and *Enterococcus faecium.*
3. High-level gentamicin resistance (HLGR) in *Enterococcus faecalis.*

1. Among Gram-positive bacteria probably the most important and worrying aspect of antimicrobial resistance relates to the development of resistance in *Staphylococcus aureus*. *S. aureus* lives as the part of the normal skin flora in the nose or on the skin, and is considered to be a major pathogen capable of causing relatively mild infections such as furuncles, post-operative wound infections up to life-threatening invasive infections such as sepsis and endocarditis [20]. As previously mentioned, infections with *S. aureus* were a major cause of morbidity and mortality in the 1930s and 1940s, though the introduction of penicillin initially greatly decreased the mortality rate. However, by the 1950s penicillinase-producing hospital strains of *S. aureus* (that were coincidently also resistant to other commonly available antimicrobials of the time) were becoming a major problem, responsible for significant numbers of infections [21]. Further, though the introduction of new antimicrobial agents such as methicillin, and the synthetic penicillins, cloxacillin and flucloxacillin, initially helped combat the problems conferred by penicillinase positive *S. aureus* [22], resistance to these new antibiotics was quickly reported, with methicillin resistance being reported in *S. aureus* (MRSA) isolates from England as early as 1960 [23].

2. In subsequent decades, nosocomial infections caused by methicillin resistant *S. aureus* (MRSA) continued to increase and an intense search was made for new antibiotics that were effective against MRSA isolates. In this respect, vancomycin, a glycopeptide antimicrobial agent was introduced to combat the treatment problems associated with MRSA. Again, though initially successful, intermediate vancomycin resistant *S. aureus* isolates were soon

reported worldwide [23], with the fear that transfer of high-level vancomycin resistance, via the *vanA* gene complex of glycopeptide-resistant enterococci into *S. aureus*, would occur. This fear became a reality when small numbers of clinical isolates of vancomycin resistant *S. aureus* (VRSA), carrying the *vanA* gene complex, were reported from the USA between 2002 and 2007. Other high-level VRSA isolates were subsequently reported in India [24-26], with the *vanA* gene complex found to be carried on transposon Tn*1546* [24], a transposon reported in *vanA* positive *E. faecium* [27]. In fact, the treatment of nosocomial VRSA infections remains problematic, with the wide scale use of antibiotics effective against VRSA most probably leading to the acquisition of new resistance phenotypes.

Vancomycin resistance is also a problem often associated with nosocomial infections by enterococci. Enterococci are prevalent in environmental, human and nosocomial settings, being found as commensals of the gastrointestinal tract of both humans and animals [28-31], and generally exhibiting only low virulence. Nevertheless, colonization followed by infection can readily occur in immuno-compromised patients and those with serious injuries within the nosocomial environment, particularly if the patient has previously been treated with antibiotics (for example in an attempt to treat an infection caused by an unrelated bacterial species). Among the genus *Enterococcus*, *Enterococcus faecalis* and *Enterococcus faecium* are the species most commonly found to be associated with human nosocomial infection [32], with *E. faecalis* accounting for approximately 75% of all enterococcal infections, and *E. faecium* accounting for the majority of the remainder [33]. These are particularly associated with infections such as urinary tract infections, bacteremia, intra-abdominal infections, skin and soft tissue infections and endocarditis [34-37]. The most important aspect of clinically relevant antimicrobial resistance among enterococci was the appearance of vancomycin-resistant *Enterococcus faecium* in 1986, which lead to clonal expansion of this highly resistant and potentially difficult-to-treat pathogen across the world [38]. Further, the spread of nosocomial vancomycin resistant enterococci has been promoted via the extensive use of vancomycin within hospitals [39, 40], as well as the use of the animal growth promoter avoparcin in Europe. With respect to phenotype, enterococci may actually exhibit intrinsic (VanC) or acquired resistance to vancomycin, with 6 acquired vancomycin resistance genotypes reported namely *vanA, vanB, vanD*, *vanE*, *vanG* and *vanL* [41, 42]]. Of these subtypes, the *vanA* genotype (VanA phenotype) is the most common type of acquired glycopeptide resistance found in vancomycin resistant enterococci within hospitals. Further, it is generally accepted that a single focal group of *vanA*-type VRE (clonal complex 17 or CC17) is particularly associated with nosocomial infections [43].

3. High-level gentamicin resistant *Enterococcus faecalis* (HLGRE) were first reported in 1979 in France [44]. Subsequently, HLGRE were described from clinical samples of hospitalized patients in several countries [45, 46]. High-level aminoglycoside resistance in enterococci is mediated by aminoglycoside-modifying enzymes (AMEs) and may be facilitated by several related AMEs that are expressed from a series of aminoglycoside resistance genes, usually carried on transposons. HLGRE is mediated by the AME *aac (6')-Ie-aph (2")-Ia*, *aph (2")-Ib*, *aph (2")-Ic*, and *aph (2")-Id* [47, 48]. *E. faecalis* is capable of acquiring and mobilizing transposon-related drug resistance determinants from, and into, other bacterial species [49, 50]. Clinically, it is particularly important to investigate the mechanism and prevalences of high-level aminoglycoside resistance in *E. faecalis*, as aminoglycosides are important antimicrobial agents when administered in combination with other cell wall-active agents such as β-lactams, this produces a synergistic bactericidal effect especially useful in nosocomial infections associated with bacteremia and endocarditis.

"Biological" mechanisms of antimicrobial resistance

Generally, microorganisms exhibit two types of antimicrobial resistance mechanisms i.e. intrinsic and acquired resistance: intrinsic resistance is a natural phenomenon that is exhibited by many microorganisms and is conferred by the analogous genetic make-up of a particular species. Acquired resistance is the result of active or passive acquisition of genes that encode antimicrobial proteins, which then protect the host bacterium from the effects of antibiotics. In some cases of acquired resistance, the genes involved in antibiotic resistance arise by mutation of a regulatory gene and in others, they are acquired from bacteria co-habitating within their environment that are already resistant to antibiotics [51].

There are 6 "biological" mechanisms associated with intrinsic and acquired antimicrobial resistance (Figure 1).

These are: a) altered target sites, b) bypass pathways, c) decreased uptake, d) active efflux system, e) enzymatic inactivation or modification, and f) overproduction of target [52].

a) Alteration in antibiotic target sites are usually brought about by point mutations at the regions of a gene necessary for antibiotic activity, resulting in lowered binding affinity between the antibiotic and its target. For example, point mutations in the gene encoding DNA gyrase can alter the binding efficiency of quinolone antibiotics, thereby reducing their efficacy. In addition, not only single mutations, but multiple point mutations may also occur, eventually leading to higher levels of resistance, as observed for the quinolone resistance-determining region (QRDR) of DNA topoisomerase genes (*gyrA*, *gyrB*, and *parC*), where multiple mutations lead to higher minimum inhibitory concentrations (MICs) and therefore decreased susceptibility to quinolone antimicrobial agents [53].

b) Bypass pathways play an important role in developing antimicrobial resistance as they allow bacterial cells to protect themselves by the production of an alternative target (usually an enzyme) that is resistant to inhibition by a particular antibiotic, while still continuing to produce the original sensitive target. One well-known example of this particular mechanism is vancomycin resistance in enterococci, which are normally susceptible to vancomycin. The normal target of vancomycin is a cell wall precursor that contains a pentapeptide that has a D-alanine-D-alanine terminus to which the vancomycin binds, thereby preventing cell wall synthesis. When an enterococcus acquires the *vanA* gene cluster, it generates an alternative cell wall precursor ending in D-alanine-D-lactate, to which vancomycin can not bind [54].

c) A decreased uptake of antimicrobial agents across the bacterial cell membrane is usually caused by lowered cell permeability; in a bacterial cell, outer membrane proteins (OMPs) provide channels of entry for molecules to the cell membrane (including antimicrobial agents) based on charge, shape, and size of the entering molecule. Loss of function of one of these porins due to a mutation event can cause loss of function of that particular porin, possibly leading to antibiotic resistance [55-57]. For example, loss of the D2 porin due to mutation causes imipenem resistance in *Pseudomonas aeruginosa* [58].

d) Enzymatic inactivation or modification of antimicrobial agents is a common mechanism of resistance that reduces or eliminates antimicrobial activity. One of the most common mechanisms involved in enzymatic modification of antimicrobial agents is acetylation. One classical example of such modification involves aminoglycoside acetyltransferase, that

acetylates and inactivates aminoglycosides such as gentamicin [59]. In addition, this mechanism is also involved in the chloramphenicol resistance [60].

e) The overproduction of an antibiotic binding target protein. One example of such type of antimicrobial resistance is β-lactam resistance in enterococci. Enterococci can overproduce low-affinity penicillin-binding proteins, leading to high-level resistance to β-lactam antibiotics.

f) The active efflux of antimicrobial agents is associated with enzymes that possess the ability to bind to antimicrobial agents and pump them out of the bacterial cell before they can reach their site of action. Examples of active efflux mediated antimicrobial resistance include, resistance to tetracycline and fluoroquinolones in enterobacteriaceae and high intrinsic resistance to penem antibiotics in *Pseudomonas aeruginosa* due to the composition of the cellular membrane and an active efflux system [61, 62].

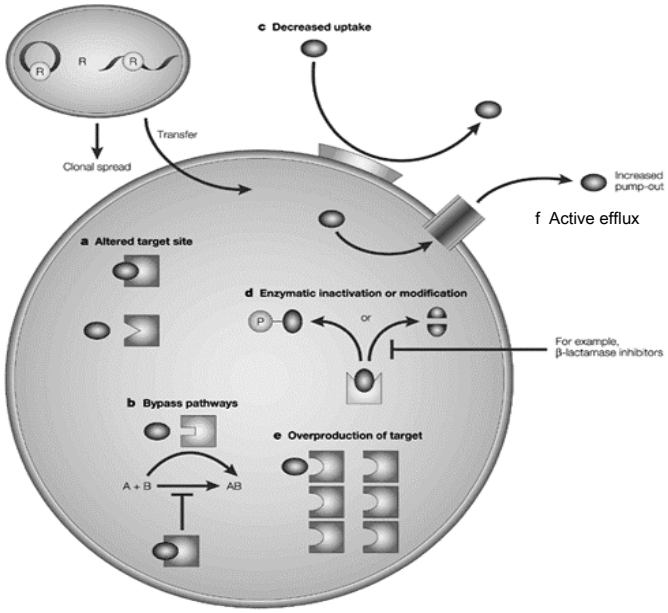

Figure 1. Different mechanisms involved in antimicrobial resistance. (Adapted with permission from: Coates A, Hu Y, Bax R *et al.* The future challenges facing the development of new antimicrobial drugs. Nat Rev Drug Discov 2002; 1:895-910).

Types of mobile genetic elements involved in antimicrobial resistance

Several different "molecular platforms" facilitate the spread of antimicrobial resistance genes among microorganisms. The different platforms associated with the transmission of antimicrobial resistances in both Gram-negative and Gram-positive bacteria are described briefly below:

Plasmids:

Plasmids are extra-chromosomal, circular, double-stranded units of DNA, that are transferable and capable of autonomous replication within a suitable host [63, 64]. They have proven to be the ideal vehicle for the dissemination of genes responsible for antimicrobial resistance. Plasmids usually do not contain any of the core genes needed by a bacterial cell for basic growth and multiplication, instead they tend to carry genes that enable the cell to circumvent particular unfavorable environmental conditions, for example when in the presence of a potentially lethal antibiotics or heavy metals. Basically, there are many types of plasmids, which are widespread in all bacterial populations studied, and which may generally be divided, into five main classes:

a) Fertility or F-plasmids, are capable of conjugation i.e. transfer of genetic material between bacteria via a conjugation tube. An F-plasmid contains *tra* (for "transfer") genes and a number of other genes responsible for compatibility and replication.

b) Resistance or R-plasmids contain genes that code for resistance to antibiotics. R-plasmids tend to harbour a variety of genes encoding not only resistance to a wide spectrum of antimicrobial agents, but also resistance to heavy metals, mutagenic agents such as ethidium bromide, and disinfectant agents including formaldehyde [65, 66]. Some examples of R-plasmid mediated antimicrobial resistance include those that mediate resitance to cephalosporins, fluoroquinolones, glycopeptides and aminoglycosides. For instance, A/C and FIA plasmids harbour genes responsible for extended-spectrum β-lactam antibiotics in enterobacteriaceae [67], whilst, pMG252 is responsible for carrying *qnr* genes, which are involved in fluoroquinolone resistance in enterobacteriaceae [68]. Additionally, pLRM19 carries *vanB* resistance genes in enterococci [69], and pRE25 harbours resistance genes against 12 antibiotics including aminoglycosides [70].

c) Col-plasmids contain genes that code for bacteriocins, i.e. bacterial proteins that possess the capacity to kill bacteria of different species.

d) Degradative plasmids enable the digestion of unusual aromatic substances e.g. toluene or salicylic acid.

e) Virulence plasmids enable bacteria to increase their pathogenic potential.

However, this division of plasmids into functional classes has tended to be replaced by a system relying on different Inc groups, involving characterization of plasmid-associated recombinases (Rec) and relaxases (Rel). More recent plasmid classification systems include factors relating to copy number (cop) and maintenance (toxin-antitoxin, or post segregational killing (PSK), systems) [71].

Figure 2. shows the typical structure of the simple resistance plasmid R100 from *E. coli*.

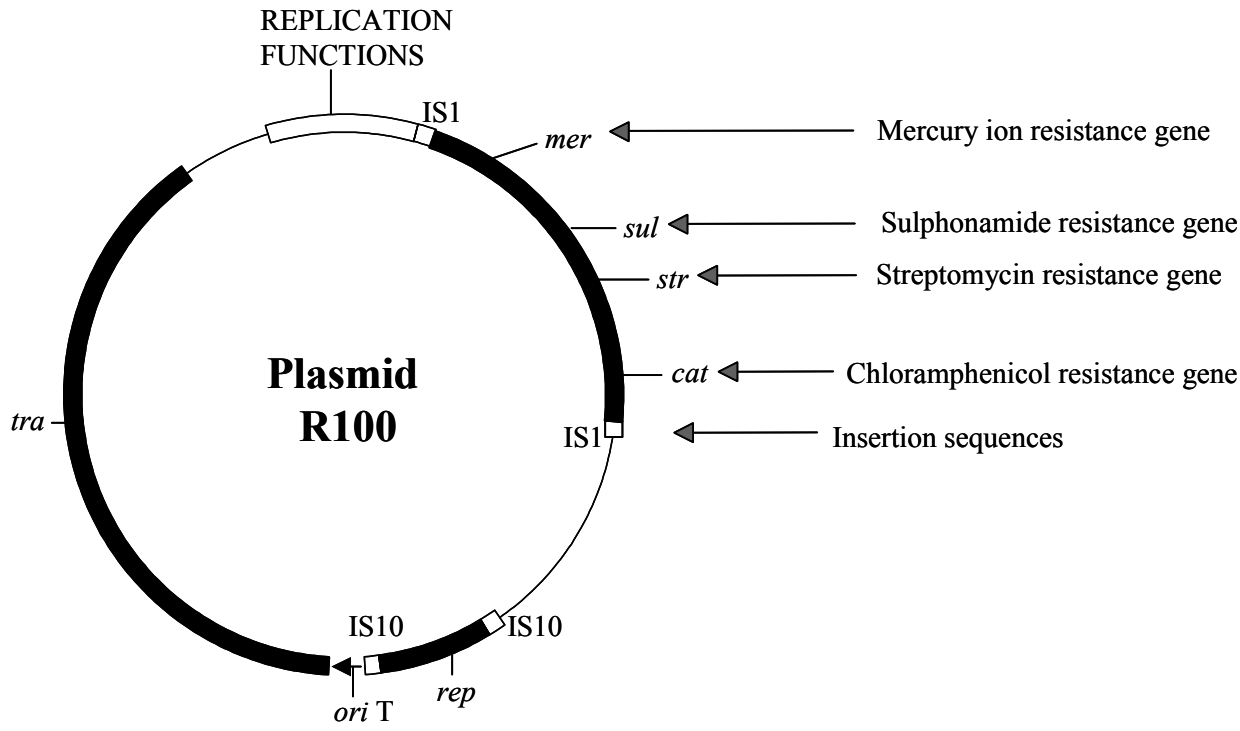

Figure 2. Typical structure of a simple antibiotic resistance plasmid R100

Transposons:

Transposons were discovered by Barbara McClintock in the 1940s [72], she was subsequently awarded a Nobel prize in 1983. Transposons belong to the set of mobile genetic elements called transposable elements. In bacteria, transposons can "jump" from chromosomal DNA to plasmid DNA and back, allowing the transfer and permanent addition of accessory genes, such as those encoding antimicrobial resistance, between bacterial species. This process may lead to the development of multi-antimicrobial resistant bacterial strains. Bacterial transposons can be divided in to four classes, Class I (composite transposons). The mobility of these transposons is associated with plasmids or phages. Common examples of Class I transposons are, Tn*5*, Tn*1525* and Tn*10*. Class II transposons are more frequently found in enterobacteriaceae, but are *not* directly transmissible. The typical example is Tn*3*. Class III transposons (Transposons of the family Tn*5090*-Tn*7*) are phage-associated. One typical example is Tn*552* from *S. aureus*. Fourth group of transposons are also called as "conjugative transposons", or ICEs (integrative and conjugative elements) or CONSTINs (conjugal self-transmissible, integrating elements), to stress its frequent interaction with the bacterial chromosome. Typical example is Tn*916*.

Class I mobile genetic elements or retrotransposons are first transcribed to RNA, and then reverse transcribed back to DNA by reverse transcriptase, before being inserted at another position in the genome. Class II transposons move directly from one position to another using a transposase to "cut and paste" themselves within the genome. Class II transposon are directly transmissible between bacteria, particularly between Gram-negatives. Class III transposons consist of bacteriophage Mu and related temperate phages. The entire phage genome functions as a transposon, and replication of the phage DNA during vegetative

growth occurs by replicative transposition facilitated by a transposase. Example of a Class II transposon, Tn*3* is shown in Figure 3. Tn*3* contains terminal inverted repeats, a transposase, a resolvase and ampicillin resistance genes. The inverted repeat sequences are recognised by the transposase and act as recognition sequences for transposase digestion. The transposase enzyme is responsible for the excision and re-integration of transposed DNA. Resolvase enzyme is involved in the separation of transposons into their component replicons during copying, cutting and pasting.

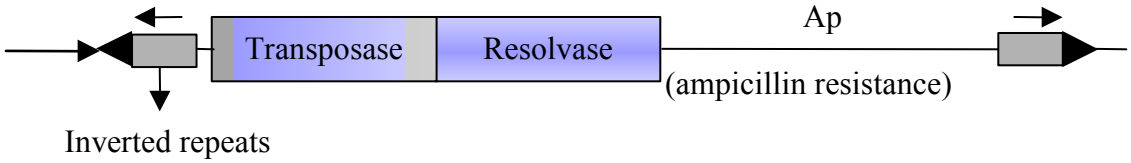

Figure 3. Tn*3* transposon found in enterobacteriaceae

Integrons:

The number of platforms involved in antibiotic resistance dissemination was further expanded by the discovery of a novel genetic system for the movement of antibiotic resistance genes called the "integron" [73]. Integrons are mobile DNA elements with a specific structure consisting of two conserved segments flanking a central region in which antimicrobial resistance "gene cassettes" are integrated [73, 74]. The important components of an integron include, an *int* gene which codes for a site-specific recombinase (involved in recombination), an adjacent recognition site for integrase called *attI* (also acts as a receptor site for the gene cassettes), and a promoter required for expression of the gene cassette. The gene cassettes are mobile elements that usually carry an antibiotic resistance gene, and an integrase-specific recombination site, a 59-base element [75]. There are four classes of integrons currently described that encode antibiotic resistance genes. The majority of integrons described belong to class 1 (Figure 4), and are associated with *sulI* gene conferring resistance to sulphonamides) [75]. The most important epidemiological feature of class 1 integrons is their association with Tn*3* transposons. Class 2 integrons are associated with Tn*7*-family of transposons. Class 3 integrons are similar to class 2, and are associated with conjugative plasmids. An example of a Class 3 integron consists of an *intI3* integrase gene, two (Pc and Pint) promoter regions, an *attI3* recombination site, a $bla_{\text{GES-1}}$ gene cassette, and a fused $bla_{\text{OXA-10}}$-type/*aac (6')-Ib* gene cassette [76]. Class 4 integrons are part of the SXT element encoding antibiotic-resistance in *Vibrio cholerae* [77].

Integrons are widespread versatile genetic elements, their ability to integrate gene cassettes (and especially gene cassettes encoding resistance to antimicrobial agents) makes them key elements in the dissemination of antibiotic resistance. Since many integrons possess multiple resistance genes, selection for one antimicrobial resistance determinant (via antibiotic treatment), may actually select for a range of antibiotic resistances.

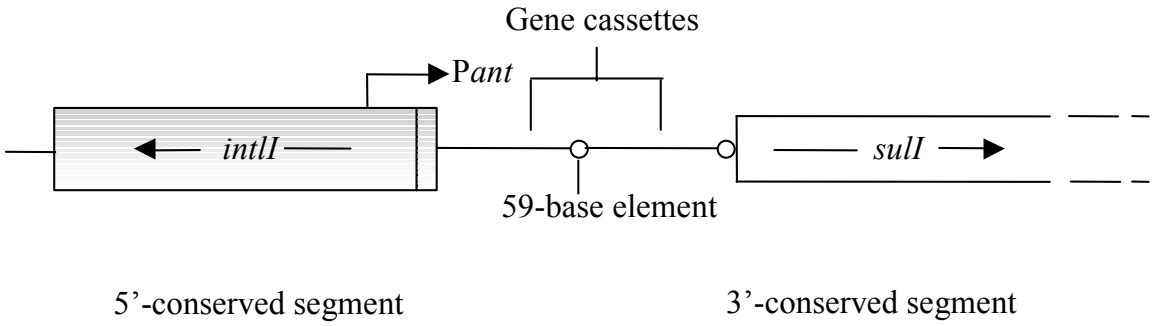

Figure 4. Schematic representation of *sulI* associated integron. Adapted from: Hall RM and Collis CM. Mobile gene cassettes and integrons: capture and spread of genes by site-specific recombination. Mol Microbiol 1995; 15:593-600 [75].

Chromosomes:

Antibiotic resistance genes present on chromosomes may transfer to other bacterial isolates via a process called transformation, in which naked DNA of a lysed bacterium is taken up across the cell membrane of another "competent" bacterial cell. This new DNA may then be incorporated into the chromosome at regions of sequence homology via a process called "homologous recombination". Antibiotic resistant genes present on bacterial chromosomes are usually mutated versions of normal, chromosomal bacterial genes. For example, a mutation may alter the ribosomal structure and hence alter the active site of action for an antimicrobial agent such as erythromycin making this particular antimicrobial agent no longer effective. Additionally, resistance to β-lactam antibiotics in some naturally transformable bacterial pathogens has arisen by inter-species recombinational events that have generated hybrid penicillin-binding proteins with reduced affinity for β-lactam antibiotics. This type of resistance is of particular concern in pneumococci, with resistance to β-lactam antibiotics rapidly increasing worldwide [78]. However, chromosomally resistant mutants are typically resistant to only a single type of antibiotic.

Modes of antimicrobial resistance transfer and dispersal

The dissemination of mobile genetic element platforms (plasmids, transposons, and integrons) has significantly contributed to the rapid spread of antimicrobial resistance. These platforms utilize 3 different modes of transmission to facilitate their dispersal, namely i) transformation, ii) conjugation and iii) transduction [79]. These modes of dispersal are shown in Figure 5.

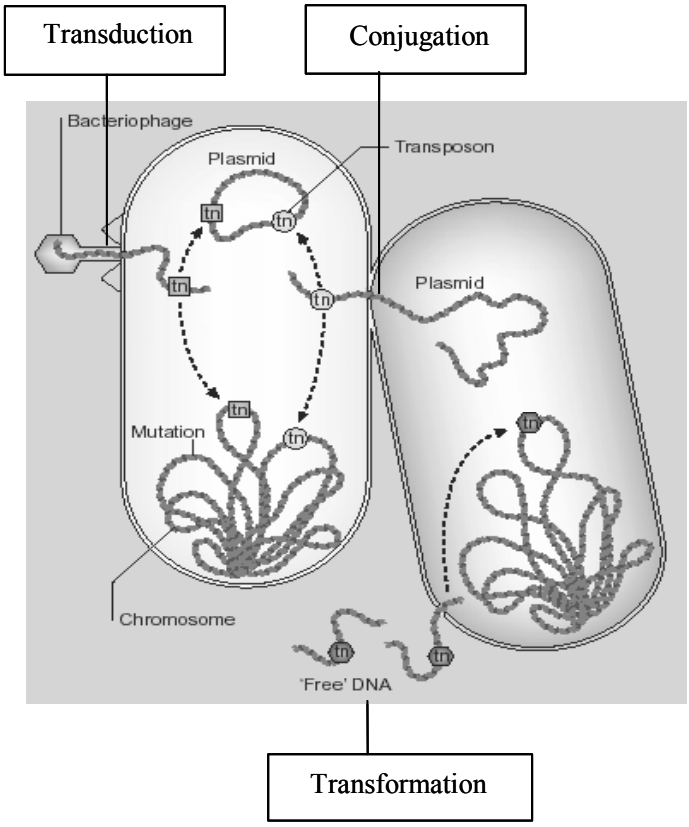

Figure 5. Different modes of antimicrobial resistance transfer (Adapted with permission from: Levy SB and Marshall B. Antibacterial resistance worldwide: causes, challenges and responses. Nat Med 2004; 10:S122-129).

i) Bacterial transformation involves a stable genetic change brought about by the uptake of naked DNA (DNA without associated cells or proteins). In the transformation process, so called "competent" bacteria acquire and incorporate DNA segments from bacteria that have died, lysed, and released their DNA into the environment ready for uptake. Clinically important examples of transformation-mediated antibiotic resistance include the acquisition of penicillin and quinolone resistance genes by pneumococci [80, 81].

ii) During conjugation, a bacterium may transfer plasmid DNA to an adjacent bacterium, via an elongated proteinaceous structure termed a "conjugation tube", which is attached to, and couples together two separate bacterial cells [82]. Conjugation among Gram-positive bacteria is usually initiated by production of sex pheromones, which facilitate the clumping of donor and recipient organisms, as well as conjugation tube attachment. Examples of conjugation-mediated antimicrobial resistance in Gram-negative bacteria include extended-spectrum β-lactam resistance [83], and in Gram-positive bacteria, high level vancomycin resistance, which is carried by F-plasmids in both enterococci and staphylococci [84].

iii) During transduction, genes are transferred from one bacterium to another via a bacteriophage (bacterial virus), though this is now thought to be a relatively rare event. Genetic exchange via transduction involves bacteriophage infection of a bacterium, phage replication, and packaging of some of the bacterial DNA (including antibiotic resistance determinants) within the phage DNA. This is followed by lysis of the bacterium and infection

of subsequent bacteria by new phages, with subsequent transfer of antibiotic resistance genes to newly infected bacteria [85].

Causes of antimicrobial resistance

Antimicrobial resistance is a natural biological phenomenon that can be accelerated by repeated exposure to antibiotics, with several studies having confirmed that increased human and veterinary antibiotic consumption has led to the emergence of antibiotic resistance worldwide [5, 6, 86-91]. Repeated exposure to antibiotics, results in the selection of pre-existing resistant variants that ultimately become fixed in the population. In addition, antibiotic pressure may also select for bacteria with an increased frequency of mutation (hypermutators) [92], that tend to possess an increased frequency of recombination. Hypermutation has only an increased frequency of homologous recombination (among not too distant sequences). Probably the fact that in most cases, hypermutation is due to defects in the MMR (methyl-mismatch-repair system). Hypermutators are selected by antibiotic exposure by "hitch-hiking", that is, hypermutators have more frequently mutations leading to antibiotic-resistance [92-96]. This selection occurs because "mutator clones" have a higher probability of producing mutants that survive the strong selection pressure of antibiotic administration. This allows their frequency to increase during treatment, which is particularly important in chronic infections when repeated antibiotic administrations are required [92, 94, 95]. In addition to antibiotic prescription policies, factors contributing to the emergence of resistance include dose and duration of antibiotic therapy and cross-selection between different antibiotic classes [97].When antimicrobial agents are used incorrectly, for too short a time, at too low a dose, at inadequate potency, or for the wrong diagnosis, the likelihood that bacteria will adapt and replicate, rather than be killed, is greatly enhanced. In fact, the World Health Organization (WHO) estimates that 50 percent of all medicines are inappropriately prescribed, dispensed, or sold, and that 50 percent of all patients fail to take their medicine properly [98]. Other major factors identified by WHO in initiating and promoting antimicrobial resistance include:

a) The unnecessary use of antibiotics by humans
b) The misuse of antibiotics by health professionals
c) Over-the-counter availability of antibiotics in many countries
d) Patient failure to follow the prescribed course of treatment
e) The use of antibiotics in animal feeds as growth hormones

Consequences of antimicrobial resistance

The consequences of antimicrobial resistance not only have a profound impact on healthcare systems as a whole, but also on patients, society and the general economy.

The major problem associated with the development of antimicrobial resistance is treatment failure which in turn leads to increased patient morbidity, mortality and cost of therapy [99, 100]. Treatment failure delays the successful response to antibiotics, and results in longer length of disease, increased patient morbidity as well as costs. It has been shown that the primary cause of initial treatment failure is suboptimal dosing [101], often compounded by treatment failure due to inappropriate choice of antibiotic therapy. For example, if a hospital pharmacy only offers a limited range of antimicrobial agents to clinicians for treatment, subsequent treatment failures may occur due to the lack of (more expensive) appropriate antibiotics. Thus healthcare policies and directives may influence

antimicrobial treatment options if they prefer older and more selective antimicrobial agents in order to reduce the cost of therapy. These economical and selective antimicrobial agents may be effective against non-resistant microorganisms [102], but may not be effective against existing or newer and emerging resistant strains.

Another consequence of antimicrobial resistance is that those infected with resistant bacterial isolates may have a prolonged illness with longer periods of infection, increasing exposure of others to drug-resistant strains of the disease. One of the best examples is tuberculosis (TB). While drug-susceptible TB can be cured within six months by the use of standard antimicrobial agents such as isoniazid, rifampicin and ethambutol, drug-resistant TB requires extensive chemotherapy for as long as two years [103]. Not only are increased costs associated with prolonged care, but patients are also at an increased risk of dying due to treatment failure. A patient infected with multi-drug-resistant TB can be expected to receive additional expensive diagnostic evaluation including extra cultures and laboratory studies, and will likely require a more potent, and usually more expensive, antimicrobial treatment regimen. For example, the drugs that are needed to treat multidrug-resistant forms of tuberculosis (streptomycin, isonicotinyl hydrazine, rifampin, ethambutol, pyrazinamide, moxifloxacin and cycloserine) are over 100 times more expensive than the first-line drugs that are used to treat the non-resistant forms of TB. In addition, if appropriate, ICU and overall hospital length-of-stay will almost certainly be extended [104, 105] if resistant TB is diagnosed. In fact, in many developing countries including Russia, the high cost of such replacement drugs is unaffordable, with the result that some diseases can no longer be treated in areas where resistance to first-line drugs is widespread.

Surveillance studies

Epidemiological surveillance is the systematic collection, analysis and dissemination of health data for the planning, implementation and evaluation of public health programmes. In this respect, antimicrobial resistance surveillance studies are one of the main means of gathering important information about:

a) Existing trends in pathogen incidence and antimicrobial resistance mechanisms.

b) The appearance of novel resistance types.

c) The prediction of future trends in antimicrobial resistance.

To be most effective, surveillance studies should be conducted at local, national and international levels. Local surveillance studies are the most important studies for clinicians needing guidance for empirical therapy and in the management of antimicrobial resistance in the nosocomial environment. Global surveillance of antibiotic resistance on the other hand is necessary, since antimicrobial resistance genes are able to cross both national and international boundaries. For example, the spread of drug-resistant tuberculosis, penicillin-resistant pneumococci and ESBLs are just a few examples of this phenomenon.

Currently, several international surveillance studies have been established. These include:

1) European Antimicrobial Resistance Surveillance System (EARSS): EARSS is the first publicly funded monitoring system for antimicrobial resistance in the European region

established in January 1999. EARSS is a European surveillance and information system that provides validated data on the prevalence and spread of major disease-causing bacteria with resistance to one or more antibiotics. Antimicrobial resistance is monitored by a network of national centres in 31 European countries. These countries sytematically collect data from clinical laboratories in their own countries. The national centres send this data to the EARSS Management Team at National Institute for Public Health and the Environment (RIVM), The Netherlands, who check them for consistency and publish, approved results. The guidelines set for EARSS were published in 2001 [106].

2) The Alexander project, established in 1992 to examine antimicrobial susceptibilities in bacterial isolates from community-acquired infections of the lower respiratory tract. Isolates of *S. pneumoniae*, *H. influenzae* and *M. catarrhalis* were collected from geographically separated areas in countries in the European Union and various states in the USA, Mexico, Brazil, Saudi Arabia, South Africa and Hong Kong. Testing of a range of compounds was undertaken in a central laboratory funded by the pharmaceutical company "GlaxoSmithKline Beecham".

3) The PROTEKT study, a longitudinal, global, multicenter surveillance study involving 69 centers from 29 countries, which was started in 1999. Antimicrobial resistance profiles were determined in microorganisms involved in community-acquired respiratory tract infections. The different classes of antimicrobial agents were used to determine antibiotic susceptibility profiles, among them the β-lactams, cephalosporins, quinolones and glycopeptides. The PROTEKT surveillance study is funded by Aventis pharma [107].

Examples of national surveillance studies include:

1) The Danish Integrated Antimicrobial Resistance Monitoring and Research Program (DANMAP).

2) NETHMAP (The Netherlands antimicrobial surveillance program).

With respect to this particular thesis, a surveillance program exclusively concentrating on the molecular mechanisms and epidemiology involved in the spread of antimicrobial resistance in clinically important bacteria was started in 2006. This surveillance program, called DRESP2 for "Drug Resistance Spread 2" was funded by a European Union grant (FP6), and involves 10 academic research institutions and 1 small medium enterprise (SME) across the European Union. The main objective of the project was to provide information on the mechanisms and "signatures" associated with the molecular mechanisms (platforms) responsible for the spread of antimicrobial resistance. This information was then to be used in predicting future trends in antimicrobial resistance.

Aim and outline of this thesis

The aim of the research performed in this thesis is to increase our current knowledge regarding the molecular mechanisms involved in the spread of antimicrobial resistance. The focus of the research was fixed on the characterization of antimicrobial resistance in clinically relevant Gram-negative and Gram-positive bacteria, in order to identify new molecular "signatures" and antibiotic resistance profiles that could provide information on the future impact of antibiotic resistances. Geographical regions were particularly targeted, where information on the molecular mechanisms and fingerprints of existing antimicrobial resistances is currently limited. The Medical Microbiology and Infectious Diseases department of the Erasmus University Medical Center was one of the partners in the

collaboration. This thesis forms part of the results obtained by the DRESP2 consortium (http:// www.dresp2.com/).

The following topics were specifically addressed in this thesis.

- **β-lactamase MIC distribution and correlation to other antibiotic resistances in *Moraxella catarrhalis* isolates.**

In this study we investigated correlation of different antibiotics in 1440 global *M. catarrhalis* isolates obtained from 7 world regions (chapter 2).

- **Production of extended-spectrum β-lactamses (ESBLs) in enterobacteriaceae from Paraguay and India.**

We characterized ESBL types in *E. c*oli and *K. pneumoniae* from Paraguay (chapter 3), as well as the prevelance and characterization of ESBLs among, *K. peumoniae* and non-typhoidal salmonellae from India (chapter 4, 5 and 6).

- **High-level vancomycin resistance in *Enterococcus faecium* isolates from Saudi Arabia and Paraguay.**

High-level vancomycin resistant *E. faecium* (*vanA* type), isolates from Saudi Arabia and Paraguay (chapter 7 and 8) were studied.

- **High-level aminoglycoside resistance in *Enterococcus faecalis* from patients attending a Dutch hospital.**

High-level gentamicin resistance among *E. faecalis* isolates obtained from patients during and after hospitalization in a Dutch teaching hospital were studied (chapter 9).

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Chapter 2

bro β-lactamase and antibiotic resistances in a global cross-sectional study of *Moraxella catarrhalis* from children and adults

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Abstract

Objectives: To compare and contrast the geographic and demographic distribution of *bro* β-lactamase and antibiotic MIC50/90 for 1440 global *Moraxella catarrhalis* isolates obtained from children and adults between 2001 and 2002.

Methods: One thousand four hundred and forty *M. catarrhalis* isolates originating from seven world regions were investigated. The isolates were recovered from 411 children <5 years of age and 1029 adults >20 years of age. PCR-restriction fragment length polymorphism (RFLP) was performed to determine *bro* prevalence and to distinguish between *bro* types. MIC values of 12 different antibiotics were determined using the CLSI (formerly NCCLS) broth microdilution method.

Results: Of the 1440 isolates, 1313 (91%) possessed the *bro-1* gene and 64 (4%) possessed the *bro-2* gene. Additionally, the prevalence of *bro* positivity between the child and adult age groups was significantly different (P<0.0001), though *bro-1* and *bro-2* prevalences within age groups were not significantly different. Consistently higher β-lactam MICs were observed for *M. catarrhalis* isolates originating in the Far East. Significant correlations in MICs were observed for several antibiotic combinations, including all five β-lactams with each other, and among the two quinolones.

Conclusions: The worldwide prevalence of *bro* gene carriage in clinical isolates of *M. catarrhalis* is now approaching 95%, with children significantly more likely to harbour *bro*-positive isolates than adults. Further, statistically significant differences in the distribution of β-lactam MICs were observed between different world regions, particularly with respect to the Far East.

Introduction

Moraxella catarrhalis is a Gram-negative diplococcal commensal of the respiratory tract of humans that is also frequently implicated as a pathogen in human disease [1, 2]. The species is frequently associated with respiratory tract infections, including acute and chronic otitis media, sinusitis, acute bronchitis and pneumonia [3]. However, a general division tends to exist between the frequency of *M. catarrhalis* isolation in upper and lower respiratory tract infection between children and adults, e.g. otitis media predominates in children compared with exacerbations of chronic obstructive pulmonary disease (COPD) in adults [4].

The first β-lactamase-producing *M. catarrhalis* isolate was reported from Sweden in 1976, and in the last 30 years there has been a dramatic increase in the percentage of β-lactamase positive *M. catarrhalis* clinical isolates. Indeed, although *M. catarrhalis* is still susceptible to the majority of antimicrobial agents, >95% of global clinical isolates are now resistant to penicillin [5-8]. In addition, *M. catarrhalis* resistance to cefaclor and cefuroxime has recently been reported, with 80% of *M. catarrhalis* isolates tested from the UK and Ireland showing resistance to cefaclor and 5% showing resistance to cefuroxime [9].

In 1977, it was reported that *M. catarrhalis* produces a single β-lactamase, designated BRO, which was subsequently found to be located on the bacterial chromosome [10]. Later research indicated the presence of two BRO types, named BRO-1 and BRO-2, encoded by *bro-1* and *bro-2* genes. The two BRO enzymes are distinguishable by their level of β-lactamase production, and different isoelectric focusing patterns (resulting from a single amino acid substitution). The *bro-2* gene has a 21 bp deletion in its promoter region compared with *bro-1* [3, 10, 11]. Further genetic analysis of the *bro* gene indicated that it may have a Gram-positive origin, due to the fact that there is no significant sequence similarity between *bro* and the β-lactamase genes of other Gram-negative bacteria, and that the gene possesses an LPXTG signal sequence motif, characteristic for Gram-positive bacteria [12, 13]. This finding, coupled with the relatively conserved nature of the immediate sequences flanking the *bro* gene, suggests that the gene may have been fortuitously acquired by *M. catarrhalis* via interspecies gene transfer, with 'promoter-up' mutations leading to the appearance and eventual dissemination of the *bro-1* variant [12]. However, this interpretation of the appearance and spread of the *bro* genes is still open to debate [14].

At the clinical level, β-lactamase production by *M. catarrhalis* has been reported to indirectly benefit other bacterial species present in mixed bacterial infections (e.g. *Streptococcus pneumoniae* and *Haemophilus influenzae*), by virtue of production of a β-lactamase whose activity allows susceptible isolates to evade antibiotic therapy with β-lactam antibiotics (specifically penicillin), possibly facilitating treatment failure [15, 16].

The aim of this study was to determine the distribution of antibiotic MIC50/90 in a cross-sectional population of global *M. catarrhalis* isolates obtained from children and adults between 2001 and 2002. Special emphasis was placed on β-lactam antibiotic resistance phenotypes and *bro* β-lactamase genetic variation due to the clinical importance of β-lactamase resistance within this species. Antimicrobial MICs of 12 different antibiotics for a set of 1440 global *M. catarrhalis* isolates were obtained. Information was also collected regarding age (of patients from which the specimen was obtained), world region and specimen type.

Materials and methods

Bacterial strains

A total of 1440 *M. catarrhalis* isolates were used in the analysis, comprising 411 isolates obtained from children (<5 years of age) and 1029 isolates obtained from adults (>20 years of age) during the years 2001–2002 (see Table 1). Samples were collected consecutively within countries, but collection times were not globally uniform; thus, the study shares characteristics with a global 'point prevalence' study where the 'point' is 1–2 years. The isolates originated from a total of 30 countries, namely Argentina (34), Australia (36), Austria (9), Belgium (40), Brazil (25), Canada (160), China (8), Ecuador (15), Eire (45), France (36), Germany (158), Hong Kong (24), Hungary (15), Italy (70), Japan (224), the Netherlands (18), Peru (7), Poland (19), Portugal (20), Russia (1), South Africa (115), South Korea (12), Spain (127), Sweden (23), Switzerland (23), Taiwan (44), Turkey (3), the UK (32), the USA (86) and Venezuela (11), where the numbers in parentheses represent the number of isolates. With respect to specimen type, the isolates were cultured from bronchiolar lavages (125), blood (9), ear swabs (48), middle ear fluids (17), nasopharyngeal swabs (199), sinus fluids (68), sputa (950) and throat swabs (20). Four isolates were cultured from unknown specimen types. Isolates were received and tested at a central laboratory. All isolates were collected as part of the PROTEKT study using sampling and identification methods as published by Felmingham [17]. There was a significant difference in sample type (lower respiratory tract versus upper respiratory tract) between the age groups (Fisher's exact test $P<0.0001$), which reflected the difference in disease states associated with *M. catarrhalis* between the two age groups.

bro gene PCR-restriction fragment length polymorphism (RFLP) screening and β-lactamase testing

To differentiate between *bro-1* and *bro-2* isolates, a PCR was first performed using primer pair BROF 50-TRGTGAAGTGATTTT KRRMTTG-30 and BROR 50-GCAATTTATTAACTGGATGTA-30, which yielded amplicons differing in size by 21 bp (*bro-1* 165 bp and *bro-2* 144 bp). The PCR thermocycler parameters used were 94ºC for 5 min, followed by 94ºC for 30 s, 51ºC for 30 s and 72ºC for 30 s for 35 cycles, and then 72ºC for 7 min. To confirm *bro-1* or *bro-2*, 5 μL of PCR product was digested using 2.5 U of the restriction enzyme Tsp509i (New England Biolabs, MA, USA) for 30 min at 65ºC. Electrophoresis was performed on the enzyme digests using 3.5% agarose at 6 V/cm for 25 min. Tsp509i cleaved the *bro-1* region of interest into two visible fragments of 55 and 91 bp, while *bro-2* was left with a visible fragment of 91 bp. The agarose gel was stained with ethidium bromide and visualized on a UV transilluminator. All isolates (including *bro*-negative isolates) were investigated for the presence of β-lactamase production using the nitrocefin disc test (Oxoid, Basingstoke, UK).

Antibiotic resistance testing

MIC values were determined using the CLSI (formerly NCCLS) broth microdilution method with lyophilized microtitre plates (Sensititre system, Trek Diagnostics) with an inoculum of $3\text{-}7\text{x}10^4$ cfu in 100 μL of medium [17, 18].

Statistical methods

The Pearson correlation coefficient was used to assess associations of $\log_2$ MICs among antibiotics. Differences in frequencies were assessed using the Fisher's exact or x^2 tests. Multiway analysis of variance (ANOVA) was performed in a linear regression framework to test the contribution of potential predictor variables to ampicillin MICs. β-

lactamase-positive isolates were included in the analysis (n=1377). The analysis was based on log_2-transformed MICs using the General Linear Model procedure in SAS version 9.1. Four main factors were included in the model: age; source; infection type; and region. First, an analysis was carried out on a model that included all main effects, the nested effect of countries within regions, and all two- three- and four-way interactions. As none of the three- or four-way interactions was significant, they were removed from subsequent analyses. For countries with samples sizes of ≥25 isolates, separate ANOVAs were conducted to compare differences among countries within regions followed by the Student–Newman–Keuls *a posteriori* test to compare means.

Results

Distribution of bro β-lactamase types

The results of PCR-RFLP screening of 1440 global *M. catarrhalis* isolates revealed that 1377 (96%) isolates contained the *bro* β-lactamase gene, whilst 63 (4%) isolates were PCR and β-lactamase negative (Table 1). Among the 1377 *bro*-positive isolates, 1313 (95%) isolates were found to carry the *bro-1* gene and 64 (5%) isolates were found to carry the *bro-2* gene. With respect to age, the prevalence of *bro-1, bro-2* and *bro*-negative isolates in the child group was 96% (396/411), 3% (12/411) and 1% (3/411), respectively; whilst for the adult group the prevalence was 89% (917/1029), 5% (52/1029) and 6% (60/1029). The prevalence of *bro* positivity between the two age groups was significantly different (x^2 P<0.0001), with a relatively increased number of *bro-2* and *bro*-negative isolates being found in isolates cultured from the adult group. However, the prevalence of *bro-1* and *bro-2* alone between the age groups was not significant (Fisher's exact test $P = 0.067$). All isolates found to be *bro* PCR positive were also found to produce β-lactamase enzyme using the nitrocefin disc test. All nitrocefin disc test positive isolates were found to be *bro* PCR positive.

Individual antimicrobial MICs

The MIC50 and MIC90 values obtained for 1440 *M. catarrhalis* isolates from seven world regions using 12 antibiotics, and divided into *bro-1*, *bro-2* and *bro*-negative types, are shown in Tables 2, S1 and S2 [Tables S1 and S2 are available as Supplementary data at *JAC* Online (http://jac.oxfordjournals.org/)], respectively. For antibiotics with more than minimal variation in MICs, isolates from the Far East had consistently higher 50% and 90% MICs of β-lactam antibiotics compared with the rest of the world. This was particularly pronounced for cefaclor where the 90% MIC was 32 mg/L in the Far East, but only 4 mg/L in all other regions tested. Unlike β-lactams, no difference was observed in non-β-lactam antibiotic MIC50/90 ranges in any geographical region, except for the 90% MICs of tetracycline observed in Eastern European isolates and 90% MICs of telithromycin observed in Far East and South African isolates. Though relatively few isolates were tested from Eastern European countries (which could have influenced the statistical validity of the results), Eastern European MICs of the remaining 12 antibiotics tested were similar to those observed for the majority of other regions tested.

Inter-relationships between antibiotics

Significant correlations were observed between: (i) all five of the β-lactam antibiotics (all 15 pairs of antibiotic combinations in both children and adults at $P<0.0001$); (ii) the quinolones ciprofloxacin and levofloxacin ($P<0.0001$) in both child and adult age groups;

(iii) all five β-lactams and erythromycin, tetracycline, chloramphenicol and sulfamethoxazole/trimethoprim (all at $P<0.01$ in children and adults); (iv) tetracycline, chloramphenicol and sulfamethoxazole/trimethoprim (all at $P<0.05$) with each other; and (v) sulfamethoxazole/trimethoprim and telithromycin (P=0.01). With particular respect to worldwide patterns, $\log_2$ MICs of ampicillin were significantly correlated with: (i) tetracycline (r=0.12), erythromycin (r=0.18) and sulfamethoxazole/trimethoprim (r=0.14) in the Far East; (ii) chloramphenicol (r=0.21), erythromycin (r=0.17) and sulfamethoxazole/ trimethoprim (r=0.20) in North America; (iii) sulfamethoxazole/ trimethoprim (r=0.23) and telithromycin (r=0.20) in Latin America; (iv) tetracycline (r=0.46) and chloramphenicol (r=0.37) in Eastern Europe; and (iv) erythromycin (r=0.13) and sulfamethoxazole/trimethoprim (r=0.09) in Western Europe, where, interestingly, there was significant ($P<0.01$) negative correlation between $\log_2$ ampicillin MICs and telithromycin (r=20.11) as well as the quinolones, ciprofloxacin (r=20.10) and levofloxacin (r=-0.15).

With regard to ampicillin MICs, there were significant differences among regions ($P=0.04$), among countries within regions (P<0.0001) as well as 'age x source' ($P<0.01$) and 'age x region' ($P=0.03$) interactions. The 'age x source' interaction reflects differences between children and adults in carriage sites for *M. catarrhalis*; children are more likely to have *M. catarrhalis* associated with the inner ear compared with the more prevalent colonization of the lower respiratory tract observed in adults. The 'age x region' interaction was caused by a higher proportion of the South African isolates being derived from children (66%), compared with isolates from all other regions (18%–36%).

The Far East region had significantly higher ampicillin MICs than all other regions (1.67 mg/L versus 0.28–0.83 mg/L). Significant differences among countries within regions were present in the Far East ($P<0.01$) where Hong Kong had the lowest MICs, North America ($P<0.001$) where MICs were significantly higher in the USA compared with Canada, and Latin America where MICs were significantly higher in Brazil compared with Argentina ($P<0.05$).

For non-β-lactam antibiotics, differences among countries within regions were present in: the Far East where there were significantly higher MICs in Taiwan of sulfamethoxazole/trimethoprim ($P<0.0001$), tetracycline ($P<0.0001$) and the quinolones ($P<0.001$), and Hong Kong had significantly lower MICs of chloramphenicol ($P<0.0001$) and the cephalosporins cefaclor $P<0.01$), cefpodoxime ($P<0.0001$) and cefuroxime $P<0.0001$); Western Europe where MICs of sulfamethoxazole/trimethoprim ($P=0.001$), levofloxacin ($P=0.02$), cefazolin ($P=0.001$) and tetracycline ($P=0.03$) were lowest in France; and in Latin America where Argentina had higher MICs of tetracycline ($P<0.001$) and levofloxacin ($P=0.01$) compared with Brazil.

Table 1. Distribution of *bro-1*, *bro-2* and *bro*-negative isolates in 1440 *M. catarrhalis* isolates obtained from children and adults in seven world regions during the years 2001 and 2002

	Children				Adults			
Region	*bro-1*	*bro-2*	*bro-neg*	*total*	*bro-1*	*bro-2*	*bro-neg*	*total*
Australia	12	-	-	12	24	-	-	24
Eastern Europe	6	1	-	7	24	4	3	31
Far East	111	2	-	113	176	12	11	199
Latin America	28	1	-	29	55	6	2	63
North America	51	1	1	53	176	5	12	193
South Africa	74	2	-	76	31	8	-	39
Western Europe	114	5	2	121	431	17	32	480
Total	396	12	3	411	917	52	60	1029

Children=isolates from children < 5 years of age.
Adults=isolates from adults > 20 years of age.
The prevalence of bro types between age groups was significantly different (P<0.0001).

Discussion

There has been a rapid rise in the prevalence of worldwide β-lactamase-resistant *M. catarrhalis* isolates (which now approaches 90%–99% of all clinical isolates), since the first reports appeared in the late 1970s. This is considered to be the fastest dissemination of any known bacterial β-lactamase [13]. In the current analysis, >90% of isolates were found to be β-lactamase and *bro* gene positive, with the great majority of isolates carrying the *bro-1* type (increased β-lactamase production compared with *bro-2*), a finding consistent with previous studies. However, when taking account of demographics, a significant difference in the distribution of *bro* types was observed, with adults being more likely to be colonized by *bro-2* or *bro*-negative isolates. This observation may reflect increased global β-lactam use in children compared with adults, or indicate that different genotypes (associated with particular *bro* phenotypes) circulate within child and adult populations. However, previous studies have reported no linkage between *M. catarrhalis* genotypes and *bro* type, with at least one study reporting ampicillin MIC variation in apparently identical *M. catarrhalis* isolates cultured from an otitis media patient in Japan [19]. Further, a recent global survey of 195 *M. catarrhalis* isolates revealed no association between *M. catarrhalis* genotypes and child or adult age groups [4]. Perhaps differences in the global prescribing of β-lactam antibiotics between child and adult age groups are responsible for the difference in *bro* type carriage observed in this study.

Table 2. Antibiotic MICs obtained for 1313 *bro-1*-positive *M. catarrhalis* isolates originating from seven world regions during the years 2001 and 2002

Country (number of isolates)	MIC (mg/L)		
	MIC_{50}	MIC_{90}	range
Australia (36)			
AMP	4	8	0.5–16
AMC	0.12	0.25	0.12–0.5
CXM	1	2	0.12–4
CEC	1	4	0.5–32
CPD	0.5	1	0.03–1
ERY	0.25	0.25	0.25–0.25
CIP	0.03	0.03	0.015–0.06
LVX	0.03	0.06	0.03–0.06
TET	0.25	0.5	0.12–0.5
CHL	0.5	0.5	0.12–0.5
SXT	0.12	0.25	0.03–0.25
TEL	0.006	0.06	0.03–0.12
Eastern Europe (30)			
AMP	4	8	0.25–8
AMC	0.12	0.25	0.12–0.25
CXM	1	2	0.25–4
CEC	2	4	0.5–4
CPD	0.5	1	0.12–1
ERY	0.25	0.25	0.25–0.5
CIP	0.03	0.03	0.015–0.06
LVX	0.03	0.03	0.003–0.06
TET	0.25	4	0.12–4
CHL	0.5	0.5	0.12–1
SXT	0.12	0.25	0.06–1
TEL	0.06	0.12	0.03–0.12
Far East (287)			
AMP	8	16	0.25–32
AMC	0.25	0.25	0.12–0.25
CXM	2	8	0.25–16
CEC	4	32	0.5–64
CPD	1	1	0.03–4
ERY	0.25	0.5	0.25–1
CIP	0.03	0.03	0.008–1
LVX	0.03	0.06	0.015–2
TET	0.25	0.5	0.12–32
CHL	0.5	0.5	0.12–2
SXT	0.12	0.5	0.03–4
TEL	0.06	0.12	0.008–0.5
Latin America (83)			
AMP	4	16	0.5–32
AMC	0.12	0.25	0.12–0.5
CXM	1	2	0.5–2
CEC	1	4	0.5–16
CPD	0.5	1	0.12–1
ERY	0.25	0.25	0.25–0.5
CIP	0.03	0.06	0.015–0.06
LVX	0.03	0.06	0.015–0.06
TET	0.25	0.5	0.12–1
CHL	0.5	0.5	0.25–4
SXT	0.25	0.5	0.03–0.5
TEL	0.06	0.12	0.03–0.25
North America (227)			
AMP	4	16	0.12–32
AMC	0.25	0.25	0.12–0.5
CXM	1	2	0.25–4
CEC	2	4	0.5–16
CPD	0.5	1	0.06–2
ERY	0.25	0.25	0.25–0.5
CIP	0.03	0.03	0.015–4
LVX	0.03	0.03	0.05–2
TET	0.25	0.5	0.12–0.5
CHL	0.5	0.5	0.12–1
SXT	0.12	0.25	0.03–1
TEL	0.06	0.12	0.008–0.5
South Africa (105)			
AMP	4	16	1–32
AMC	0.12	0.25	0.12–2
CXM	2	2	0.25–4
CEC	2	4	0.5–32
CPD	0.5	1	0.1–2
ERY	0.25	0.25	0.25–0.25
CIP	0.03	0.03	0.015–0.12
LVX	0.03	0.03	0.015–0.12
TET	0.25	0.5	0.25–1
CHL	0.5	0.5	0.25–1
SXT	0.25	0.5	0.06–1
TEL	0.06	0.12	0.004–0.25
Western Europe (545)			
AMP	4	8	0.25–32
AMC	0.12	0.25	0.12–0.5
CXM	1	2	0.12–8
CEC	2	4	0.5–16
CPD	0.5	1	0.06–2
ERY	0.25	0.25	0.25–1
CIP	0.03	0.03	0.008–0.12
LVX	0.03	0.03	0.008–0.12
TET	0.25	0.5	0.12–1
CHL	0.5	0.5	0.12–1
SXT	0.12	0.25	0.03–2
TEL	0.06	0.12	0.004–0.25

AMP, ampicillin; AMC, amoxicillin/clavulanate; CXM, cefuroxime; CEC, cefaclor; CPD, cefpodoxime; ERY, erythromycin; CIP, ciprofloxacin; LVX, levofloxacin; TET, tetracycline; CHL, chloramphenicol; SXT, sulfamethoxazole/trimethoprim; TEL, telithromycin.
MIC_{50} = MIC where 50% of the isolates were inhibited.
MIC_{90} = MIC where 90% of the isolates were inhibited.
Range = range of MICs.

The statistical interpretation of our results must be viewed cautiously because the sample isolates were not randomly selected. While use of convenience samples in surveillance studies is not uncommon, its impact is most probably greatest within countries of small geographical extent and those with minimal socioeconomic differentiation where residents have equal access to healthcare. With geographic and cultural homogeneity, clonal outbreaks are more likely to dominate a convenience sample. Conversely, the timing of outbreaks and the underlying genotypes involved are much less likely to be coincident across large regions and, thus, regional patterns of differentiation should be viewed with more confidence. With respect to geographical region, it is obvious from this study that consistently increased *bro-1* β-lactam MICs are observed in *M. catarrhalis* isolates

originating from the Far East, and especially Japan (which provided the majority of isolates collected from the Far East in this study). Though some of these results have been published separately by Inoue *et al.* [20] and Canton [21], we are the first to show in detail that *bro-1*-positive *M. catarrhalis* isolates from the Far East represent a phenotypically distinct subset of global isolates.

Mechanisms not associated with the *bro* gene *per se* may be involved in the increased β-lactamase resistance observed in isolates from the Far East carrying the *bro-1* gene. To support this hypothesis, a study of 818 *M. catarrhalis* isolates from five European countries and the USA, collected between 1992 and 1993, indicated 8% penicillin and 4% amoxicillin resistance in β-lactamase-negative *M. catarrhalis* isolates, though this activity was limited to isolates from Europe alone [22]. Another study of 90 *M. catarrhalis* isolates from Turkey and published in 2007 found a single BRO-negative isolate (1/5) that exhibited penicillin resistance [23]. A third study reported that 16% of BRO negative isolates collected from a single hospital over a 10 year period (1984–1994) in Tennessee, USA, exhibited reduced susceptibility to penicillin [14]. Finally, Jetter *et al.* [24] recently published evidence linking the *M. catarrhalis* outer membrane porin M35 to aminopenicillin susceptibility. Taken together, these results indicate that non-*bro*-related mechanisms of β-lactamase resistance exist within some isolates of the *M. catarrhalis* species, and that these mechanisms may be associated with geographical and/or temporal factors.

The question remains as to whether regional differences in β-lactam MICs are actually clinically relevant. In this respect, it has been reported that *bro*-positive *M. catarrhalis* isolates may protect penicillin-susceptible *Streptococcus pneumoniae* isolates from antibiotic therapy [15]. Therefore, It would be interesting to investigate whether the prevalence of *S. pneumoniae* β-lactam treatment failures in the Far East could be related to co-colonization between *S. pneumoniae* and *bro*-positive *M. catarrhalis* isolates, and whether any increased prevalence in treatment failure was due to increased β-lactam MICs in the Far East.

Interestingly, studies have indicated an increase in MICs of both cefuroxime and cefaclor, as well as an overall decrease in susceptibility to cephems [20, 22, 25-28], though in our study this effect was observed for Far East isolates only. Based on past experience, with respect to the rapid dissemination of penicillin resistance in *M. catarrhalis*, it seems logical that the global monitoring of these antibiotic resistances in *M. catarrhalis* should continue.

The majority of global *M. catarrhalis* isolates circulating during the years 2001–2002 were susceptible to most non-β-lactam antibiotics. These results are consistent with previous studies investigating antimicrobial susceptibility from *M. catarrhalis* isolates cultured in 1999–2000 and 1999–2007 [9, 20, 29]. Occasionally, resistance to tetracycline, erythromycin, streptomycin and fluoroquinolones has been reported [30], though in this study the only exception to non-β-lactam antibiotic susceptibility appeared to be a relative 8-fold increase in tetracycline MICs to 4 mg/L (intermediate resistance) at the 90% inhibition level, for isolates recovered from Eastern Europe. However, the number of isolates tested from Eastern Europe was relatively low, which could have affected the statistical reliability of our results. In this respect it is worthwhile noting that the MICs obtained for all other antibiotics (including β-lactam antibiotics) in this region matched the MICs obtained for the majority of other geographical regions. Further analysis of interregional differences revealed that 30% (6/20) of isolates from Poland possessed tetracycline MICs of 4 mg/L, with neither Hungary nor Turkey (the other countries included in this global region) possessing tetracycline MICs >1 mg/L. Further research is therefore necessary to determine whether tetracycline use in Poland is particularly high compared with the rest of the world, or whether our finding represents a statistical anomaly, perhaps resulting from a sample with over-representation of a single clone.

Not unexpectedly, strong correlations in MICs were found among functionally related antibiotics, i.e. β-lactams or quinolones. MIC correlations for antibiotics with a shared mechanism action are most probably caused by a specific resistance determinant. Thus, the action of a β-lactamase is the likely explanation for worldwide correlations among the five β-lactam agents. Similarly, several known resistance factors can account for the correlations between the two quinolones. While correlations in MICs were stronger among antibiotics with shared mechanisms of action, significant correlations were prevalent even among functionally and structurally unrelated antibiotics. An association of susceptibility phenotypes for antibiotics whose modes of action have no evident relationship is more likely to be caused by genetic linkage of resistance determinants it may derive from a non-specific resistance mechanism, such changes in efflux pumps or membrane permeability. Either may explain the correlations between β-lactam agents and four antibiotics possessing diverse mechanisms of action. However, *M. catarrhalis*, resistance to non-β-lactam antibiotics is rare, and plasmids, particularly multidrug resistance plasmids, are uncommon. Thus, the significant correlations involving non-β-lactam agents most probably represent small differences in susceptibility to the non-β-lactam agents. Such incremental differences that influence susceptibility to multiple agents may be caused by genetic mutations whose individual phenotypic effects may be small but that in concert with other factors may bring about marked reductions in susceptibility. Again, these multifactorial effects may arise from changes in the efficiency and specificity of efflux pumps and membrane permeability.

In this study, significant correlations were observed between tetracycline, chloramphenicol, sulfamethoxazole/trimethoprim and telithromycin, as well as β-lactam MICs. These significant correlations may reflect the preferred antibiotic prescribing practices associated with the treatment of *M. catarrhalis* infections in different world regions, or possibly the preferred antibiotic prescribing policies for infections where *M. catarrhalis* is a co-colonizing bacterium. In any case, β-lactam antibiotics, with or without the inclusion of a β-lactamase inhibitor (e.g. amoxicillin/clavulanate), remain the antimicrobial drugs of choice for treating *M. catarrhalis* infections [31].

Finally, it would be interesting to know whether differences in β-lactam prescribing policies in the Far East (compared with the rest of the world) are providing the selection pressure towards increased β-lactam MICs in this world region, as β-lactams are commonly used for the empirical treatment of community-acquired respiratory tract infections in the Far East [32]. If this is indeed the case, then changes in antibiotic prescribing policy in the Far East may be warranted.

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Abstract 1134_04_123. European Society of Clinical Microbiology and Infectious Diseases, Basel, Switzerland.

Supplementary data

Table S1. Antibiotic MICs obtained for 64 *bro-2*-positive *M. catarrhalis* isolates originating from seven world regions during the years 2001 and 2002

	MIC (mg/L)		
Country (number of isolates)	MIC_{50}	MIC_{90}	range
Australia (0)			
AMP	-	-	-
AMC	-	-	-
CXM	-	-	-
CEC	-	-	-
CPD	-	-	-
ERY	-	-	-
CIP	-	-	-
LVX	-	-	-
TET	-	-	-
CHL	-	-	-
SXT	-	-	-
TEL	-	-	-
Eastern Europe (5)			
AMP	2	8	1-8
AMC	0.12	0.12	0.12-0.12
CXM	0.5	0.5	0.5-0.5
CEC	1	2	0.5-1
CPD	0.25	0.25	0.12-0.25
ERY	0.25	0.5	0.25-0.5
CIP	0.03	0.06	0.03-0.06
LVX	0.03	0.06	0.015-0.06
TET	0.5	1	0.25-1
CHL	0.5	1	0.5-1
SXT	0.5	4	0.25-4
TEL	0.06	0.12	0.06-0.12
Far East (14)			
AMP	2	4	0.5-4
AMC	0.12	0.25	0.12-0.25
CXM	0.5	2	0.25-2
CEC	1	2	0.5-8
CPD	0.25	0.5	0.06-0,05
ERY	0.25	0.25	0.25-0.25
CIP	0.03	0.06	0.015-0.5
LVX	0.03	0.06	0.015-1
TET	0.25	0.5	0.25-0.5
CHL	0.5	0.5	0.5-0.5
SXT	0.12	0.25	0.03-0.25
TEL	0.06	0.06	0.06-0.12
Latin America (7)			

AMP	1	8	0.5-8
AMC	0.12	0.25	0.12-0.25
CXM	0.5	1	0.25-1
CEC	1	2	0.5-2
CPD	0.12	0.25	0.12-0.25
ERY	0.25	0.25	0.25-0.5
CIP	0.03	0.03	0.015-0.03
LVX	0.03	0.06	0.03-0.06
TET	0.25	1	0.25-1
CHL	0.5	0.5	0.5-0.5
SXT	0.25	4	0.12-4
TEL	0.06	0.12	0.03-0.12

North America (6)

AMP	0.5	4	0.25-4
AMC	0.12	0.25	0.12-0.25
CXM	0.5	1	0.25-1
CEC	0.5	1	0.5-1
CPD	0.12	0.25	0.12-0.25
ERY	0.25	0.25	0.25-0.5
CIP	0.03	0.06	0.015-0.06
LVX	0.03	0.06	0.03-0.06
TET	0.25	0.5	0.25-0.5
CHL	0.5	0.5	0.25-0.5
SXT	0.12	0.25	0.12-0.25
TEL	0.06	0.06	0.03-0.6

South Africa (10)

AMP	1	2	0.25-4
AMC	0.12	0.12	0.12-0.25
CXM	0.5	0.5	0.25-1
CEC	1	2	0.5-2
CPD	0.12	0.25	0.12-0.5
ERY	0.25	0.25	0.25-0.25
CIP	0.03	0.03	0.015-0.03
LVX	0.03	0.03	0.015-0.06
TET	0.25	0.5	0.25-0.5
CHL	0.5	0.5	0.5-0.5
SXT	0.25	0.25	0.12-1
TEL	0.06	0.06	0.03-0.12

Western Europe (22)

AMP	1	2	0.25-4
AMC	0.12	0.12	0.12-0.25
CXM	0.5	1	0.25-2
CEC	1	2	0.5-2
CPD	0.12	0.5	0.12-0.5
ERY	0.25	0.25	0.25-0.25
CIP	0.03	0.06	0.03-0.06
LVX	0.03	0.06	0.03-0.06
TET	0.25	1	0.25-1

CHL	0.5	1	0.25-1
SXT	0.25	0.5	0.06-1
TEL	0.06	0.12	0.03-0.12

AMP, ampicillin; AMC, amoxicillin/clavulanate; CXM, cefuroxime; CEC, cefaclor; CPD, cefpodoxime; ERY, erythromycin; CIP, ciprofloxacin; LVX, levofloxacin; TET, tetracycline; CHL, chloramphenicol; SXT, sulfamethoxazole/trimethoprim; TEL, telithromycin.
MIC_{50} = MIC where 50% of the isolates were inhibited.
MIC_{90} = MIC where 90% of the isolates were inhibited.
Range = range of MICs.

Table S2. Antibiotic MICs obtained for 63 *bro*-negative *M. catarrhalis* isolates originating from seven world regions during the years 2001 and 2002

	MIC (mg/L)		
Country (number of isolates)	MIC_{50}	MIC_{90}	range
Australia (0)			
AMP	-	-	-
AMC	-	-	-
CXM	-	-	-
CEC	-	-	-
CPD	-	-	-
ERY	-	-	-
CIP	-	-	-
LVX	-	-	-
TET	-	-	-
CHL	-	-	-
SXT	-	-	-
TEL	-	-	-
Eastern Europe (3)			
AMP	0.12	0.12	0.12-0.12
AMC	0.12	0.12	0.12-0.25
CXM	0.25	0.5	0.25-0.5
CEC	0.5	0.5	0.5-0.5
CPD	0.12	0.12	0.06-0.12
ERY	0.25	0.25	0.25-0.25
CIP	0.03	0.06	0.03-0.06
LVX	0.03	0.06	0.03-0.06
TET	0.25	0.25	0.25-0.25
CHL	0.5	0.5	0.25-0.5
SXT	0.12	0.25	0.06-0.25
TEL	0.015	0.03	0.008-0.03
Far East (11)			
AMP	0.12	0.12	0.12-0.12
AMC	0.12	0.12	0.12-0.12
CXM	0.5	0.5	0.25-0.5
CEC	0.5	1	0.5-1
CPD	0.12	0.12	0.06-0,12
ERY	0.25	0.25	0.25-0.25
CIP	0.03	0.03	0.015-0.03

LVX	0.03	0.06	0.03-0.06
TET	0.25	0.25	0.12-0.5
CHL	0.5	0.5	0.5-0.5
SXT	0.12	0.12	0.06-0.25
TEL	0.06	0.06	0.06-0.12
Latin America (2)			
AMP	0.12	0.12	0.12-0.12
AMC	0.12	0.12	0.12-0.12
CXM	0.5	0.5	0.5-0.5
CEC	0.5	1	0.5-1
CPD	0.12	0.12	0.06-0,12
ERY	0.25	0.25	0.25-0.25
CIP	0.03	0.03	0.03-0.03
LVX	0.03	0.06	0.015-0.06
TET	0.25	0.5	0.25-0.5
CHL	0.5	0.5	0.5-0.5
SXT	0.06	0.12	0.06-0.12
TEL	0.06	0.06	0.06-0.06
North America (13)			
AMP	0.12	0.12	0.12-0.12
AMC	0.12	0.12	0.12-0.12
CXM	0.25	0.5	0.25-0.5
CEC	0.5	1	0.5-2
CPD	0.12	0.25	0.12-0.25
ERY	0.25	0.25	0.25-0.25
CIP	0.03	0.03	0.015-0.03
LVX	0.03	0.06	0.015-0.06
TET	0.25	0.5	0.25-0.5
CHL	0.5	0.5	0.25-0.5
SXT	0.12	0.25	0.06-0.5
TEL	0.06	0.06	0.04-0.06
South Africa (0)			
AMP	-	-	-
AMC	-	-	-
CXM	-	-	-
CEC	-	-	-
CPD	-	-	-
ERY	-	-	-
CIP	-	-	-
LVX	-	-	-
TET	-	-	-
CHL	-	-	-
SXT	-	-	-
TEL	-	-	-
Western Europe (34)			
AMP	0.12	0.12	0.12-0.12
AMC	0.12	0.12	0.12-0.12

CXM	0.5	0.5	0.12-2
CEC	0.5	1	0.5-1
CPD	0.12	0.25	0.06-0.5
ERY	0.25	0.25	0.25-0.25
CIP	0.03	0.06	0.015-0.06
LVX	0.03	0.06	0.03-0.06
TET	0.25	0.5	0.12-1
CHL	0.5	1	0.25-1
SXT	0.12	0.25	0.06-01
TEL	0.06	0.12	0.04-0.12

AMP, ampicillin; AMC, amoxicillin/clavulanate; CXM, cefuroxime; CEC, cefaclor; CPD, cefpodoxime; ERY, erythromycin; CIP, ciprofloxacin; LVX, levofloxacin; TET, tetracycline; CHL, chloramphenicol; SXT, sulfamethoxazole/trimethoprim; TEL, telithromycin.
MIC_{50} = MIC where 50% of the isolates were inhibited.
MIC_{90} = MIC where 90% of the isolates were inhibited.
Range = range of MICs.

Chapter 3

Dominance of CTX-M-2 and CTX-M-56 among extended-spectrum β-lactamases produced by *Klebsiella pneumoniae* and *Escherichia coli* isolated in hospitals in Paraguay

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Sir,

Extended-spectrum β-lactamases (ESBLs) have been reported from all the continents of the world including South America [1, 2]. However, there is currently a lack of knowledge regarding ESBL carriage in many South American countries, including Paraguay. Therefore, the aim of this study was to investigate the molecular characteristics and clonality of ESBL-positive *Klebsiella pneumoniae* and *Escherichia coli* isolates from Asunción, Paraguay.

One hundred and eight clinically relevant ESBL-positive *K. pneumoniae* (83) and *E. coli* (25) isolates, obtained from a total of 1006 *K. pneumoniae* (428) and *E. coli* (578), were collected during an 8 week period from April to June 2005 from 12 collaborating laboratories representing different major semi-public and private hospitals in the region of Asunción.

Isolates were initially tested for resistance to ceftazidime and cefotaxime using CLSI guidelines [3], and antibiotic susceptibility profiling was performed using the VITEK 2 system (bioMérieux, Marcy l'Éetoile, France). Among the 83 *K. pneumoniae* isolates tested, the proportions resistant to gentamicin, ciprofloxacin, piperacillin/tazobactam and co-trimoxazole were 75%, 72%, 99% and 55%, respectively. Among the 25 ESBL-positive *E. coli*, the proportions resistant to the same antibiotics were 72%, 68%, 96% and 56%. As well as resistance to ceftazidime and cefotaxime, 58% (48/83) of the Paraguayan ESBL-positive *K. pneumoniae* and 44% (11/25) of *E. coli* isolates were found to be resistant to both ciprofloxacin and gentamicin. Multidrug resistance (i.e. expression of both fluoroquinolone and aminoglycoside resistance) among ESBL-positive *K. pneumoniae* and *E. coli* is already well documented [4]. All of the ESBL-positive isolates tested were susceptible to imipenem.

Genotyping by PFGE revealed a lack of clonality, and a high degree of genotypic diversity in both *K. pneumoniae* and *E. coli* isolates, with no association between genotype and hospital of isolation, type of ESBL or associated antibiotic resistance profile (Figure 1).

PCR screening and sequencing of β-lactamase genes of the ESBL-positive isolates were performed using standard methods and primers [Table S1, available as Supplementary data at *JAC* Online (http://jac.oxfordjournals.org/)] [5]. With respect to the 83 ESBL-positive *K. pneumoniae* isolates, 83% produced CTX-M-2 and 11% produced CTX-M-56. Interestingly, both CTX-M-2 and CTX-M-56 belong to the same group of β-lactamase gene variants, the 'CTX-M-2 group'. Among the CTX-M-2 isolates, 88% also produced SHV-1 while 32% were positive for TEM-1, while 56% of the isolates producing CTX-M-56 were also positive for SHV-1 and TEM-1. Five (6%) ESBL-positive *K. pneumoniae* isolates lacked genes encoding CTX-M enzymes but were positive for SHV-2. Among the 25 isolates of ESBL-positive *E. coli*, 16 (64%) produced CTX-M-2 (10 of which also produced TEM-1) and 9 (36%) produced CTX-M-56, of which 8-produced TEM-1. These data show that 100% of clinical ESBL *E. coli* and 94% of clinical ESBL *K. pneumoniae* isolates cultured in Asunción, Paraguay carried CTX-M-2 group ESBL enzymes, which is higher than the previously published data from Brazil, Bolivia and Peru [1, 6, 7].

Plasmids were characterized using the method of Carattoli *et al.* [8]. Primer pairs used to detect A/C, FIA, I1, F1B and $F11_s$ replicons are listed in Table S1. Plasmid analysis revealed that 48% of *K. pneumoniae* isolates carried A/C plasmids, with 52%, 24% and 12% of *E. coli* isolates carrying A/C, FIA and both A/C and FIA plasmids, respectively. In this study, 44% of CTX-M-2-positive isolates and 78% of CTX-M-56-positive isolates were associated with possession of an A/C type plasmid. The association between CTX-M-56 (but not CTX-M-2) and A/C plasmid carriage was significant (Fisher's exact test P=0.01 versus P=0.1, respectively). To our knowledge, this is the first report associating CTX-M-56 ESBL resistance with A/C replicon type plasmid carriage. This study highlights the important role played by CTX-M enzymes in ESBL phenotype Enterobacteriaceae within Paraguayan hospitals.

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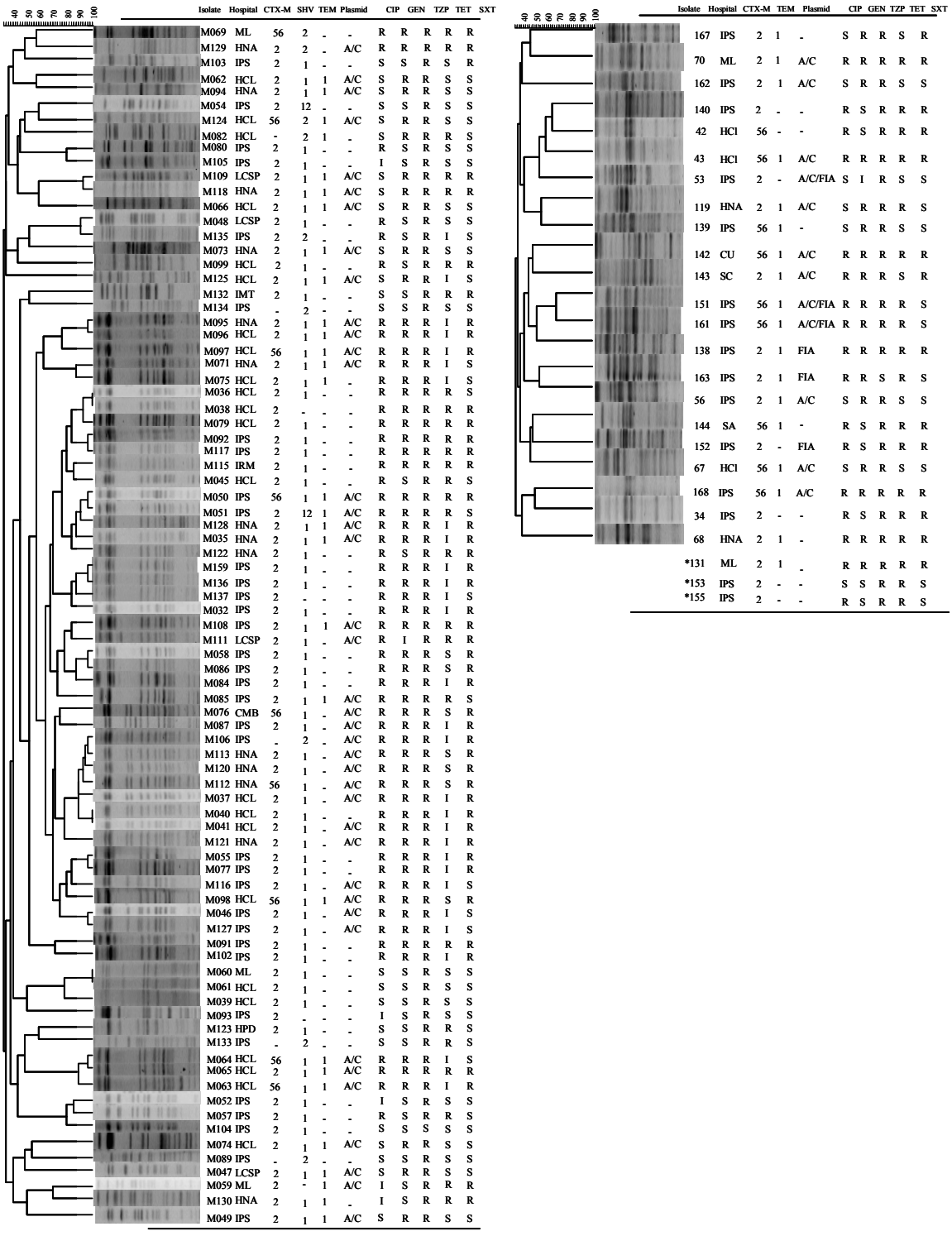

Figure 1. PFGE patterns obtained from 83 ESBL-positive *K. pnumoniae* (lef) and *E. coli* (right) isolates obtained from Paraguay during 8 weeks from April to June 2005. Cluster analysis was performed using the method of Dice with UPGMA ('unweighted pair group method with arithmetic mean'), with band tolerance set to 1.5%. All isolates were resistant to ampicillin, amoxicillin/clavulanic acid, cefotaxime, ceftazidime, cefazolin and piperacillin. All isolates were sensitive to imipenem. IPS, Instituto de Prevision Social; HNA, Hospital Nacional; HCL, Hospital de Clinicas; LCSP, Laboratorio Central de Salud Publica; IMT, Instituto de Medicina Tropical; IRM, Ineram; CMB, Centro Me´dico Bautista; HPD, Hospital General Pedia´trico; ML, Meyer Lab; CU, Laboratorio Curie; SC, Saint Carla; SA, Saint America; CIP, ciprofloxacin; GEN, gentamicin; TZP, piperacillin/tazobactam; TET, tetracycline; SXT, co-trimoxazole. *Non-typeable isolates.

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Supplementary data

Table S1. Primer sequences used for detection of different β-lactamase genes in *E. coli* and *K. pneumoniae* isolates

Primer	Nucleotide sequence (5'-3')	Target gene	Expected size
Bla-1 (fw)	ATAAAATTCTTGAAGACGAAA	TEM_{bla}	1079
Bla-2 (rev)	GACAGTTACCAATGCTTAATC		
TEM seq	TTACTGTCATGCCATCC	TEM	
TEM seq	AGAGAATTATGCAGTGC		
SHV-1 (fw)	GCCCGGGTTATTCTTATTTGTCGC	SHV	1017
SHV-2 (rev)	TCTTTCCGATGCCGCCGCCAGTCA		
CTX-M-1m (fw)	AAAAATCACTGCGCCAGTTC	CTX-M-1 group	415
CTX-M-1m (rev)	AGCTTATTCATCGCCACGTT		
CTX-M-2m (fw)	CGACGCTACCCCTGCTATT	CTX-M-2 group	552
CTX-M-2m (rev)	CCAGCGTCACATTTTTCAGG		
CTX-M-9m (fw)	CAAAGAGAGTGCAACGGATG	CTX-M-9 group	205
CTX-M-9m (rev)	ATTGGAAAGCGTTCATCACC		
CTX-M-8m (fw)	TCGCGTTAAGCGGATGATGC	CTX-M-8 group	666
CTX-M-8m (rev)	AACCCACGATGTGGGTAGC		
CTX-M-25m (fw)	GCACGATGACATTCGGG	CTX-M-25 group	327
CTX-M-25m (rev)	AACCCACGATGTGGGTAGC		
CTX-M-2 (fw)	TTAATGACTCAGAGCATT	CTX-M-2	899
CTX-M-2 (rev)	GATACCTCGCTCCATTTATTG		
I1 (fw)	CGAAAGCCGGACGGCAGAA	I1 plasmid	139
I1 (rev)	TCGTCGTTCCGCCAAGTTCGT		
FIA(fw)	CCATGCTGGTTCTAGAGAAGGTG	FIA plasmid	462
FIA(rev)	GTATATCCTTACTGGCTTCCGCAG		
FIB(fw)	GGAGTTCTGACACACGATTTTCTG	FIB plasmid	702
FIB(rev)	CTCCCGTCGCTTCAGGGCATT		
A/C(fw)	GAGAACCAAAGACAAAGACCTGGA	A/C plasmid	465
A/C(rev)	ACGACAAACCTGAATTGCCTCCTT		
FII_S (fw)	CTGTCGTAAGCTGATGGC	FII_S plasmid	270
FII_S (rv)	CTCTGCCACAAACTTCAGC		

Chapter 4

Important methodological considerations with respect to the differentiation of CTX-M-15 and CTX-M-28 extended-spectrum β-lactamases

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Dear Editor,

The acquisition and expression of β-lactamases by bacteria is a major health concern in the treatment of infectious disease, with the increase in carriage of extended-spectrum β-lactamases (ESBLs) being of particular concern. CTX-M β-lactamases constitute one family of the these rapidly disseminating ESBL enzymes, having been identified in numerous countries within Africa, Asia, Europe, South America and the USA, also including India [1, 2]. Phylogenic studies have revealed 5 major groups of acquired CTX-M enzymes, CTX-M-1, CTX-M-2, CTX-M-8, CTX-M-9 and CTX-M-25, with CTX-M-15 and CTX-M-28 (the subject of this letter) belonging to the CTX-M-1 group [3]. More specifically, CTX-M-15 has been frequently isolated from India [4], whilst CTX-M-28 is much less common, only recently having been identified in India and Tunisia [5, 6]. Further, these two enzymes only differ by a single amino acid substitution, which is present at the C-terminus of the protein (3'-end of the CTX-M gene), and it is usual to distinguish between these two CTX-M types (as well as other CTX-M types) using nucleotide sequencing (see GenBank accession numbers AY044436 and AJ549244).

In this respect, we would like to caution researchers to use at least one CTX-M flanking gene primer (3'-end) in order to obtain sequencing products that accurately differentiate between CTX-M-15 and CTX-M-28. In particular, if nucleotide polymorphisms exist in the PCR primers used to amplify CTX-M genes, and the resultant PCR products are subsequently sequenced, then these nucleotide polymorphisms will be present in any subsequent sequence data obtained from the original PCR product. For example, Achour *et al.* [5] used four CTX-M group-specific primer sets to screen for CTX-M genes, and then cloned and sequenced the resulting PCR products. However, the use of primers CTX-1F and CTX-1R generates a PCR product with 5'- and 3'- termini comprising CTX-M-28-specific sequences (Figure 1). Moreover, even if the gene present was actually CTX-M-15, subsequent PCR screening, cloning and sequencing would generate a CTX-M-28 sequence, due to the use of non-homologous primers. On the other hand, the use of CTX-M-15 specific primers and sequencing of subsequent PCR products will mis-identify any CTX-M-28 sequences (Figure 1). This may have been the case in publications by Moubareck *et al.* (2005) [6] and Weill *et al.* (2004) [7].

In fact, the mis-identification of CTX-M-15 and CTX-M-28 enzymes is due to the fact that the nucleotide sequences are very similar, with only 2 single nucleotide substitutions. Moreover, these sequence differences occur close to the 5'-end (position 21) and 3'-end (position 865) of the CTX-M gene, the 5'-end and 3'-end of genes usually being chosen as sites to design "specific" PCR screening primers (Figure 1).

We therefore caution researchers against using CTX-M gene 3'-end PCR screening primers to generate PCR product that will be later used in sequencing reactions, especially in regions where CTX-M- 15 and CTX-M-28 are present.

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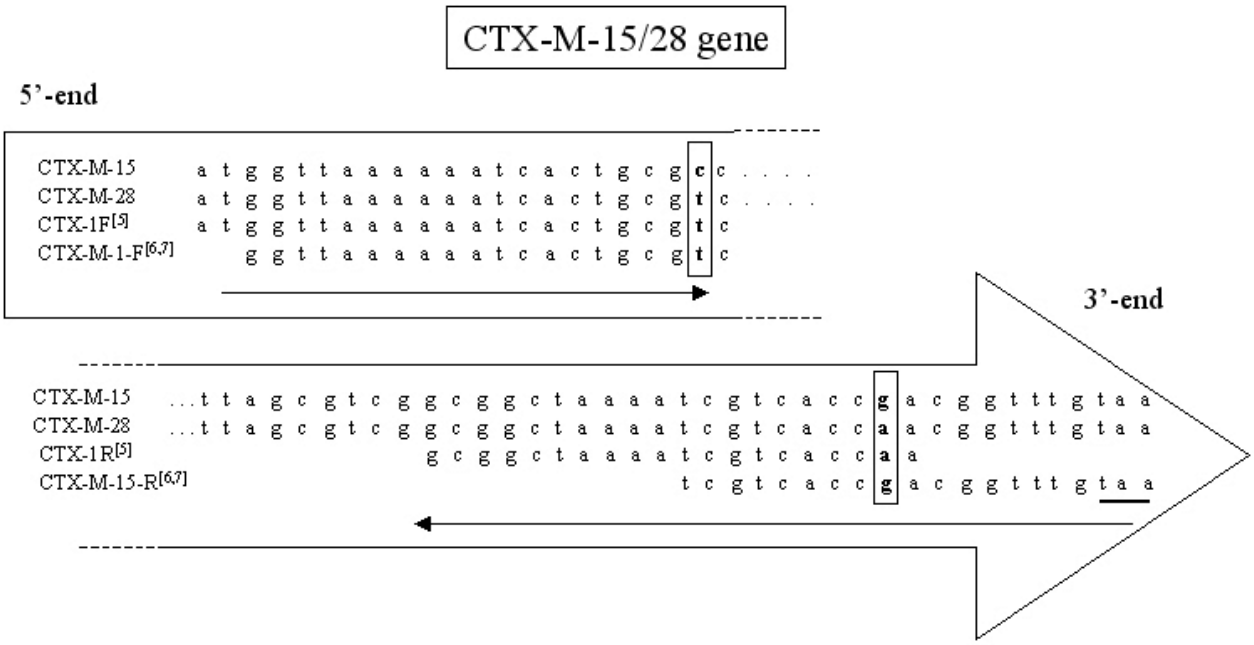

Figure 1. Problems associated with using non-homologous PCR primers to generate CTX-M sequencing products to differentiate between CTX-M-15 and CTX-M-28. CTX-M-15, reference CTX-M-15 sequence (GenBank Accession number AY044436). CTX-M28, reference CTX-M-28 sequence (GenBank Accession number AJ549244). CTX-1F and CTX-1R, PCR primers used by Achour *et al.* (2008) [5]. CTX-M-1-F and CTX-M-15-R, PCR primers used by Moubarek *et al.* (2005) [6] and Weill *et al.* (2004) [7]. Arrows represent direction of PCR amplification. Underlined nucleotides are the stop codon. The cytosine to thymidine nucleotide substitution (position 21) is silent. NB: The reverse primers have been reverse complemented to match the direction of the gene sequence.

Chapter 5

Molecular characterization of extended-spectrum β-lactamase producing *Klebsiella pneumoniae* isolated from blood cultures in Pondicherry, India

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Abstract

Background & objective: Extended-spectrum β-lactamase (ESBL)-producing strains of *Klebsiella pneumoniae* continue to cause major problems in hospitals in India. Molecular characterization of such ESBL-carrying isolates is essential in allowing hospitals to identify the source of these pathogenic bacteria, whilst providing useful information regarding the distribution of clonally related ESBL genotypes at both national and international levels. Further, monitoring of the spread of individual β-lactamase genes and their associated genetic platforms (like plasmids) provides a means to monitor for the appearance of new ESBLs, or to establish the dominance of older ESBL enzymes. In this respect, we characterized *K. pneumoniae* isolates associated with serious infections in a tertiary care hospital in Pondicherry, India, and compared the results to previous publications, focusing on India.

Methods: A total of 36 ESBL-phenotype and 3 non-ESBL phenotype *K. pneumoniae* isolates were characterized in this study. The isolates were obtained from blood cultures taken from 39 patients admitted to 8 different wards, at a single tertiary hospital in Pondicherry, India. Isolate characterization was performed using pulsed field electrophoresis (PFGE), ESBL gene screening, ESBL gene sequencing, and PCR-based plasmid profiling.

Results: Ninety-two percent (36/39) of isolates were positive for $bla_{\text{CTX-M-15}}$, 82% (32/39) for bla_{TEM}, 46% (18/39) for bla_{SHV}, and 82% (32/39) for bla_{OXA}. PFGE identified 3 major clusters of *K. pneumoniae* with no relationship between PFGE type and ward.

Interpretation & conclusions: A very high incidence of multiple ESBL-gene carriage was detected among the isolates, with the most notable result being the presence of CTX-M-15 in 92% of isolates. This study emphasizes the major role that CTX-M-15 plays in facilitating antimicrobial resistance in Indian hospitals, as well as its rapid spread throughout India, since its first identification in 1999.

Introduction

Extended-spectrum β-lactamases (ESBLs) are enzymes that confer resistance to all penicillins and cephalosporins, including the sulbactam and clavulanic acid combinations and monobactams such as aztreonam. ESBL production is seen predominantly in *Escherichia coli* and *Klebsiella* species (ESBL-EK) but may also be seen in other enterobacteriaceae [1]. ESBL producing *Klebsiella pneumoniae* were first reported in 1983 from Germany and since then a steady increase in resistance against cephalosporins has been seen. ESBLs are encoded by transferable conjugative plasmids, which also quite often code resistance determinants to other antimicrobials [2].

Currently, the ESBLs associated with third-generation cephalosporin-resistant (3GC-resistant) enterobacteriaceae can be divided into the 'big three' families of TEM, SHV and CTX-M- type β-lactamases. TEM and SHV variants are reliant on key amino acid substitutions to increase their substrate profile to include the 3GCs, whereas the CTX-Ms have an intrinsic extended-spectrum profile [3]. More than 100 ESBL variants from different types are known, the most abundant types are represented by SHV, TEM, OXA and CTX-M [4]. Production of these enzymes is either chromosomal or plasmid mediated [5]. The plasmid mediated nature of some of these enzymes facilitating their rapid spread among bacterial species and isolates, and is important for infection control, clinical and therapeutic applications [6].

Interestingly, ESBLs are most commonly detected in *K. pneumoniae,* it being an opportunistic pathogen causes severe infections in hospitalized patients, including immunocompromised hosts with severe underlying diseases [7]. Bloodstream infections associated with *K. pneumoniae* may arise as a consequence of pneumonia (community- and ventilator-acquired), the urinary tract, intra-abdominal pathologies, and central venous line-related infections. However, though associated with nosocomial infections worldwide, relatively little information is currently available regarding the molecular biology of ESBL positive *K. pneumoniae* isolates from India, the second most populated country in the world. In order to provide more information from this country, we performed molecular characterization of ESBLs from bloodstream isolates of *K. pneumoniae* collected from a tertiary care hospital in Pondicherry, India.

Materials and methods

Bacterial isolates

A total of 39 single blood culture isolates of *K. pneumoniae* were obtained from 39 patients admitted to 8 different wards at JIPMER (Jawaharlal Institute of Postgraduate Medical Education & Research), Pondicherry, India, during the year 2008. Blood culture was performed using biphasic medium consisting of Brain Heart Infusion (BHI) agar and BHI broth with sodium polyanethol sulphonate as an anticoagulant. *K. pneumoniae* were identified using standard microbiological procedures [8].

Antimicrobial susceptibility testing

The antimicrobial resistance profile of *K. pneumoniae* isolates was obtained using the disk diffusion method on Mueller Hinton agar (Hi-Media, Mumbai), following the zone size criteria recommended by the Clinical and Laboratory Standards Institute (CLSI) [9]. The antibiotics (μg) tested included ampicillin (10), amikacin, gentamicin, piperacillin (100),

piperacillin/ tazobactum (100/10), cefoperazone/ sulbactum (75/ 10), cefoxitin (30), cefotaxime (30), ceftazidime (30), ceftriaxone, ciprofloxacin (5) and meropenem (10).

ESBL screening and confirmation by phenotypic methods

Isolates showing resistance to one or more third generation cephalosporins (3GCs) were tested for ESBL production by the combination disk method using cefotaxime (CTX) (30 µg) cefotaxime / clavulanic acid (10 µg), cefatzidime (CAZ) (30 µg), and ceftazidime/clavulanic acid (10 µg). A ≥5mm increase in diameter of the inhibition zone of the cephalosporin-plus-clavulanate disc, when compared to the cephalosporin disc alone, was interpreted as phenotypic evidence of ESBL production (CLSI guidelines) [9]. *K. pneumoniae* ATCC 700603 was used as positive control and *Escherichia coli* ATCC 25922 was used as negative control.

PCR screening and sequence analysis

All the 39 isolates were subjected to molecular analysis. PCR screening and sequencing was performed to identify the β-lactamase resistance genes; bla_{TEM}, bla_{SHV}, bla_{OXA-1} group and bla_{CTX-M} as previously described [10-13]. Sequencing was performed using both forward and reverse PCR primers and standard methods on a 3130X1 Genetic Analyzer (ABI PRISM). The BLASTN program was used for database searching (http://www.ncbi.nlm.nih.gov/BLAST/). Additional sequencing primers were required for *bla*TEM PCR product sequencing (Lagging strand 7, 5'-TTACTGTCATGCCATCC-3' and Lagging strand 3, 5'-AGAGAATTATGCAGTGC-3'). PCR primers corresponding to sequences downstream (ORF 1) of the bla_{CTX-M} genes (M3 int upp, 5'-TCACCCAGCCTCAACCTAAG-3' and ORF1 pol M3, 5'-GCACCGACACCCTCACACCT-3' were also used [14]. Finally, PCR products of blaCTX-M were subjected to sequencing using primers, CTX-M-1 fw multi 5'-AAAAATCACTGCGCCAGTTC-3', CTX-M-1 multi (REV) F seq 5'-AACGTGGCGATGAATAAGCT-3' and ORF1 pol M3, 5'-GCACCGACACCCTCACACCT-3'. The presence of individual plasmid types was determined by PCR screening of FIA, FIB, FIIs, A/C and I1replicons [15].

Genotyping

Isolate genotyping was performed using pulsed field gel electrophoresis (PFGE) with *Xba*I restriction enzyme. Briefly, isolates were incubated overnight at 37^0C in 7mL Mueller Hinton broth. After incubation, 1mL of bacterial cells were harvested, pelletted and washed three times using 1ml EET (Na_2EDTA 100mM, EGTA 10mM, Tris HCl 1M) buffer, before being adjusted to a cell density of 0.5 at 560nm. A 100µL of cell suspension and 100µL of 1.4% PFGE grade agarose in EET buffer were mixed and poured into PFGE plug moulds. The plugs were incubated at 4^0C for 30 minutes to harden and 1ml of lysozyme (5mg/ml) was added before incubation at 37^0C for 3 to 4 hours. Lysozyme was removed from the plugs and 1ml each of deproteinising solution (per plug 3 mg/mL proteinase K and 1 % SDS) was added prior to overnight incubation at 37^0C. Next, the plugs were washed 6 times every 30 minutes with $T_{10}E_1$ (10mM Tris, 1mM EDTA) buffer, and then soaked in $T_{10}E_{0.1}$ (10mM Tris, 0.1mM EDTA) buffer for 30 minutes. Restriction digestion was carried out using 40U of *Xba*I (Fermentas) at 37^0C. PFGE was performed in 1% agarose gel in a CHEF DR-II system (BioRad) with the following conditions 0.5X Tris- Borate EDTA buffer, 14^0C, 6 V/cm for 22

h (with switch times ranging from 5 to 40 s). Lambda ladder PFGE marker (BioRad) was used as a molecular weight standard.

Results

Of the 39 isolates investigated, 37 (94.8%) were found to be resistant to at least one of the 3GCs. Among these 37 isolates, 36 (97.2%) were found to be ESBL positive by phenotypic testing. Antibiotic susceptibility revealed that majority of them was multidrug resistance showing 95%, 87%, 92% resistance to gentamicin, ciprofloxacin and ceftriaxone. Twenty-one percent showed resistance to amikacin and only 5% to meropenem.

Among the 39 isolates, 32 (82%) were positive for bla_{TEM}, 18 (46%) for bla_{SHV}, 36 (92%) for $bla_{\text{CTX-M}}$, and 32 (82%) for bla_{OXA}, respectively. Sequencing of $bla_{\text{CTX-M}}$ amplicons revealed the presence of $bla_{\text{CTX-M-15}}$ in all isolates.

Using PCR-based replicon typing, only a single isolate harbored both FIA and FIB replicons that carried $bla_{\text{CTX-M-15}}$. Plasmids harboring FIIs, A/C and I1 replicons types were not detected. Finally, PFGE identified 3 major clusters of *K. pneumoniae* with no relationship between PFGE type and ward (Figure 1).

Discussion

In recent years, a significant increase in ESBL producing *Klebsiella* spp. has been reported in India [16-20], with results from our Indian *K. pneumoniae* blood culture isolates adding extra evidence for this trend. For example, with respect to "classical" ESBL enzymes, in one study in north India 67% of *Klebsiella* isolates possessed both bla_{TEM} and bla_{SHV} genes, with 20% possessing a bla_{TEM} gene alone, and 8.4% a bla_{SHV} gene [21]. From our study, 44% of *K. pneumoniae* isolates carried bla_{TEM} and bla_{SHV} genes, 41% a bla_{TEM} gene only, and only 5.1% a bla_{SHV} gene. This difference was statistically significant (Chi square test $P<0.01$), though these results do not take into account the added association observed between bla_{TEM}, bla_{SHV} and $bla_{\text{CTX-M}}$ genes. However, the significance of this finding is not yet apparent, though geographical and chronological differences may have played a part in the different results obtained.

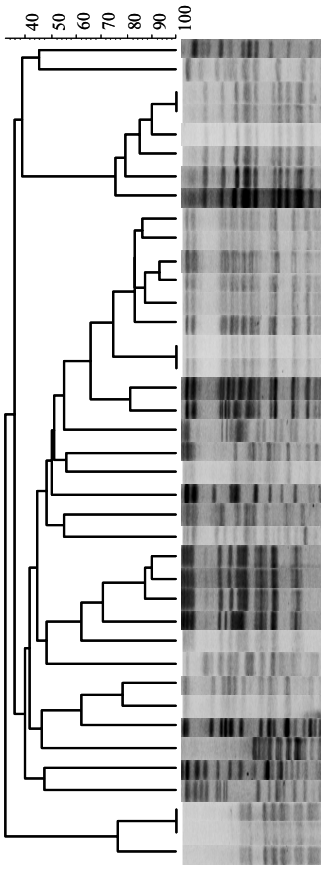
The most notable result from the study was the presence of CTX-M-15 in 92% (36/39) of isolates, as well as the combination of CTX-M-15 resistance and OXA resistance in 82% (32/39) of isolates. Further, 36% (14/39) of isolates possessed CTX-M, TEM, SHV, and OXA resistance. CTX-M-type ESBLs have become increasingly prevalent worldwide in the past 15 years [22, 23], with CTX-M-15-positive *K. pneumoniae* (first identified in India in 1999 [24] being recently found in Portugal [25], Korea [26] and Western Africa [27], as well as Bulgaria, Canada, France, Italy, Japan, Poland, Romania, Russia and Turkey [28]. Worryingly, 2 of our isolates (5%) possessed TEM, SHV, CTX-M, OXA and meropenem resistance genes, which seriously limits treatment options using extended-spectrum β-lactams and carbapenem antimicrobials, leaving tigecycline as the only clinically relevant antimicrobial agent for these isolates. Taken together, this study emphasizes the major role that CTX-M-15 plays in facilitating ESBL-mediated antimicrobial resistance in Indian hospitals, and reiterates its association with multiple antibiotic resistance determinants, including carbapenem resistance.

The *K. pneumoniae* isolates cultured in this study were found to possess a range of genotypes, and were not clonal in nature, though some dendrogram clustering of isolates was observed e.g. isolates 79 to 40, 27-86 etc in Figure 1. However, no relationship was observed between clustering and type of infection, or antibiotic resistance profile, except that 3 CTX-

M-15 positive isolates were genotypically distinct from the remaining (CTX-M-15 positive) isolates. The 2 meropenem resistant isolates belonged to the same genotypic cluster and hospital ward (NICU), possibly indicating noscomial transmission of this potentially lethal multi-antimicrobial resistant *K. pneumonia* strain. Therefore, surveillance and infection prevention measures should be considered as a priority to limit further nosocomial spread.

The extensive use of antibiotics has contributed greatly to the emergence of multidrug-resistant microbial isolates that possess the ability to cause nosocomial infections. The spread of antimicrobial resistance in *K. pneumoniae* isolates is complicating the treatment of serious nosocomial infection in India, not least because resistance in *K. pneumoniae* is typically caused by the acquisition of plasmids containing multiple antimicrobial resistances (including genes coding for ESBL resistance) [29]. Further, though carbapenems are currently considered to be the preferred agent for the treatment of serious infections caused by ESBL-producing *Klebsiella* strains, our study showed that 5% (2/39) of isolates were also resistant to meropenem, which causes great concern with respect to the choice of an effective treatment option, and with respect to the spread of combined ESBL and carbapenem resistant *K. pneumoniae* isolates within Indian hospitals.

Extended-spectrum β-lactamase (ESBL)-producing strains of *K. pneumoniae* continue to cause major therapeutic concerns in intensive care units, medical wards and surgical wards throughout India. Molecular characterization of such ESBL-carrying isolates is essential in allowing hospitals to identify the source of these pathogenic bacteria, whilst providing useful information regarding the distribution of clonally related ESBL genotypes at both national and international levels. Further, monitoring of the spread of individual β-lactamase genes and their associated genetic platforms (e.g. plasmids) provide a means to monitor for the appearance of new ESBLs, or to establish the dominance of older ESBL enzymes.



Isolate	Age	Unit	CE	CA	CF	G	AK	CI	M	A	TEM	SHV	CTX-M-15	OXA
28	20	MED	R	S	S	S	S	R	S	S	+		+	
83	35	CAS	R	S	S	S	S	I	S	S	+	+	+	+
74	NB	NICU	R	R	I	R	S	R	S	R	+		+	+
75	NB	NICU	R	R	S	R	S	R	S	R	+		+	+
46	NB	NICU	R	R	I	R	S	R	S	R	+	+	+	+
70	NB	NICU	R	R	R	R	S	R	S	R	+		+	+
23	NB	NICU	R	R	R	R	S	R	S	R	+		+	+
30	NB	NICU	R	I	I	R	S	R	S	R	+		+	+
79	NB	NICU	R	S	R	R	S	R	S	R	+		+	+
84	NB	NICU	R	I	R	R	S	R	S	R	+	+	+	+
8	NB	NICU	R	S	R	R	S	R	R	R	+	+	+	+
71	NB	NICU	R	R	R	R	S	R	S	R	+		+	+
77	NB	NICU	R	R	R	R	S	R	R	R	+	+	+	+
65	NB	NICU	R	I	R	R	S	R	S	R	+	+	+	+
52	NB	NICU	R	S	I	R	S	R	S	R	+	+	+	+
57	NB	NICU	R	S	S	R	S	R	S	R	+		+	+
34	NB	NICU	R	S	R	R	S	R	S	R	+		+	+
40	NB	NICU	R	S	R	R	S	R	S	R	+		+	+
43	40	SURG	R	R	I	R	S	R	S	R	+		+	+
20	7	PAED	I	R	R	R	S	R	S	R			+	+
87	NB	PICU	R	R	R	R	S	R	S	R		+	+	+
39	26	OG	R	R	R	R	R	R	S	R	+	+	+	+
25	48	MICU	R	R	R	R	S	R	S	R	+	+	+	+
80	30	MED	R	R	R	R	R	R	S	R	+		+	+
27	NB	NICU	R	R	R	R	S	R	S	R	+	+	+	+
29	NB	NICU	R	R	R	R	S	R	S	R	+	+	+	+
24	NB	NICU	R	R	I	R	S	R	S	R	+		+	+
37	NB	NICU	R	R	I	R	I	R	S	R	+	+	+	+
86	NB	NICU	R	R	R	R	I	R	S	R	+	+	+	+
66	NB	NICU	R	S	R	R	R	I	S	R	+	+	+	+
13	NB	MED	R	R	R	R	R	R	S	R		+	+	
85	NB	PICU	R	R	R	R	R	R	S	R			+	
31	NB	NICU	R	R	R	R	S	R	S	R			+	+
15	2	PAED	R	R	R	R	R	R	S	R			+	
35	12	NICU	R	R	R	R	S	R	S	R	+	+	+	+
42	NB	NICU	R	R	R	R	S	R	S	R	+	+	+	+
58	70	MEDI	R	S	R	R	S	S	S	R				
60	NB	NICU	S	S	R	R	S	S	S	R	+			
69	50	SURG	S	S	S	R	S	S	S	R	+			

Figure 1. Relationship between PFGE genotype and antimicrobial susceptibility profiles for *Klebsiella pneumoniae* isolates from Pondicherry. Antimicrobial drugs to which isolates were resistant are listed: Cefotaxime (Ce), ceftazidime (Ca), ciprofloxacin (Cf), gentamicin (G), amikacin (Ak), ceftriaxone (Ci), meropenem (M) and ampicillin (A). NB: new born. Unit: MED = medicine, CAS = casualty, NICU = neonatal intensive care unit, SURG = surgery, PAED = paediatrics.

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Chapter 6

Molecular characterization of antimicrobial resistance in non-typhoidal salmonellae from India

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Abstract

Objectives: Extended-spectrum cephalosporins and fluoroquinolones are essential antimicrobials for treating invasive salmonellosis, though emerging resistance to these antimicrobials is of growing concern, especially within India. Therefore, a study was set up to characterize the antimicrobial susceptibility phenotypes, types of ESBL genes and plasmids, and genotypic relationships of 23 non-typhoidal salmonellae isolated from patients admitted to four different hospitals in India.

Methods: Non-typhoidal *s*almonellae isolates were investigated using serotyping; antimicrobial susceptibility testing; PCR screening for β–lactamase genes, *qnr* genes, *aac (6')-Ib* and integrons; sequencing for quinolone resistance mutations; and plasmid replicon typing. An antimicrobial resistance microarray was used for preliminary screening and identification of bla_{TEM} & bla_{SHV} genes. Phenotypic testing for the presence of efflux pumps, as well as genotypic characterization, were also performed.

Results: Twelve out of 23 isolates possessed the cephalosporin resistance phenotype, and PCR screening revealed that isolates possessed TEM-1, SHV-12, DHA-1, OXA-1-like and CTX-M-15 ESBL genes. FIIS plasmid replicons were detected in the majority of isolates. The presence of efflux pumps was detected in 4 isolates resistant to ciprofloxacin. Isolates from the same geographical region were genotypically diverse, though some clustering of identical serotypes was observed.

Conclusions: A very high proportion (52%) of ESBL producing non-typhoidal salmonellae were isolated from 4 hospitals in India. Such pathogens could jeopardize classical antibiotic therapy. Continued surveillance for the presence of ESBL producing (non-typhoidal) salmonellae in India is essential.

Introduction

Non-typhoidal *s*almonellae are one of the principal pathogens implicated in food-borne gastroenteritis worldwide. In severe infections such as meningitis and septicemia, antibiotic treatment is essential, with extended-spectrum cephalosporins being preferentially used to treat for example salmonellosis in children [1, 2]. However, treatment failures due to the in-vivo acquisition of extended-spectrum β-lactamase (ESBL) or fluoroquinolone resistance genes in salmonellae isolates are now well established [3-5]. *Salmonella* spp. resistant to extended-spectrum cephalosporins have been known since 1988 [6] and may be isolated from hospitals in many different continents including Europe [7], North and South America [8], North Africa, India and South Korea [9, 10]. Salmonellae have been found to possess a wide variety of ESBL enzymes, including TEM, SHV, PER, CTX-M [11-13], as well as plasmid mediated AmpC enzymes (e.g. CMY, DHA, ACC-1) [14]. CTX-M enzymes are particularly important in facilitating the ESBL resistance phenotype and have been described in several *Salmonella* serotypes [15]. In fact, 12 out of more than 50 currently known CTX-M enzymes (which are divided into 5 major phylogenetic groups) have been identified in the *Salmonella* genus [16].

Fluoroquinolone resistance is also a growing problem in non-typhoidal salmonellae, with the first report of ciprofloxacin resistance in *Salmonella enterica* infection (eventually leading to treatment failure) being published in 1990 [17]. Since then, there have been reports of ciprofloxacin resistant isolates being found in many countries, including India, Pakistan, Vietnam and Spain [18-26].

Combined ESBL and fluoroquinolone resistance are major problems in the effective treatment of bacterial infections, both in the community and in the nosocomial setting. This problem is greatly increased when bacterial pathogens such as non-typhoidal salmonellae acquire both ESBL and fluoroquinolone resistance, as the consequences could be difficult for both livestock and in the community [27]. In order to manage and prevent the spread of antimicrobial resistance, both governments and clinicians require accurate information as to the prevalence and molecular mechanisms of antimicrobial resistance within currently circulating bacterial pathogens. At the present moment in time, this information tends to be lacking, especially in countries with large populations and unrestricted "over the counter" prescription policies, such as India. Therefore, this study was set up to investigate the extent of antimicrobial resistance, including ESBL and fluoroquinolone resistance, in non-typhoidal salmonellae originating from patients admitted to four hospitals in India. Information was also obtained regarding the circulating serotypes and genotypes of non-typhoidal salmonellae in order to relate these characteristics to specific antimicrobial phenotypes.

Materials and methods

Bacterial isolates and serotyping

Twenty-three isolates of non-typhoidal salmonellae were collected from patients attending four different hospitals in India (Pondicherry, Bangalore, Mangalore and Delhi) during 2007–2008. Non-typhoidal salmonellae were isolated from 19 stool cultures, 3 blood cultures and 1 CSF. Isolates were initially identified biochemically [28], followed by confirmation using specific antisera. Serovar determination was performed at the Rijksinstituut voor Volksgezondheid en Milieu (RIVM), Bilthoven, The Netherlands.

Antimicrobial susceptibility

Antibiotic susceptibility profiles were obtained against 38 antibiotics using the Vitek (bioMerieux) system. The MICs of ceftriaxone and ciprofloxacin were determined using the E-test (AB Biodisk). Isolates were confirmed as ESBL produces via the combination disk method using ceftazidime (30µg) and ceftazidime-plus-clavulanate (30µg plus 10µg) disks. A ≥5mm increase in diameter of the inhibition zone of the ceftazidime-plus-clavulanate disc, when compared to the ceftazidime disc alone, was interpreted as phenotypic evidence of ESBL production. AmpC β-lactamase production was measured using cefoxitin (zone diameter <18mm was considered AmpC positive). The AmpC disk test for plasmid mediated AmpC β-lactamase detection was carried out according to the procedure previously described [29]. For the detection of efflux pumps, a 2-step dilution series of the antibiotics nalidixic acid and ciprofloxacin was made to detect minimum inhibitory concentration (MIC) in the absence and presence of the efflux pump inhibitor PABN (phenyl-arginine-β-naphthylamide). MIC values were expressed as the highest dilution to inhibit growth, as determined by a lack of opacity in the respective wells of the microtitre plate used. All tests were performed according to CLSI guidelines where applicable [30].

PCR screening and sequence analysis

Isolates were initially screened for the presence of TEM and SHV β-lactamases using a commercially available antimicrobial resistance gene microarray (Check-Points BV). PCR screening and sequencing was performed to identify the β-lactamase resistance genes; bla_{TEM}, bla_{SHV}, bla_{OXA-1} group, bla_{CTX-M} and *amp*C as previously described [31-35]. The mechanism of quinolone resistance was determined by investigating mutations in the DNA gyrase *(gyrA* and *gyrB)* and DNA topoisomerase IV (*parC* or *parE*) genes [36-38]. Sequencing was performed using both forward and reverse PCR primers and standard methods on a 3130X1 Genetic Analyzer (ABI PRISM)**.** Additional sequencing primers were required for bla_{TEM} PCR product sequencing (Lagging strand 7, 5'-TTACTGTCATGCCATCC-3' and Lagging strand 3, 5'-AGAGAATTATGCAGTGC-3'). PCR primers corresponding to sequences downstream (ORF 1) of the *bla*CTX-M genes (M3 int upp, 5'-TCACCCAGCCTCAACCTAAG-3' and ORF1 pol M3, 5'-GCACCGACACCCTCACACCT-3' were also used [40]. Finally, PCR products of bla_{CTX-M} were subjected to sequencing using primers, CTX-M-1 fw multi 5'-AAAAATCACTGCGCCAGTTC-3', CTX-M-1 multi (REV) F seq 5'-AACGTGGCGATGAATAAGCT-3' and ORF1 pol M3, 5'-GCACCGACACCCTCACACCT-3'. Plasmid-mediated quinolone resistance, *qnr* was detected as previously described [40]. PCR screening for *aac (6')-Ib* was performed according to Robicsek *et al*., 2006 [41]. The presence of individual plasmid types was determined by PCR screening of I1, FIA, FIB, FIIs, A/C, HI1, Frep, K/B, and B/0 replicons [42]. The presence or absence of integrons was determined using 5'-CS and 3'-CS primers specific for the variable regions of integrons as described by Levesque *et al*. 1995 [43].

Iso-electric focusing

Isoelectric focusing of β-lactamase enzymes was performed using the following procedure. Briefly, an overnight culture of the relevant isolate was made in 5 mL Brain Heart Infusion (BHI) broth with 100 mg/L ampicillin. After overnight incubation the culture was diluted 20 times in 5mL fresh BHI and incubated at 37^0C for 4 hours and with shaking at 200rpm. The culture tubes were centrifuged for 5 minutes at 4000rpm at 4^0C, the pellet resuspended in 200µL of 50mM tris-HCl, pH 7.4. Five-microlitre lysozyme solution was added (40mg/mL), and the mix incubated for 60 minutes at 37^0C. After 60 minutes, 10 µL of

0.5 M EDTA was added and the mix incubated for a further 10 minutes at room temperature. The mix was finally centrifuged for 5minutes at 14000 rpm and the supernatant transferred to a clean tube [44]. The isoelectric point (pI) of the β-lactamase was determined by isoelectric focusing, applying the supernatants of crude cell extracts to Phast gels (GE HealthCare, Fairfield, CT, USA) with a pH gradient of 3–9 in a Phast system (GE HealthCare). Extended-spectrum β-lactamases (ESBLs) with known pI values (TEM-1, SHV-2) were included as pI markers.

Genotyping

Isolate genotyping was performed using pulsed field gel electrophoresis (PFGE) with 2 restriction enzymes *Xba*I and *Spe*I, as well as ERIC PCR. Briefly, isolates were incubated overnight at 37^0C in 7mL Mueller Hinton broth. After incubation, 1mL of bacterial cells were harvested, pelletted and washed three times using 1mL EET (Na_2EDTA 100mM, EGTA 10mM, Tris HCl 1M) buffer, before being adjusted to a cell density of 0.5 at 560nm. A 100µL of cell suspension and 100µL of 1.4% PFGE grade agarose in EET buffer were mixed and poured into PFGE plug moulds. The plugs were incubated at 4^0C for 30 minutes to harden and 1mL of lysozyme (5mg/mL) was added before incubation at 37^0C for 3 to 4 hours. Lysozyme was removed from the plugs and 1mL each of deproteinising solution (per plug 3 mg/mL proteinase K and 1% SDS) was added prior to overnight incubation at 37^0C. Next, the plugs were washed 6 times every 30 minutes with $T_{10}E_1$ (10mM Tris, 1mM EDTA) buffer, and then soaked in $T_{10}E_{0.1}$ (10mM Tris, 0.1mM EDTA) buffer for 30 minutes. Restriction digestion was carried out using 40U of *Xba*I or *Spe*I (Fermentas) at 37^0C. PFGE was performed in a 1% agarose gel in a CHEF DR-II system (BioRad) with the following conditions 0.5X Tris- Borate EDTA buffer, 14^0C, 6 V/cm for 22h (with switch times ranging from 5 to 40 s). Lambda ladder PFGE marker (BioRad) was used as a molecular weight standard. ERIC PCR was performed on extracted DNA using PCR primers ERIC-1: 5'-ATGTAAGCT CCTGGGGATTCAC-3' and ERIC-2: 5'-AAGTAATGACTGGGGTGAGCG-3' as described by Chmielewski *et al*. 2002 [45].

Results

The 23 non-typhoidal *Salmonella isolates* investigated comprised 6 serovars, namely *Salmonella* Agona (48%), *Salmonella* Typhimurium (26%), *Salmonella* Enteritidis (13%), *Salmonella* Senftenberg (4%), *Salmonella* Lexington (4%) and *Salmonella* Kirkee (4%). Serovars were not linked to a particular hospital. Three of the of 23 (13%) *Salmonella* isolates were determined to be resistant to the following; extended-spectrum cephalosporins (cefotaxime, ceftazidime, and ceftriaxone), a monobactam (aztreonam), and a cephamycin (cefoxitin), and amoxicillin/clavulanic acid. They exhibited intermediate resistance to piperacillin/tazobactam. The three isolates were phenotypically confirmed to be ESBL and AmpC producers.

Twelve of the 23 (39%) phenotypically confirmed ESBL positive isolates were resistant to extended-spectrum cephalosporins, and 4 of these isolates were also resistant to ciprofloxacin. Seven of the 23 (30%) isolates were resistant to nalidixic acid, with the MIC of 2 of the 4 ciprofloxacin resistant isolates (A15 and A13) being >64µg/mL, and the other two (B8 and B9) being 32µg/mL. At the species level, 2 of the *Salmonella* Agona isolates were resistant to extended-spectrum cephalosporins and ciprofloxacin. No resistance to carbapenems was recorded. Interestingly, 11 (92%) of the 12 ESBL-positive isolates belonged to the *S.* Agona serovar, with 9 (75%) of these 11 *S.* Agona isolates being genotypically clonal, and isolated from 2 different hospitals. ESBL Array analysis (Check-

Points BV) revealed the presence of 12 TEM and 11 SHV positives within the 23 isolates, with all TEM-positive isolates being TEM-1 positive, and all SHV isolates being either SHV12 or SHV5 positive. IEF experiments showed that the cell extracts of these isolates contained β-lactamases with a corresponding pI of 5.4 (the pI of TEM-1) and 11 β-lactamases with a corresponding pI of 8.2 (the pI of SHV-12). Of the 12 ESBL isolates, 12 (100%), 11 (91.6%), 3 (25.0%), 2 (16.7%) and 1 (8.3%) isolates were found to be TEM-1, SHV-12, DHA-1, OXA-1-like and CTX-M-15 positive, respectively. Additionally, 3 ESBL producers (25%) were co-producers of plasmid-mediated AmpC β-lactamase (Figure 1). Phenotypic evidence for the involvement of efflux pumps was detected in 4 isolates that were ciprofloxacin resistant. The range of mutations found in the DNA gyrase *(gyrA* and *gyrB)* and DNA topoisomerase IV (*parC* or *parE*) genes of the ciprofloxacin (quinolone) resistant isolates are shown in Table 1. A single ciprofloxacin resistant isolate harbored no mutations in the *gyrA* gene, but contained mutations in the QRDR (quinolone resistance determining region) region of the *parC* gene. All the isolates were negative for qnr gene and all 4 ciprofloxacin resistant isolates carried the *aac (6')-Ib* gene. Plasmids containing FIIS replicons were the most frequent plasmid types found (7/23 isolates), with multiple plasmid types being observed in 3 isolates only. Twelve isolates were negative for all of the replicons tested (Figure 1). There was no correlation between carriage of plasmid types and *Salmonella* serovar. Integrons were detected in 35% (8/23) of the isolates, all ESBL resistant, but were not associated with a particular serovar.

Though genotypic diversity was observed between the different serovars, some intra-serovar clonality was observed, particularly among similar serovars isolated from the same hospital (Figure 2). The results from the ERIC-PCR tended to correlate with the PFGE genotyping results. Finally, antibiotic resistance profiles between ESBL producers tended to be similar, with non-ESBL producing isolates being much more susceptible to a range of common, clinically useful, antibiotics. In contrast, ESBL producing isolates tended to be resistant to most antibiotics tested, except amikacin, ciprofloxacin, meropenem, nalidixic acid and piperacillin/tazobactam.

Discussion

In India, non-typhoidal salmonellae constitute approximately 20% of the *Salmonella* serovars currently circulating [46], and dissemination of isolates resistant to extended-spectrum β-lactamases is frequently observed. Indeed, in this particular study, 52% (12/23) of non-typhoidal salmonellae isolated harbored ESBL genes and exhibited an ESBL phenotype, representing a very high percentage of isolates. Though several different serovars were identified, the great majority of ESBL producers belonged to the *Salmonella* Agona serovar, which also represented the most abundant serovar isolated in this study. In fact, *S.* Agona was reported for the first time in Ghana in 1961 [47], though this particular serovar has since been reported in many countries worldwide in both humans and animals [48]. For example, in Brazil, *S.* Agona has been reported as the being the fourth most common *Salmonella* serotype isolated from non-human sources, and among the top 10 serotypes associated with human disease [49]. Further, multidrug-resistant *S.* Agona has been responsible for at least two hospital outbreaks in paediatric wards [50, 51]. In both cases, the strains were found to harbor large plasmids that conferred resistance to multiple antibiotics. At the current moment in time, there are very few publications that have investigated antibiotic resistance in non-typhoidal salmonellae [52]. From our results, it appears that *S.* Agona may also play an important role in non-typhoidal salmonellae ESBL-mediated resistance in India. More importantly, of particular concern is the fact that 2 isolates of *S.* Agona recovered in this

study possessed both ESBL and fluoroquinolone resistance genes, seriously limiting antimicrobial treatment options, and possibly providing a reservoir for the spread of this combination of antibiotic resistances to other pathogenic bacteria.

Eleven of the 23 non-typhoidal *Salmonella* isolates in this study were found to possess the SHV-12, ESBL phenotype and gene. The SHV-12 ESBL enzyme was first described in a *Klebsiella pneumoniae* strain isolated in Switzerland and differs from the SHV-1 enzyme by three amino acids substitutions [53]. It is currently one of the most common non-CTX-M ESBLs circulating and has been identified in many Gram-negative species, including *Salmonella* species [10, 54]. All of the 12 ESBL producers in our study also possessed the TEM-1 (non-ESBL) β-lactamase. TEM-1 is the most commonly encountered β-lactamase in the enterobacteriaceae, being able to hydrolyze narrow-spectrum penicillins and cephalosporins, such as cephalothin and cephaloridine [8].

Among the isolates studied, 3 were positive for DHA-1, a plasmid-mediated AmpC enzyme first identified in *Salmonella enteritidis* isolates from Saudi Arabia in 1992 [55], but is now reported in several other countries [56, 57]. Finally, a single isolate was positive for the CTX-M-15 ESBL enzyme. CTX-M-15 β-lactamase is now the most common CTX-M type reported worldwide, being first identified in enterobacterial isolates from India in 1999. The carriage of CTX-M-15 ESBL enzymes is a cause of concern for India [58].

The ESBL enzymes that tend to be responsible for cephalosporin resistance in the genus *Salmonella* are TEM and SHV β-lactamase derivatives, though CTX-M type ESBLs have recently (though infrequently) been reported. In a study by Rotimi *et al*., 3.4% of the *Salmonella s*pp. studied were found to be CTX-M-15 positive [59]. Further, the association of the CTX-M-15 gene with integrons, which can facilitate the rapid spread of resistance, is worrying [60], and studies have ascertained that most CTX-M gene-carrying plasmids possess replicons belonging to the incompatibility group (Inc) FII, or possess multi replicons of Inc FII associated with Inc FIA and FIB [61-65].

Replicon type FIIs plasmids are associated with *Salmonella* virulence [66]. In this study, FIIs replicons plasmids were found in 7 (30.4%), and B/O replicons in 3 (13%), of serovars. Both FIA and FIB replicons were positive in 3 (13.0%) of the isolates. Plasmids harboring I1, A/C, HI1, Frep, and K/B replicons types were not detected. Plasmids have been classified according to their incompatibility (Inc) with other plasmids [67], Inc typing is based on the fact that two plasmids sharing common replication and partitioning elements meaning that they are unable to proliferate stably within the same bacterium [67]. Sorting of plasmids into Inc groups is advantageous because specific plasmid types have been associated with virulence and/or antimicrobial resistance [63, 68-73].

Integrons are genetic agents known to harbor and spread antibiotic resistance determinants in pathogenic bacteria [59]. Interestingly, in our set of non-typhoidal salmonellae*,* all isolates containing integrons were ESBL producing isolates, and integrons have previously been associated with the carriage and spread of ESBL enzymes. The association of particular β-lactamase genes with class 1 integrons (*bla*$_{VIM}$, *bla*$_{IMP}$, *bla*$_{GES}$, *bla*$_{VEB}$, *bla*$_{CTX-M-2/-9}$, and *bla*$_{CMY}$) or by sharing the same plasmid (*bla*$_{TEM}$ and *bla*$_{SHV}$), has been reported in enterobacteriaceae [74-81], but the overall occurrence of integrons among ESBL-producing isolates has not been thoroughly analyzed [82, 83]. Therefore, the exact role of integrons in facilitating non-typhoidal *Salmonella* antibiotic resistance does require further investigation, not least in order to determine the importance of these mobile genetic elements in spreading antimicrobial resistance within the *Salmonella* genus.

The antimicrobial resistance ESBL Array (Check-Points BV) is a DNA micro-array system that offers accurate and rapid detection of ESBL genes. The use of such DNA micro-arrays for rapid genotypic detection of β-lactamases could be an important tool for monitoring the spread of ESBL resistance genes within the hospital setting of the future [84].

Genotyping of our non-typhoidal salmonellae revealed variation in PFGE profiles within identical serovars, and no conclusive association between hospital and genotype. However, it was interesting to note that *S.* Agona isolates from Delhi were all clonal, indicating that this particular *S.* Agona strain may be endemic within this region of India. Further, the fact that this genotype also carries ESBL resistance, means that further studies into the current extent of infection with this genotype, as well as its potential to spread into surrounding regions, should be more closely investigated.

The presence of ESBL and fluoroquinolone antibiotic resistance in non-typhoidal *Salmonella* isolates within resource poor countries is jeopardizing classical antibiotic therapy. Continued surveillance of circulating ESBL producing, as well as fluoroquinolone resistant, salmonellae is warranted throughout India.

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Table 1 . Mutations found in the DNA gyrase *(gyrA* and *gyrB)* and DNA topoisomerase IV (*parC* or *parE*) genes of 4 quinolone resistant non -typhoidal salmonellae from India.

Isolate No	g*yrA*	g*yrB*	p*arC*	p*arE*
A15	Ser 83 → Tyr Asp 87 → Gly	nd	Ser 80 → Ile	nd
A13	Ser 83 → Tyr Asp 87 → Gly	nd	Thr 57 → Ser Ser 80 → Ile	nd
B8	nd	nd	Thr 57 → Ser	nd
B9	Ser 83 → Le Asp 87 → Asn	nd	nd	nd

All 4 of the above isolates were resistant to ciprofloxacin. Two of the nalidixic acid sensitive control strains (included as negative controls) had no mutations (data not shown)
nd = mutations not detected

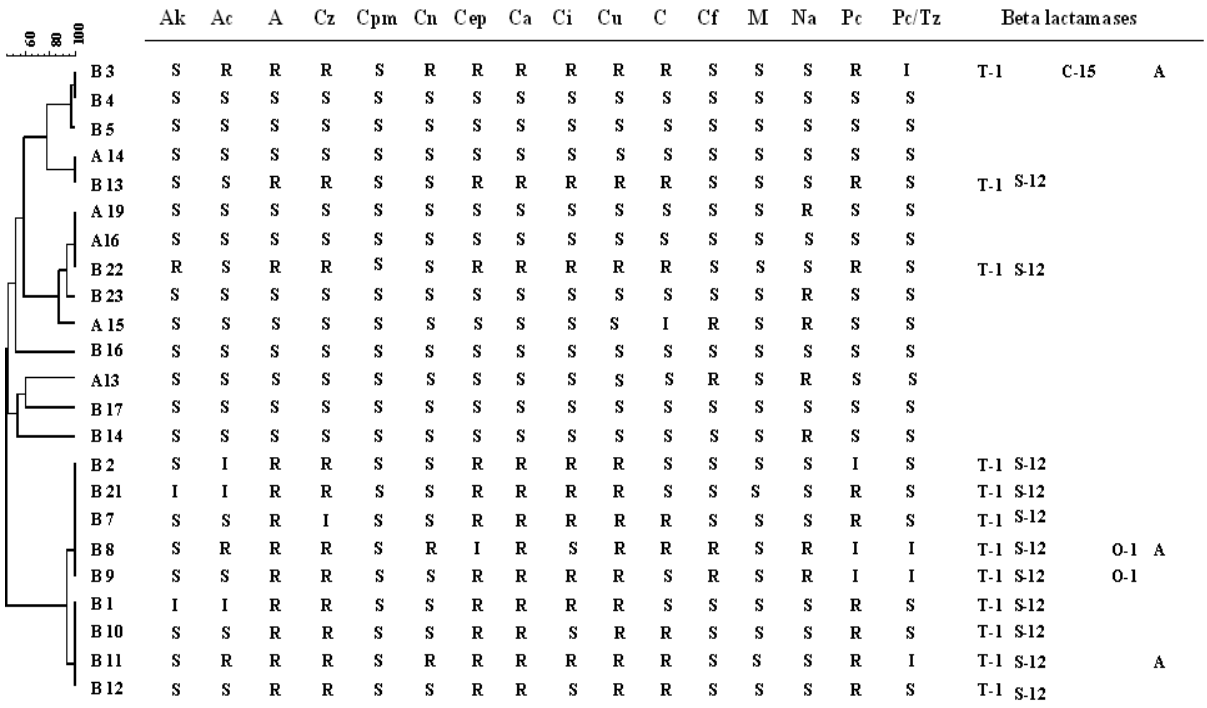
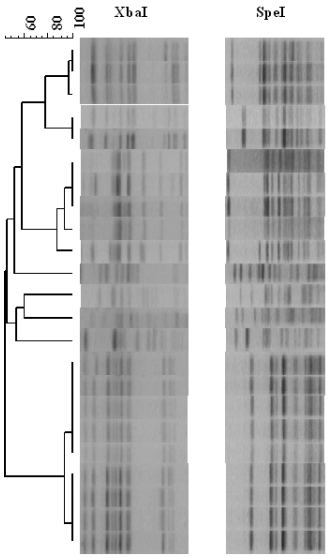


Figure 1. Relationship between PFGE genotype and antimicrobial susceptibility profiles for 23 non-typhoidal *Salmonella* isolates from India. Antimicrobial drugs to which isolates were resistant are listed: amikacin (Ak), amoxycillin/clavulanic acid (Ac), ampicillin (A), cefazolin (Cz), cefepime (Cpm), cefoxitin (Cn), cefpodoxime (Cep), ceftazidime (Ca), ceftriaxome (Ci), cefuroxime (Cu), chloramphenicol (C), ciprofloxacin (Cf), meropenem (M), nalidixic acid (Na), piperacillin (Pc), piperacillin/tazobacatam (Pc/Tz). T-1 = TEM-1, S-12 = SHV-12, C-15 = CTX-M-15, O-1= OXA-1 group, A = AmpC (DHA-1).



Isolate	Serovar	Place	Year	Beta-lactamases				Ep	Int	ERIC	Plasmids
B3	Typhimurium	P	2008	T-1		C-15	A		Int	C	FIA & FIB, B/O
B4	Typhimurium	P	2008							C	FIIS
B5	Typhimurium	P	2008							C	FIIS
A14	Typhimurium	M	2006							A	FIIS
B13	Agona	P	2007	T-1	S-12				Int	E	
A19	Agona	P	2007							H	FIIS
A16	Enteritidis	M	2006							B	FIIS
B22	Agona	P	2007	T-1	S-12					E	FIIS
B23	Enteritidis	P	2008							B	FIIS
A15	Enteritidis	M	2006					Ep		A	
B16	Lexington	B	2008							D	
A13	Senftenberg	M	2006					Ep		A	
B17	Kirkee	B	2008							G	
B14	Typhimurium	D	2007							F	B/O
B2	Agona	D	2008	T-1	S-12					G	
B21	Agona	D	2006	T-1	S-12					G	
B7	Agona	D	2006	T-1	S-12				Int	G	
B8	Agona	D	2008	T-1	S-12	O-1	A	Ep	Int	G	FIA & FIB
B9	Agona	B	2008	T-1	S-12	O-1		Ep	Int	I	FIA & FIB, B/O
B1	Agona	D	2005	T-1	S-12					G	
B10	Agona	B	2008	T-1	S-12				Int	G	
B11	Agona	D	2006	T-1	S-12		A		Int	G	
B12	Agona	D	2007	T-1	S-12				Int	G	

Figure 2. PFGE patterns of 23 non-typhoidal salmonellae obtained from India between 2005 and 2008, using restriction enzymes *Xba*I and *Spe*I. Cluster analysis was performed using the method of DICE with UPGMA with band tolerances set to 1.5%. All isolates were cultured from stool specimens apart from isolates B4 (CSF), A19, B5 and B13 (blood culture). Place: P = Pondicherry, M = Mangalore, B = Bangalore, D = Delhi. T-1 = TEM-1, S-12 = SHV-12, C-15 = CTX-M-15, O-1= OXA-1 group, A = AmpC (DHA-1), Ep = Efflux pump positive based on antibiotic testing (see methods), Int = Integrons PCR, ERIC = ERIC PCR type, Plasmids = Plasmids PCR (Carattoli *et al.,* 2005), FIA, FIB, FIIS, B/O = major plasmids incompatibility groups.

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Chapter 7

Analysis of VanA vancomycin-resistant *Enterococcus faecium* isolates from Saudi Arabian hospitals reveals the presence of clonal cluster 17 and two new Tn*1546* lineage types

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Abstract

Objectives: The aim of this study was to characterize 34 vancomycin-resistant VanA *Enterococcus faecium* isolates obtained from two hospitals in Saudi Arabia and to assess Tn*1546* variation within these isolates.

Methods: PFGE and multilocus sequence typing (MLST) genotypes, antibiotic susceptibility patterns, the presence of enterococcal surface protein (*esp*) and hyaluronidase (*hyl*) genes and conjugation frequencies were determined. In addition, Tn*1546* elements were characterized.

Results: PFGE and MLST analysis revealed the presence of 31 and 6 different genotypes, respectively. Further, three new ST types were discovered. Ninety-seven percent (33/34) of the isolates were associated with clonal complex 17 (CC17), with all isolates but one being resistant to ampicillin and all isolates being susceptible to linezolid. The *esp* and *hyl* genes were found in 44% (15/34) and 53% (18/34) of the isolates, respectively. Tn*1546* analysis revealed that the isolates belonged to five different groups, including two new lineages. The IS-element insertions described did not abolish the transfer of VanA resistance.

Conclusions: VanA vancomycin-resistant *E. faecium* isolates obtained from Saudi Arabian hospitals include CC17 MLST types, a clonal cluster associated with *E. faecium* nosocomial infection worldwide. Novel *E. faecium* MLST types are circulating in Saudi Arabia, as well as novel Tn*1546* types. It seems likely that CC17 *E. faecium* isolates may be distributed throughout the Middle East as well as Europe, America, Africa and Australia.

Introduction

Enterococci and vancomycin-resistant enterococci (VRE) are often found in the human and animal gut and have become increasingly responsible for nosocomial infections, particularly in the USA. In Europe, however, VRE have tended to be associated with community carriage and occasional nosocomial outbreaks, although the incidence of VRE infection may be changing [1]. Enterococci often express high-level resistance to glycopeptides and aminoglycosides, with vancomycin resistance having been promoted via the extensive use of vancomycin in hospitals, as well as the animal growth promoter avoparcin.

Six different vancomycin resistance types have so far been described in enterococci, namely VanA, VanB, VanC, VanD, VanE and VanG. The VanA type is characterized by both high-level and inducible resistance to vancomycin (MICs 64–1024 mg/L) and teicoplanin (MICs 16–512 mg/L) and has been shown to be directly facilitated by the carriage of transposon Tn*1546*, a transposon widely disseminated in humans, animals and the environment [2].

Currently, there is a distinct lack of data regarding the molecular analysis of VanA *Enterococcus faecium* Tn*1546* carriage in VRE isolates originating from the Middle East, including Saudi Arabia. Therefore, the aim of this study was to determine *E. faecium* genotypic variation and VanA Tn*1546* transposon diversity in VRE isolates recovered from the Kingdom of Saudi Arabia.

Materials and methods

Bacterial isolates

Thirty-four vancomycin-resistant *E. faecium* isolates were cultured from clinical specimens obtained from two large tertiary-care hospitals in the Kingdom of Saudi Arabia, namely the King Faisal Specialist Hospital and Research Centre, Riyadh (KFSH&RC), and the King Fahad National Guard Hospital, King Abdulaziz Medical City, Riyadh (KFNGH). Isolates were collected between 2000 and2003. Two pairs of isolates (25/29 and 17/32) were isolated from two different patients, but on separate occasions.

PFGE and multilocus sequence typing (MLST)

PFGE was performed using *Sma*I-digested fragments of bacterial chromosomal DNA, with fragment separation achieved in 0.8% agarose. Electrophoresis conditions comprised using a constant voltage of 6 V/cm at 148C and pulse times of 3.5–25 s increased linearly over 12 h (block 1), followed by 1–5 s increased over 8 h. Gel patterns were analysed using BioNumerics software (Applied Maths) with the band tolerance set at 1.0%. MLST was performed using internal fragments from seven housekeeping genes. Allele numbers and sequence types were assigned after reference to the online *E. faecium* MLST database at http://efaecium.mlst.net/.

Antibiotic susceptibility testing

Antibiotic susceptibility testing was performed using the VITEK 2 automated identification and susceptibility system (bioMérieux, Marcy l'Étoile, France) according to the manufacturer's instructions.

PCR characterization of Tn1546 elements and screening for virulence genes

Primer pairs used to characterize Tn*1546* were based on those published by *Miele et al*. [3]. In addition, primer walking was utilized to define insertion site-specific sequences. PCR screening for the enterococcal surface protein (*esp*) and hyaluronidase (*hyl*) virulence genes used three different primer pairs. For the *esp* gene, primer pairs esp11 (50-TTGCTAATGCTAGTCCACGACC-30)/esp12 (50-GCGTCAACACTTGCATTGCCGAA-30) and 14F (50-AGATTTCATCTTTGATTCTTGG-30)/12R (50-AATTGATTCTTTAGCATCTGG- 30) were used, and for the *hyl* gene, primer pair HYL n1 (50-ACAGAAGAGCTGCAGGAAATG-30)/HYL n2 (50-GACTGACGTCCAAGTTTCCAA-30). A touchdown PCR protocol was used for all primer pairs, comprising an initial annealing temperature of 65ºC, which was initially reduced by 18C per cycle over 15 cycles, and then followed by 20 cycles of amplification using an annealing temperature of 55ºC. All PCRs were performed using Taq DNA polymerase (EP0402, Fermentas).

Conjugation frequency

Conjugation frequency was determined using a standard methodology [4], with *E. faecium* GE1 being used as the recipient strain, and antibiotic concentrations of 6 mg/L vancomycin, 64 mg/L rifampicin and 10 mg/L fusidic acid being used. Conjugation frequencies were calculated with reference to the donor isolate.

Results and discussion

PFGE and MLST genotyping

PFGE identified 18 'possibly related' PFGE genotypes based on a 70% similarity cut-off value (approximately equivalent to four to six PFGE fragment differences) [5], with all isolates being genotypically related at a similarity of 34% (Figure 1). MLST genotyping revealed six ST types, with three new ST genotypes (ST 358, 359 and 360) and 59% (20/34) of the isolates being ST 17 genotypes. Further, the three new ST types (four isolates) and ST 16 (two isolates) were exclusively associated with the KFSH&RC, whereas ST 18 (eight isolates) was exclusively associated with the KFNGH. In contrast, ST 17 was recovered from both hospitals. Ninety-seven percent (33/34) of the isolates belonged to clonal complex 17 (CC17), a cluster of *E. faecium* MLST genotypes previously associated with global nosocomial infections [1]. CC17 has been previously associated with ampicillin resistance and carriage of the enterococcal surface protein pathogenicity island. In Saudi isolates, ampicillin resistance and *esp* gene carriage were found in 99% and 45% of the isolates, respectively, indicating that CC17 isolates from Saudi Arabia possess similar characteristics to those obtained from other well documented regions of the world.

Antibiotic susceptibility patterns

All isolates but one were resistant to vancomycin and ampicillin, and all isolates were susceptible to linezolid (Figure 1). No pattern was observed with respect to the general expression of multiple antibiotic resistances and PFGE genotype.

Characterization of Tn1546 elements

In total, five different Tn*1546* types were observed within our 34 *E. faecium* isolates (Figure 2). In eight isolates (numbers 2, 4, 5, 6, 12, 34, 35 and 41), the PCR amplicon size analysis revealed no major insertions or deletions, compared with theoretical amplicon products generated using reference Tn*1546* BM4147 sequence. In isolates where Tn*1546* polymorphisms were found, the majority of polymorphic events involved the insertion of IS*1216V* within the intergenic regions of either *vans* and *vanH* or *vanX* and *vanY* genes, along with other less frequent insertions of IS*1485* and IS*1251*. In fact, IS*1216V* is one of the most common insertion sequences found in Tn*1546*-bearing vancomycin-resistant enterococci worldwide. Further, IS*1251* has previously been demonstrated to be present in Tn*1546* transposons present within human clinical *E. faecium* isolates from Brazil, Korea, the USA, Norway and Ireland. Our Tn*1546* results allowed us to add two new lineages to a previously published evolutionary scheme [6], namely lineage III (IS*1485* insertion in *orf1*) and lineage IV (IS*1216V* insertion between *vanS* and *vanH*).

Prevalence of esp and hyl virulence genes

Using PCR screening, the enterococcal surface protein (*esp*) gene was found to be present in 44% (15/34) and the hyaluronidase (*hyl*) gene in 53% (18/34) of the isolates tested. *esp*-positive isolates made up 46% (10/22) of the isolates from the KFNGH and 41% (5/12) of the isolates from the KFSH&RC. In contrast, *hyl*-positive isolates made up 41% (9/22) of the isolates from the KFNGH but 75% (9/12) of the isolates from the KFSH&RC. Only four *esp*-positive isolates were also found to be *hyl*-positive, whereas 14 *hyl*-positive isolates were found to be *esp*-negative. A significant difference in the prevalence of *esp* and *hyl*-positive isolates was observed with respect to PFGE clusters (Figure 1), with 71% (10/14) of the *esp*-positives belonging to the cluster bordered by isolates 19 and 40 (Fisher's exact test P = 0.02), yet 75% (15/20) of the *hyl*-positives belonging to the cluster bordered by isolates 36 and 12 (Fisher's exact test P = 0.004). The distribution of the two hospitals between the PFGE clusters defined earlier was not significant (Fisher's exact test P = 0.066). The expression of enterococcal surface protein (Esp) has been associated with both biofilm formation and nosocomial infection in *E. faecium* [7], as well as CC17 isolates [8]. Further, a recent publication indicated that clinical *E. faecium* isolates carrying the *esp* gene possess an increased conjugation frequency with respect to the ability to acquire VanA vancomycin resistance [9]. The prevalence of the *esp* gene within our 33 CC17 isolates was somewhat lower than previously reported [1], although all isolates were ampicillin-resistant. However, 91% (30/33) of the CC17 isolates possessed either the *esp* or *hyl* virulence genes. The hyaluronidase protein (Hyl) is a virulence trait also associated with clinical *E. faecium* isolates, with a ratio of 2:1 for *esp:hyl*-positive isolates reported in a sample of 577 isolates worldwide [10]. This compares with a ratio of 1:1.2 in our smaller group.

Conjugation frequency

Conjugation frequencies of representative isolates from each transposon group ranged from 2.2×10^{-4} to 1.9×10^{-6}. These frequencies lie within the range of previously published transformation frequencies observed for other global VanA-resistant, Tn*1546*-carrying *E. faecium* isolates.

Conclusions

In this study, we investigated 34 vancomycin-resistant VanA *E. faecium* isolates cultured from two hospitals situated in Riyadh, Saudi Arabia. The majority of the isolates

tested belonged to CC17 and were resistant to ampicillin, suggesting that *E. faecium* MLST types associated with nosocomial infection are also circulating in the Middle East. The Tn*1546* analysis revealed two new lineages, including two previously undescribed IS*1485* and IS*1216V* insertions.

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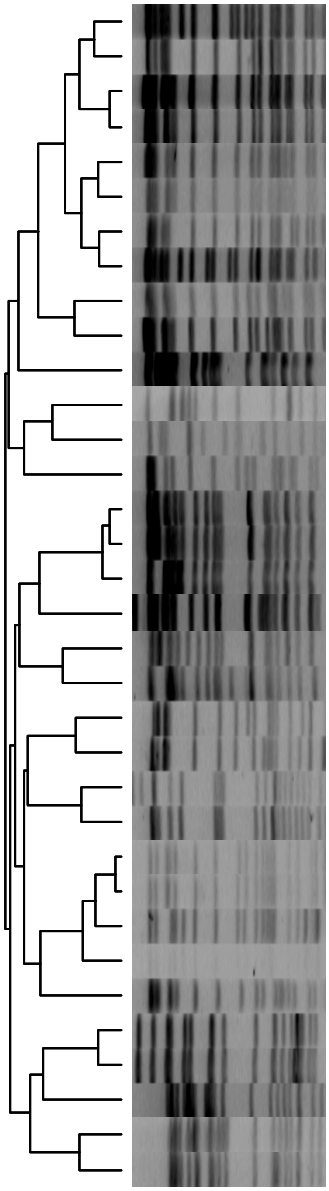

Isolate	Hosp.	*esp*	*hyl*	VAN	AMP	GEN	TET	CIP	Q/D	LZD	MLST
19	N	+	-	R	R	R	R	R	S	S	17
23	N	-	-	R	R	R	R	R	S	S	17
16	N	+	-	R	R	R	R	R	S	S	17
17	N	+	-	R	R	R	R	R	S	S	17
28	N	+	-	R	R	R	R	R	S	S	17
29	N	+	-	R	R	R	R	R	S	S	17
26	N	+	+	R	R	R	R	R	S	S	17
32	N	+	-	R	R	R	R	R	S	S	17
25	N	+	-	R	R	R	R	R	S	S	17
27	N	-	-	R	R	R	R	R	S	S	17
33	N	-	+	R	R	S	S	R	S	S	18
1	K	-	-	R	R	S	R	I	I	S	358
41	K	+	+	R	R	R	R	R	S	S	360
40	N	+	-	R	R	R	R	R	S	S	17
36	N	-	+	R	R	S	S	R	S	S	18
37	N	-	+	R	R	S	S	R	S	S	18
35	N	-	+	R	R	S	S	R	S	S	17
39	N	-	+	R	R	S	S	R	S	S	18
30	N	-	+	R	R	S	S	R	S	S	18
38	N	-	+	R	R	S	S	R	S	S	18
6	K	+	+	R	R	R	R	R	S	S	359
9	K	+	+	R	R	R	R	R	S	S	359
14	N	-	-	R	S	R	R	I	S	S	18
18	N	-	-	R	R	R	R	R	S	S	18
7	K	-	+	R	R	R	R	R	S	S	17
10	K	-	+	R	R	R	R	R	S	S	17
11	K	-	+	R	R	R	R	R	S	S	17
8	K	-	+	R	R	R	R	R	S	S	17
13	N	+	-	R	R	R	R	R	S	S	17
4	K	+	-	R	R	S	S	R	S	S	16
5	K	+	-	R	R	S	S	R	S	S	16
34	N	-	+	R	R	R	S	R	S	S	17
2	K	-	+	R	R	R	S	R	S	S	17
12	K	-	+	R	R	S	S	I	S	S	17

Figure 1. PFGE patterns obtained from 34 vancomycin-resistant *E. faecium* isolates from Saudi Arabia. Cluster analysis was performed using the method of DICE with UPGMA with band tolerances set to 1.0%. Hosp. = hospital, where K = King Faisal Specialist Hospital and Research Centre and N = King Fahad National Guard Hospital. *esp,* enterococcal surface protein PCR; *hyl*, hyaluronidase PCR; AMP, ampicillin; GEN, gentamicin; TET, tetracycline; Q/D, quinupristin/dalfopristin; LZD, linezolid; CIP, ciprofloxacin; R, resistant; I, intermediate resistance; S, susceptible.

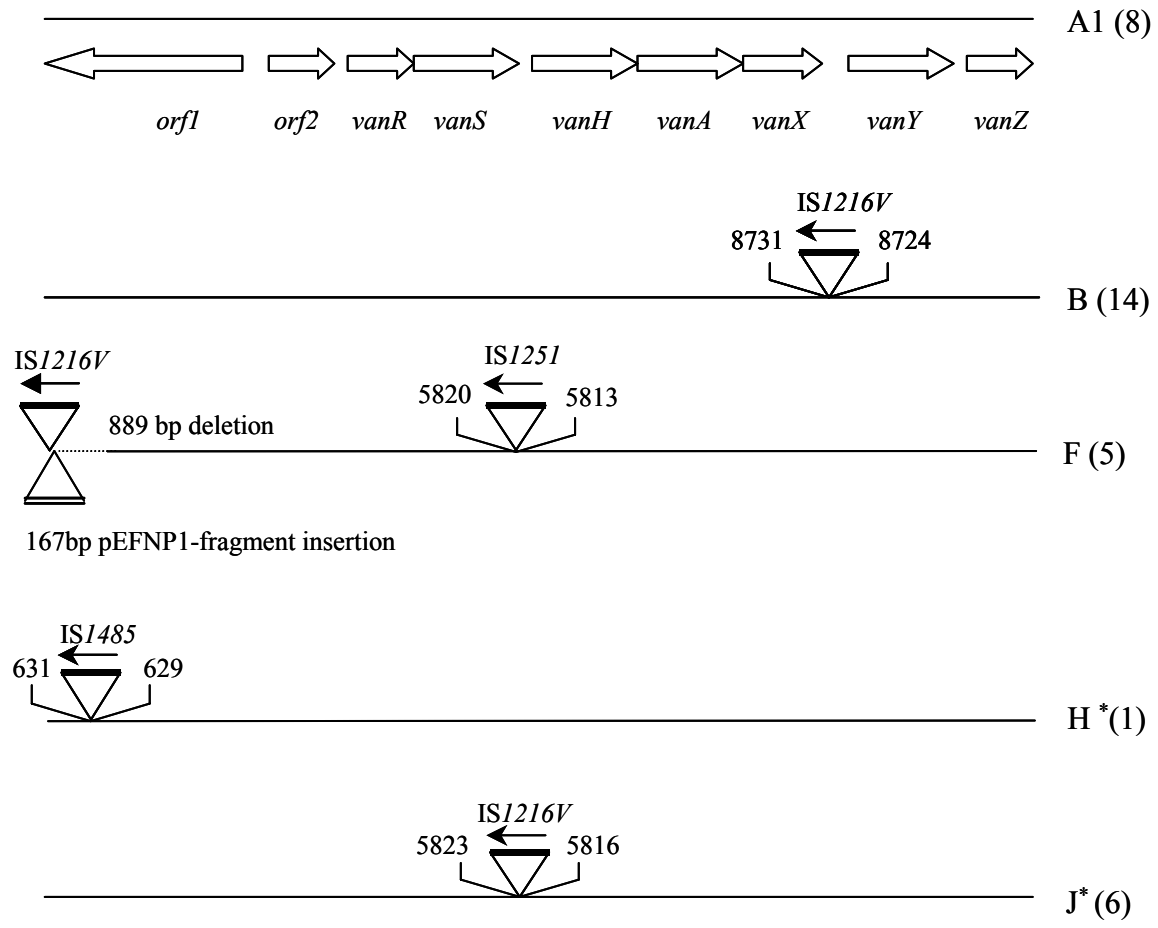

Figure 2. Genetic maps of Tn*1546* types associated with 34 *E. faecium* isolates originating from Saudi Arabia. The unbroken black lines represent reference Tn*1546* sequence BM4147. Unfilled block arrows represent the positions of genes and open reading frames (*orf1* and *orf2)*. Filled boxes represent IS elements and arrows their direction of transcription. Dotted lines represent deletions. The unfilled box represents a sequence similar to plasmid pEFNP1 (Losteinkit *et al*., 2000; GenBank accession number AB038522, unpublished results). The positions of the first nucleotides upstream and downstream of the insertion sequence are shown. Group definitions (right-hand side) are based on those published by Schouten *et al.* [6]. Asterisk indicates novel Tn*1546* group. Numbers in parentheses indicate how many isolates belonged to each Tn*1546* grouping.

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Chapter 8

High prevalence of ST-78 infection-associated vancomycin-resistant *Enterococcus faecium* from hospitals in Asunción, Paraguay

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Abstract

Forty infection-associated VanA-type vancomycin-resistant *Enterococcus faecium* (VRE) strains obtained from five collaborating hospitals in Asunción, Paraguay were investigated. Genotyping using pulsed-field gel electrophoresis (PFGE) and multilocus sequence typing revealed the presence of 17 cluster types and four STs, with 93% (37/40) of isolates comprising ST type 78. Other ST types included ST-132, ST-210 and one new ST type (ST-438). All but one isolate (ST-438) were associated with clonal complex 17 (CC17), and 97% of the total isolates carried the *esp* gene. Three Tn*1546* variants were found, including a new lineage containing an IS*Efa5* insertion in an existing IS*1251* element.

Vancomycin-resistant Enterococci (VRE) are nosocomial pathogens widely disseminated within hospitals [1], with the *vanA* genotype being the most common type of acquired glycopeptide resistance found in these enterococci. Although the first clinical isolate of VRE was detected in Europe in 1986, studies have now shown that clinical VRE may be isolated in several world regions [2]. Further, it is currently accepted that a single focal group of *vanA*-type VRE (clonal complex 17 or CC17) is particularly associated with nosocomial infections [3]. However, there is some evidence to suggest that this grouping may be influenced by the high rates of recombination associated with *E. faecium* [4].

In 1988, French researchers identified a small mobile genetic element in VanA-type *Enterococcus faecium* called *Tn1546*, which is involved in vancomycin resistance [5], and there are currently five other acquired vancomycin resistance genotypes reported in enterococci, namely *vanB, vanD*, *vanE*, *vanG* and *vanL* [5, 6].

Currently, there exist limited data regarding the incidence and types of clinical vancomycin-resistant *E. faecium* from countries in South America. Therefore, the objective of this study was to investigate the complexity of clinical *E. faecium* isolates from Paraguay, by determining *E. faecium* genotypes and VanA Tn*1546* transposon diversity, and to compare the results with isolates from both South America and the rest of the world.

Two hundred and twenty-one VanA vancomycin-resistant *E. faecium* isolates were collected between January 2005 and March 2007 from five collaborating hospitals in Asunción Paraguay. These five hospitals, the Instituto de Previsión Social (IPS), Hospital de Clinicas (HCL), Hospital Nacional (HNA), Centro Medico de Bautista (CMB) and Instituto de Medicina Tropical (IMT), are representative of the national healthcare system in Paraguay. A subset of 40 isolates was chosen for further analysis, based on their site of isolation and association with disease. These infection-associated isolates were cultured from abscess material, blood, tissue and needle aspirates, representing 7% (7/97), 20% (1/5) and 27% (32/117) of the isolates obtained from HCL, HNA, and IPS, respectively. No infection-associated isolates were obtained from CMB (0/1) and IMT (0/1) hospitals.

Antibiotic resistance profiling was performed using the VITEK 2 system (BioMérieux, Marcy l'Étoile, France) and E-test (amoxicillin/clavulanic acid), according to CLSI guidelines. All 40 infection-associated isolates tested in this study were resistant to ampicillin, amoxicillin/clavulanic acid, ciprofloxacin, erythromycin, imipenem, penicillin, teicoplanin and vancomycin. All isolates were sensitive to linezolid and tetracycline. Forty-three per cent (17/40) of the isolates were resistant to gentamicin (Figure. 1).

The enterococcal surface protein gene (*esp*) was detected by PCR screening [7], revealing that all isolates were *esp* positive with the exception of one isolate (ST132). The prevalence of *esp* in these isolates is higher than previously reported for European CC17 isolates [8].

Pulsed-field gel electrophoresis (PFGE) and multi locus sequence typing (MLST) genotyping were performed as previously described [9]. PFGE results revealed 17 cluster types with no identifiable yearly pattern per cluster. Cluster analysis was performed using the method of DICE with UPGMA, with band tolerance set at 1.5% and the threshold cut-off value at 80% [10, 11]. The results of MLST genotyping revealed three known (ST-78, ST-132 and ST-210) and one novel (ST-438) ST type, with 93% (36/40) of isolates comprising ST-78 genotypes. These ST-78 isolates comprised 100% of all infection-associated isolates from the HCL and HNA hospitals, and 87% (28/32) of the isolates from the IPS. Although this ST type has previously been reported in Europe, it has not been reported in South America, the USA or Spain, approximately 95% of Paraguayans are of mixed Spanish and Amerindian descent. Therefore, the origins of ST-78 in Paraguay remain unexplained, although it appears that the ST-78 strain has spread to many hospitals in Asunción.

In total, 97% (39/40) of the isolates tested belonged to MLST clonal complex 17 (CC17), which comprises a cluster of *E. faecium* genotypes previously associated with

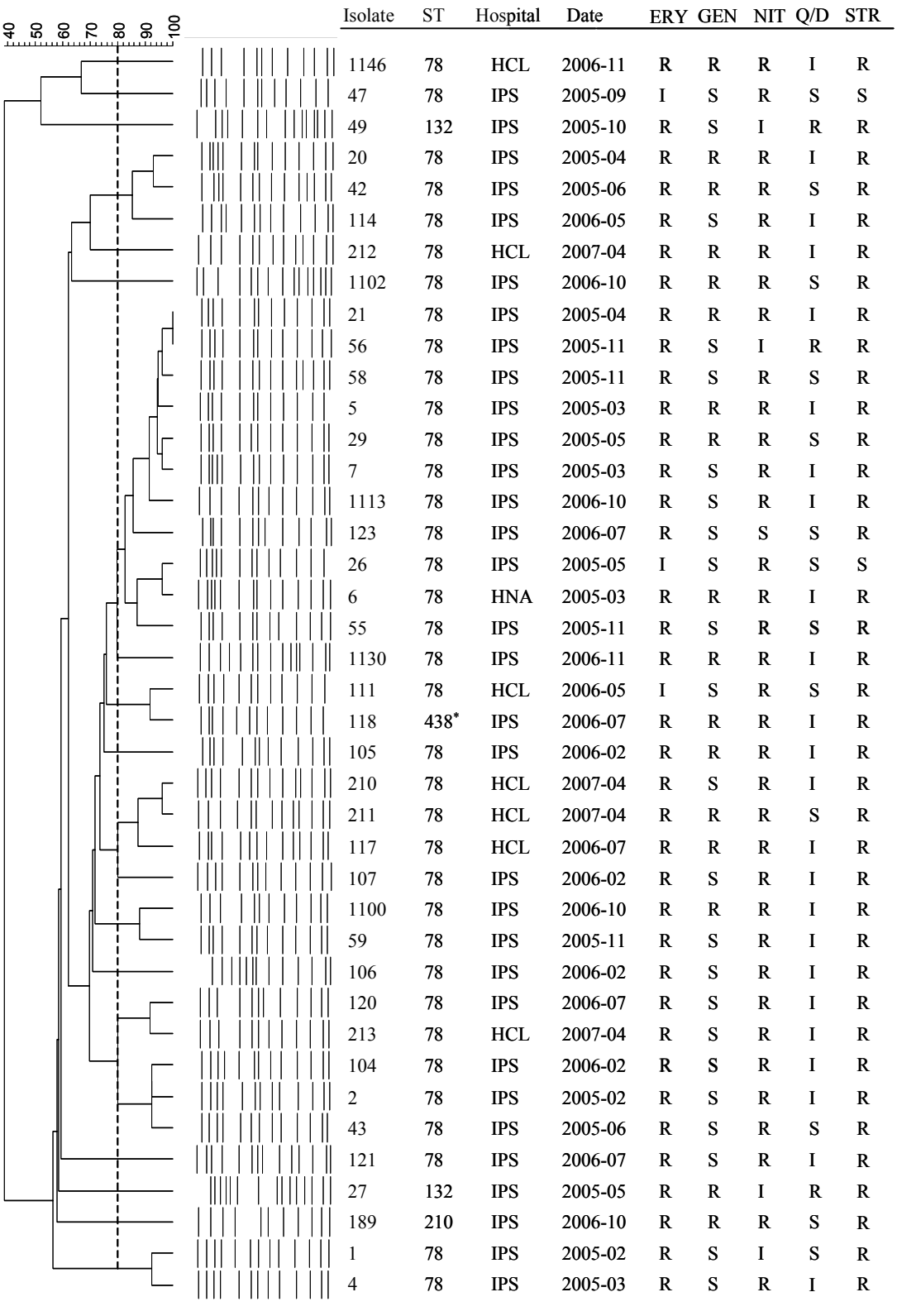
hospital infections [3, 12]. ST-438 was the only non-CC17 isolate recovered. Further analysis revealed no association between the antibiotic resistance profile and PFGE or MLST genotypes (Figure. 1).

Characterization of Tn*1546* was performed using previously published primers [13], and a touchdown PCR thermocycling protocol [7]. PCR primer walking and sequencing were used to define insertion site-specific sequences. The results of Tn*1546* characterization revealed three different variants (Figure. 2). Two of the isolates did not show any insertion or deletion compared with the classic Tn*1546* (BM4147) transposon sequence [14]. In contrast, 92% (37/40) of the isolates carried a Tn*1546* variant containing an insertion of IS*1216V* (and a resultant deletion event) in *orf1,* coupled to an IS*1251* insertion in the intergenic region of *vanS* and *vanH* (Tn*1546* type F1 described by Willems *et al*. [15]). A single isolate with a new MLST genotype (ST-438) carried a novel Tn*1546* variant of type F1 containing an IS*Efa5* insertion. This IS element has only previously been described in Brazilian *E. faecium* isolates, where the IS*Efa5* was exclusively inserted in the intergenic region of *vanX* and *vanY* [16]. In this ST-438 Paraguayan isolate the IS*Efa5* element was inserted in the left flanking region of another insertion element IS*1251*, which itself was situated in the intergenic region between *vanS* and *vanH*. Sequencing of the IS*Efa5* flanking insertion regions showed an inverted repeat of 6 bp (AGAGCC) flanked by an 8 bp direct repeat (AGAATAAT) of the IS*1251* insertion element. From current evidence, presence of the IS*Efa5* element in Tn*1546* appears to be specific for *Enterococcus* isolates originating in South America. Further, this unusual insertion event indicates that Tn*1546* may still be evolving in South American E. faecium isolates.

This is the first report of molecular investigations into vancomycin-resistant *E. faecium* from Paraguay. The results from 40 infection-associated VRE isolates obtained from hospitals in Asunción revealed that the majority of isolates were found to be MLST type ST-78, a genotype associated with MLST clonal complex 17 (hospital-associated) isolates. The majority of isolates also carried the same Tn*1546* structural variant (type F1), indicating that this combination currently represents the majority of infection-associated VanA *E. faecium* isolates currently circulating in hospitals in Asunción, Paraguay [15]. Also, although relatively little information is currently available, there appears to be an association between Tn*1546*/IS*Efa5* and enterococcal isolates circulating in South America.

Acknowledgements

The authors would like to acknowledge W. Goessens and E. Dullart of the routine microbiology laboratory of the Erasmus MC and N Zarate from Laboratorio Central de Salud Publica, Asunción, Paraguay for their assistance.



Isolate	ST	Hospital	Date	ERY	GEN	NIT	Q/D	STR
1146	78	HCL	2006-11	R	R	R	I	R
47	78	IPS	2005-09	I	S	R	S	S
49	132	IPS	2005-10	R	S	I	R	R
20	78	IPS	2005-04	R	R	R	I	R
42	78	IPS	2005-06	R	R	R	S	R
114	78	IPS	2006-05	R	S	R	I	R
212	78	HCL	2007-04	R	R	R	I	R
1102	78	IPS	2006-10	R	R	R	S	R
21	78	IPS	2005-04	R	R	R	I	R
56	78	IPS	2005-11	R	S	I	R	R
58	78	IPS	2005-11	R	S	R	S	R
5	78	IPS	2005-03	R	R	R	I	R
29	78	IPS	2005-05	R	R	R	S	R
7	78	IPS	2005-03	R	S	R	I	R
1113	78	IPS	2006-10	R	S	R	I	R
123	78	IPS	2006-07	R	S	S	S	R
26	78	IPS	2005-05	I	S	R	S	S
6	78	HNA	2005-03	R	R	R	I	R
55	78	IPS	2005-11	R	S	R	S	R
1130	78	IPS	2006-11	R	R	R	I	R
111	78	HCL	2006-05	I	S	R	S	R
118	438*	IPS	2006-07	R	R	R	I	R
105	78	IPS	2006-02	R	R	R	I	R
210	78	HCL	2007-04	R	S	R	I	R
211	78	HCL	2007-04	R	R	R	S	R
117	78	HCL	2006-07	R	R	R	I	R
107	78	IPS	2006-02	R	S	R	I	R
1100	78	IPS	2006-10	R	R	R	I	R
59	78	IPS	2005-11	R	S	R	I	R
106	78	IPS	2006-02	R	S	R	I	R
120	78	IPS	2006-07	R	S	R	I	R
213	78	HCL	2007-04	R	S	R	I	R
104	78	IPS	2006-02	R	S	R	I	R
2	78	IPS	2005-02	R	S	R	I	R
43	78	IPS	2005-06	R	S	R	S	R
121	78	IPS	2006-07	R	S	R	I	R
27	132	IPS	2005-05	R	R	I	R	R
189	210	IPS	2006-10	R	R	R	S	R
1	78	IPS	2005-02	R	S	I	S	R
4	78	IPS	2005-03	R	S	R	I	R

Figure 1. Pulsed-field gel electrophoresis (PFGE) patterns obtained from 40 infection-associated vancomycin-resistant *Enterococcus faecium* isolates obtained from Paraguay between 2005 and 2007. Cluster analysis was performed using the method of DICE with UPGMA with band tolerance set to 1.5%.

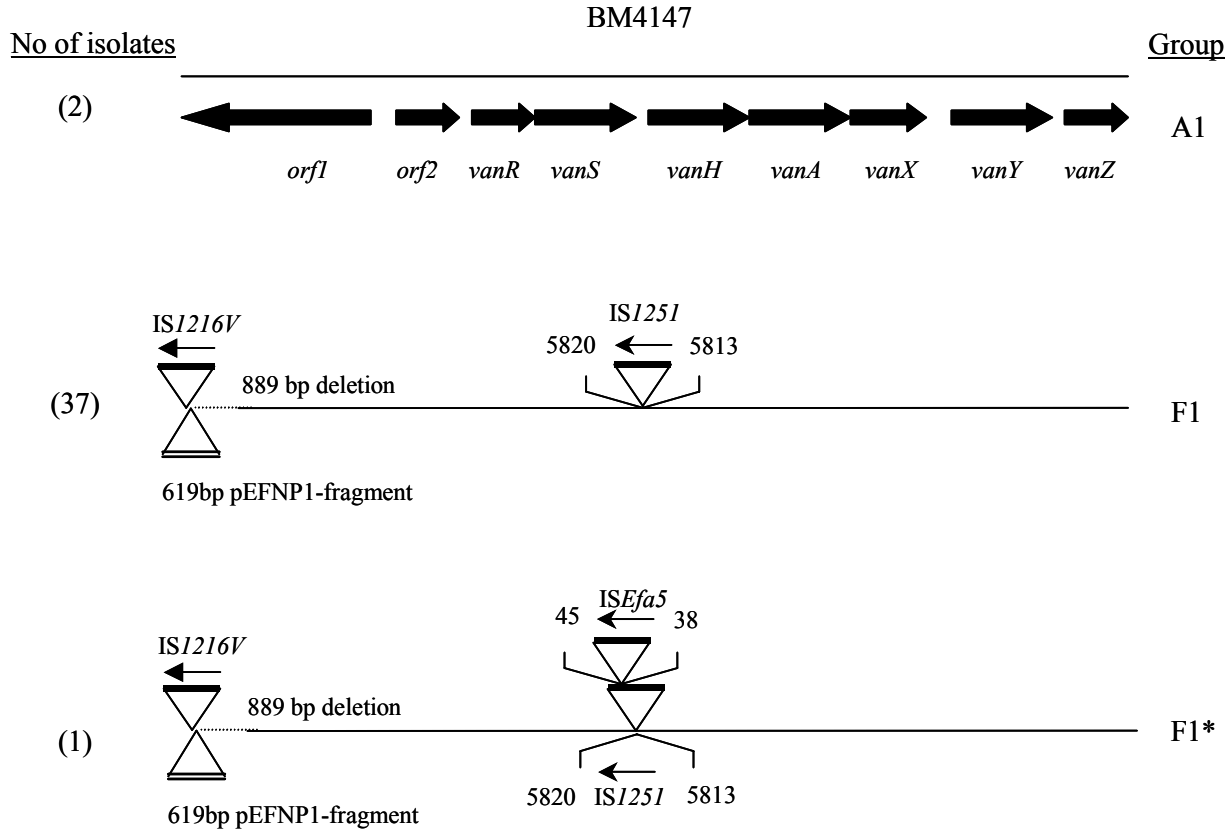


Figure 2. PCR and sequence-based characterization of Tn*1546* in 40 infection-related *Enterococcus faecium* isolates from Paraguay. Tn*1546* reference sequence BM4147 is represented by the unbroken black lines. The position of different genes and open reading frames (*orf1* and *orf2)* are shown by filled block arrows. IS elements are shown by filled boxes and their direction of transcription is represented by arrows. Deletions are represented by dotted lines. The positions of the first nucleotides upstream and downstream of the insertion sequences are shown. The groups are based on the published results of Willems *et al.* [15]. *A novel F1 variant of Tn*1546*. Numbers on the left indicate how many isolates were found to belong to each Tn*1546* variant.

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Chapter 9

Molecular epidemiology of high-level aminoglycoside resistant *Enterococcus faecalis* patients during and after hospitalisation

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Submitted for publication

Abstract

Molecular epidemiology of 53 high-level gentamicin resistant (HLGR) *E. faecalis* isolates cultured from 1,686 specimens collected from intensive care and general wards at the Erasmus Medical Centre, Rotterdam, The Netherlands, between 2000 and 2003 was studied. PFGE and MLST genotyping, antibiotic susceptibility profiling, PCR screening for the presence of virulence genes, and assessment of Tn*4001* element flanking region variability was performed. The MLST analysis revealed the presence of 3 major ST types (ST-2, ST-6 and ST-16), confirming the importance of these STs in the nosocomial setting. Genotypic analysis of 14 serial HLGR isolates obtained from 6 patients revealed that replacement of HLGR genotypes did not occur during hospital admission, suggesting that equilibrium exists between HLGR genotypes and their host. Among the HLGR *E. faecalis* isolates tested, the majority possessed *esp*, *cob*, *ccf*, *cpd*, *asI* and *cylM* genes. PCR screening for the *aac (6')-Ie-aph (2")-Ia* gene revealed that all HLGR isolates possessed this 6'-acetyltransferase-2"-phospho-transferase gene. Most isolates also showed deletions in their Tn*4001* flanking sequences. Finally, the percentage of patients colonized with HLGR *E. faecalis* on ICU wards was found to be significantly higher than patients on general wards. Our results confirm the importance of HLGR *E. faecalis* ST-2, ST-6 and ST-16 types in the nosocomial environment, particularly in ICU wards. Further, the replacement of "indigenous" HLGR *E. faecalis* genotypes, already present in patients at the time of admission to hospital, does not appear to occur. This suggests that a balance exists between pathogen and host.

Introduction

Enterococcus faecalis is the leading causative agent of human infection among the different species of enterococci, being specially associated with nosocomial bacteremia and bacterial endocarditis [1-4].

Treatment options for nosocomial infections, usually involve the use of penicillin or a glycopeptide antibiotic in conjunction with an aminoglycoside, a combination that has a synergistic effect against many nosocomially acquired enterococcal infections. However, these antibiotic treatment therapies are being compromised due to combinations of intrinsic and acquired antimicrobial resistance [2]. Indeed, the intrinsic low-level resistance of enterococci to many commonly used antimicrobial agents e.g. β-lactams and glycopeptides, may have facilitated their survival in hospital environments, allowing them to acquire other antibiotic resistance genes involved in, for example, high-level resistance to aminoglycosides [5]. In fact, one of the most frequently used aminoglycoside antibiotics is gentamicin, with high-level gentamicin resistance (HLGR) being first observed in *E. faecalis* in1979 [6]. Further, HLGR (MIC ≥500 mg/L) may be facilitated by several related aminoglycoside-modifying enzymes (AMEs) that are expressed from a series of gentamicin resistance genes usually carried on transposons, namely *aac (6')-Ie-aph (2")-Ia*, *aph (2")-Ib*, *aph (2")-Ic*, and *aph (2")-Id* [7, 8].

The ability of enterococci to acquire virulence traits helps them survive in the human host [9, 10]. Studies have reported that enterococcal virulence factors such as cytolysins and aggregation substance (AS) are among the major factors known to be associated with *E. faecalis* virulence, and have also been associated with enterococcal pathogenicity in animal models [2, 11-12]. Additional virulence factors carried by *E. faecalis* include cell wall adhesins e.g. *efaAfs* [2, 13] and the cell wall-associated cytolysin proteins that are expressed by the *cylA*, *cylB* and *cylM* genes, which are involved in immune evasion. Sex pheromone genes such as *cpd, cob, ccf* and *cad* induce chemotaxis in human leucocytes and induce the secretion of lysosomal enzymes in the human host [10, 14-16].

The aim of this study was to characterize and compare HLGR *E. faecalis* isolates circulating in both ICU and general wards at a large university medical centre in The Netherlands (i.e. Erasmus MC, Rotterdam). We also aimed to provide information regarding the prevalence of dominant genotypes, their origin, and enterococcal dynamics during hospitalisation and after discharge.

Materials and methods

Bacterial strains

The *E. faecalis* isolates investigated in this study originated from patients admitted to either intensive care wards (surgical and neurological) or general wards (internal medicine, neurology and pulmonology) of the Erasmus MC, Rotterdam, The Netherlands, between November 2000 and July 2003. All strains were isolated from stool specimens or rectal swabs of adults over 18 years of age as part of a simultaneous study investigating the colonization and resistance dynamics of Gram-negative bacteria at the Erasmus MC [17]. Stool samples and swabs were obtained within 48 hours of admission, during hospital stay (at least 4 days after admission and at weekly intervals thereafter), and 1-3 months after discharge. Samples were cultured in enterococci-selective esculin enrichment broth containing 75mg/L aztreonam (Bristol Myers Squibb) for primary isolation. Subculture was performed using Columbia blood agar.

Antibiotic susceptibility testing

The determination of antibiotic minimum inhibitory concentrations was performed using the VITEK 2 system (bioMerieux) and AST-P524 cards. *E. faecalis* ATCC 29212 was used as a reference isolate. High-level gentamicin resistant *E. faecalis* isolates were selected using the results obtained from VITEK 2 screening such that a single, randomly chosen *E. faecalis* colony (from a maximum of 5) was investigated per patient per time period (i.e. at the time of admission, during stay, and at either 1 or 3 months post-discharge). Gentamicin susceptible *E. faecalis* isolates were also analyzed from patients who had been colonized by high-level gentamicin resistant *E. faecalis* in at least 1 sample period and where any of the remaining sampling periods had recovered *E. faecalis* isolates that were gentamicin susceptible only.

Multi-locus sequence typing (MLST)

MLST PCR and sequence analysis was performed using internal fragments from seven housekeeping genes of *E. faecalis* as previously described [18]. Allele numbers were assigned after reference to the online *E. faecalis* MLST database situated at http://efaecalis.mlst.net/, and sequence types (ST) were then determined based on these allelic profiles.

Pulsed-field gel electrophoresis (PFGE)

Pulsed-field gel electrophoresis (PFGE) was performed using *Sma*I digested fragments of genomic chromosomal DNA. Fragments were separated in 0.8% agarose using a constant voltage of 6 Vcm^{-1} at 14°C and pulse times of 3.5 – 25 seconds, which increased linearly over 12 hours (block 1). This was followed by a pulse time of 1 – 5 seconds increasing over 8 hours. Gel patterns were analyzed using GelCompar software (Applied Maths) with gel lanes normalized against a lambda DNA ladder (BIO RAD) with band tolerance set at 1.5%.

PCR for virulence-associated genes

PCR screening for the presence of aggregation substance *(asI*), gelatinase *(gelE*), cytolysins (*cylA, cylB* and *cylM*), sex pheromones (*cpd, cob* and *ccf*), and enterococcal surface protein (*esp*) genes was performed using primer sequences previously published by Eaton and Gasson (2001) [10]. PCR for the hyaluronidase (*hyl*) gene was performed using primers published by Vankerckhoven *et al*. [19]. A touchdown PCR protocol was used for thermocycling [20].

PCR screening and sequencing of Tn4001 transposon

PCR screening for the presence of Tn*4001* containing gentamicin resistant gene *aac (6')-Ie-aph (2")-la* and flanking regions was performed using the primers published by Vakulenko *et al*. [8]. A touchdown PCR protocol was used for thermocycling. The primer sequences used were:

aac (6')-Ie-aph (2")-la	5'- CAGGAATTTATCGAAAATGGTAGAAAAG – 3'
	5'- CACAATCGACTAAAGAGTACCAATC – 3'
aac (6')-Ie-aph (2")-la	5'- CAGAGCCTTGGGAAGATGAAG – 3'
	5'- CCTCGTGTAATTCATGTTCTGGC – 3'

Results

Bacterial strains

The patient and sample chronology associated with high-level gentamicin *E. faecalis* colonization in ICU and general wards at Erasmus MC between 2000 and 2003 is shown in Table 1. In total, 1,686 stool samples or rectal swabs (797 ICU and 889 general wards) were obtained from 413 patients (187 ICU and 226 general ward patients), with an enterococcus species being cultured from 1,026 (61%) specimens, yielding 5,112 enterococcus colonies for investigation. The following enteroccus species were identified: 3,292 (64%) *E. faecalis*; 1,034 (20%) *E. faecium*; 320 (6%) *E. cassiflavus /gallinarum;* 280 (5%) *E. hirae;* 98 (2%) *E. durans* and 88 (2%) *E. avium*. Four hundred and eighty out of 3,292 (15%) *E. faecalis* colonies (368 ICU and 112 general wards), isolated from 57 patients (40 ICU and 17 general wards), exhibited high-level resistance to gentamicin. This translates to 20% (40/187) of patients from the ICU and 7% (16/226) of patients from general wards were colonized by HLGR *E. faecalis* isolates. Further, a significant difference was observed (Table 2) between the percentage of patients colonized with HLGR *E. faecalis* isolates in the ICU wards compared to general wards during stay but not during admission or after discharge [Fisher's Exact Test $P < 0.001$]. Fifty-three HLGR *E. faecalis* isolates were selected for further analysis from 39 patients who had been colonized by a HLGR *E. faecalis*. These 53 isolates included 12 isolates from admission, 24 isolates from intensive care ward patients, 6 isolates from patients exclusively admitted to general wards, and 11 isolates collected between 1 and 3 months after discharge from hospital. Ten gentamicin susceptible *E. faecalis* isolates were investigated from patients who had also been shown to be colonized by high-level gentamicin resistant *E. faecalis* isolates.

Antibiotic susceptibility patterns for HLGR

Antibiotic susceptibility patterns of 53 HLGR and 10 gentamicin susceptible *E. faecalis* are shown in Tables 3a and 3b, respectively. Among the 53 high-level gentamicin resistant isolates tested, 92% (49/53) were resistant to erythromycin, 72% (38/53) resistant to tetracycline and 60% (32/53) resistant to streptomycin. All 53-HLGR *E. faecalis* isolates were susceptible to ampicillin, ciprofloxacin, teicoplanin and vancomycin. Of the 10-gentamicin susceptible *E. faecalis* isolates, all were susceptible to ampicillin, ciprofloxacin, vancomycin and teicoplanin.

Multi-locus sequence typing (MLST)

The results of multi locus sequence typing are shown in Tables 1, 3a and 3b. Three major MLST-types (ST-2, ST-6 and ST-16) were isolated, representing 79% of the HLGR isolates. In contrast, MLST results for 10 gentamicin susceptible isolates cultured from the same patients revealed that all possessed different MLST-types. With respect to the relationship between MLST-type and antibiotic profiles, our ST-2 isolates were all susceptible to tetracycline, whilst ST-6 and ST-16 were all resistant. Additionally, our ST-16 isolates were all susceptible to levofloxacin, and a trend towards streptomycin susceptibility was observed for our ST-6 isolates.

PFGE results

PFGE of 14 serial HLGR isolates from 6 patients revealed that identical genotypes, present at the time of admission, could be isolated from the same patient before, during and after hospital stay (Figure 1).

PCR for virulence-associated genes

The results of PCR screening of virulence genes in 53 HLGR *E. faecalis* and 10 gentamicin susceptible *E. faecalis* isolates are shown in Tables 3a and 3b, respectively. Among HLGR *E. faecalis* isolates, 51% (27/53), 85% (45/53), 89% (47/53), 100% (53/53), 94% (50/53), 51% (27/53), 45% (24/53) and 11% (21/53) were positive for *esp*, *cob*, *ccf*, *cpd*, *asI*, *cylM*, *cylB* and *cylA* genes, respectively. All 53-HLGR isolates were PCR negative for hyaluronidase (*hyl*) and gelatinase (*gelE)*. Comparison with MLST data indicated a negative association between carriage of *cylM*, *cylB* and *cylA* genes and ST-2 isolates. Further, there was an association between ST-16 isolates and *cylA* negativity. Finally, both ST-2 and ST-6 isolates did not harbour the *esp* gene.

PCR of the Tn4001 transposon

PCR screening results for Tn*4001* revealed that all isolates possessed the *aac (6')-Ie-aph (2")-Ia* gene. In addition, deletions in the IS256 3'flanking region of Tn*4001* were observed in 68% (36/53) of isolates, and deletions in the 5'flanking region in 51% (27/53) of isolates. Twenty-one isolates (40%) possessed deletions in both 3'and 5'flanking sequences. All gentamicin susceptible isolates tested were negative for the *aac (6')-Ie-aph (2")-Ia* gene as well as for the 5'and 3'flanking IS*256* regions.

Discussion

The reason for the frequent isolation of enterococci from the nosocomial setting is that enterococci are prevalent in the human gut and possess the ability to acquire antibiotic resistance. Additionally, several factors not associated with the selective pressure of antimicrobial use could also facilitate adaptation of enterococci to the hospital environment, including factors associated with the patients themselves e.g. the immune status of the patient, and factors associated with enterococcal virulence e.g. the possession of virulence genes.

Among the antimicrobial agents currently in use in the nosocomial setting, aminoglycosides are particularly important therapeutic agents because of their ability to generate synergistic bactericidal activity in combination with other antimicrobial agents, such as various β-lactams and vancomycin [7]. However, aminoglycoside resistance genes, which encode various aminoglycoside-modifying enzymes e.g. *aac (6')-Ie-aph (2")-Ia*, *aph (2")-Ib*, *aph (2")-Ic*, and *aph (2")-Id*, are becoming widespread in the hospital environment, resulting in bacterial pathogens that possess high-level resistance to aminoglycosides. This is now a common problem in many hospitals, especially with respect to the HLGR enterococcal species *E. faecalis* and *E. faecium*, that can readily acquire transposon Tn*4001* that possess a bifunctional enzyme, 6'-acetyltransferase-2"-phospho-transferase encoded by the fused *aac6-aph2* gene [7, 8]. Further, an association between the emergence of HLGR strains and the use of other non-aminoglycoside antimicrobial agents, such as glycopeptides, cephalosporins, quinolones and carbapenems, is now well documented [21-23]. Results obtained from Dutch HLGR *E. faecalis* isolates in this study, showed a similar trend in this respect, with all the HLGR isolates being resistant to ciprofloxacin and cefuroxime. Though all the HLGR isolates in this study were susceptible to ampicillin, the latest 2009 report relating to the consumption of antimicrobial agents and antimicrobial resistance among medically important

bacteria in The Netherlands (NETHMAP) indicated that there has been an increase in the number of β-lactamase positive *E. faecalis* isolates circulating within The Netherlands, which could indicate a recent trend in the evolution of nosocomial *E. faecalis is*olates. This situation requires further monitoring.

Previous studies have reported that 2 clonal complexes (CC2 and CC9), and the singleton ST16 of *E. faecalis* are especially associated with HLGR in the nosocomial setting, with the results from our study being comparable to the results previously reported (Figure 2). Taken together, the results further verify the importance of CC2 and ST-16 genotypes in facilitating nosocomial infection [18, 24, 25].

As previously mentioned, antimicrobial resistance may not be the only factor associated with the predominance of enterococci (and especially *E. faecalis*) in the nosocomial environment. For example, several other characteristics could act to enhance the virulence of HLGR *E. faecalis* strains, including the production of extracellular surface protein (Esp), cytolysins, aggregation substance, adhesins, extracellular superoxide (ESO), and gelatinase. Of these, Esp is perhaps the best-studied example of an enterococcal virulence trait. The prevalence of *esp* gene carriage within our endemic HLGR isolates was 51%, which indicates that carriage of the *esp* gene is not specific for endemic HLGR *E. faecalis* within our hospital isolates. However, a higher level (73%) of *esp* gene carriage was observed in clinical *E. faecalis* isolates from Swedish hospitals by Hallgren *et al.* [26], who also reported that isolates possessing the *esp* gene did not show increased adherence compared with *esp* gene negative isolates. Taken together, these results suggest that antimicrobial resistance (and possibly the absence of the *esp* gene) is the most important factor in facilitating *E. faecalis* nosocomial infection. With respect to genotypes, isolates belonging to clonal complex 2 (which contains ST types 2 and 6) and originating from 7 individual patients were all found to be *esp* negative, which is in contrast to the data published by Kawalec *et al*. [24], who observed an incidence of 48% *esp* carriage in CC2 isolates. The reason for the difference between the two studies could however be related to differences in geographical region or in the year of isolation of strains. Other enterococcal virulence factors such as *asaI*, β-haemolysins (*cylM*, *cylB* and *cylA*), hyaluronidase and gelatinase have also been shown to be important in *E. faecalis* virulence [2, 11-12]. The majority of HLGR (as well as susceptible isolates) tested in our study harbored the sex pheromones *cob*, *ccf* and *cpd*, as well as the aggregation substance *asaI*. In a recent study of Hallgren *et al*, *asaI* was detected in 79% of 94 *E. faecalis* isolates, respectively, which is a high percentage and similar to our own findings. In contrast, no isolates tested possessed the hyaluronidase (*hyl*) or gelatinase (*gelE*) genes. With respect to the *cyl* genes, a high degree of diversity was observed in the incidence of *cylM*, *cylB* and *cylA* carriage between ST types, though in general, ST16 isolates tended to be more frequently positive for one or more of these genes compared to the other ST types observed in the study. However, it is not yet known whether the increased carriage of *cyl* beta-hemolysin genes increases the virulence potential of ST16 *E. faecalis* isolates.

The majority of HLGR *E. faecalis* isolates possess a plasmid that contains the transposon Tn*4001* [25]. Interestingly, all of the major HLGR *E. faecalis* ST types in our study, possessed deletion events in one or more of the Tn*4001* IS*256* flanking sequences. Casetta *et al.*[28], suggested that Tn*4001* may be stabilized when incorporated into the *E. faecalis* chromosome, with a loss of flanking IS*256* insertion sequences being associated with carriage on plasmids. This suggests that plasmids may play an important role in facilitating high-level gentamicin resistance within our hospital. However, further research is necessary to determine whether a single plasmid type or multiple plasmid types are involved in the transmission of high-level gentamicin resistance between bacterial species within the Erasmus MC or in other hospitals where HLGR *E. faecalis* is found.

Finally, several studies have reported that nosocomial infections are more frequent in intensive care units (ICUs) than general hospital wards, with enterococci being reported as one of the most common organisms isolated from ICU outbreaks [29-32]. In this study, the percentage of patients colonized with HLGR *E. faecalis* was found to be statistically significant when compared to patients who had only been admitted to general wards. The increased use of antibiotics on ICU wards is the most likely mechanism facilitating the significant incidence of HLGR *E. faecalis* carriage in ICU patients. Interestingly, we found evidence that some patients are colonized with HLGR *E. faecalis* at the time of admission to hospital, and that the same genotype persists within the patient during their hospital stay. Hence, replacement of "indigenous" HLGR *E. faecalis* genotypes, in patients that are HLGR *E. faecalis* positive at the time of admission, by other (hospital-dominant) HLGR *E. faecalis* genotypes does not appear to occur in patients already colonized with (community-acquired) HLGR *E. faecalis* at the time of admission.

Table 1. Chronology of gentamicin resistant *E. faecalis* isolates

Patient	Ward Type	Adm.	Stay	1-3 months
7	nICU	-	R (254)	ltf
23	nICU	*	R (16)	#
94	nICU	-	R (16)	ltf
106	nICU	R (16)	-	ltf
174	nICU	-	R (6)	!
207	nICU	R (16)	-	!
315	nICU	-	R (6)	ltf
6	sICU	-	R (21)	s (206)
12	sICU	-	R (2)	ltf
13	sICU	s (97)	R(2)	ltf
19	sICU	s (267)	R (2)	R (2)
20	sICU	*	R (2)	#
22	sICU	*	R (2)	#
59	sICU	s (4)	s (4)	R (253)
64	sICU	-	R (6)	#
67	sICU	s (16)	R (16)	*
131	sICU	-	R (16)	-
142	sICU	R (2)	R (2)	R (2)
162	sICU	s (116)	-	R (2)
177	sICU	R (6)	R (6)	ltf
182	sICU	R (16)	R (16)	#
188	sICU	R (6)	R (6)	#
210	sICU	-	R (159)	ltf
218	sICU	R (159)	*	ltf
281	sICU	-	R (6)	s (40)
346	sICU	-	*	R (16)
404	sICU	R (16)	R (16)	R (16)
437	sICU	s (74)	R (16)	*
440	sICU	*	R (6)	#
476	sICU	-	R (6)	ltf
44	internal	R (16)	*	*
47	internal	*	R (16)	R (16)
72	neurology	-	R (21)	*
143	neurology	R (16)	R (16)	s (251)
181	neurology	R (159)	R (159)	R (159)
189	neurology	R (16)	R (16)	R (16)
48	pulmonology	*	*	R (16)
69	pulmonology	*	R (23)	!
127	pulmonology	-	-	R (16)

sICU = surgical intensive care unit. nICU = neurological intensive care. Internal = internal medicine. - = no *E. faecalis* isolated. s = gentamicin sensitive *E. feacalis* isolated. * = Enterococcus species isolated not *E. faecalis*. ltf = lost to follow-up. # = died before final specimen could be taken. ! = refused permission once discharged. Numbers in parenthesis = ST type.

Table 2. Number of patients colonized by high-level gentamicin resistant *E. faecalis* at a Dutch hospital at different time periods.

	Admission	Stay	After Discharge
ICU	11 (6)	30 (16)*	10 (5)
General Wards	6 (3)	9 (4)*	8 (4)

() = percentage of patients colonized with high-level gentamicin resistant *E. faecalis* when compared to the total number of patients sampled on ICU (187) or general wards (226). * = statistically significant (Fishers Exact Test $P < 0.001$ comparing number of patients with and without high-level gentamicin resistant *E. faecalis* isolations between wards at each time period). Differences in Admission and After Discharge were not significant using the same test ($P = 0.09$ and $P = 0.39$, respectively). Patients serially colonized by high-level gentamicin resistant *E. faecalis* isolates were counted at each time period.

Table 3a. Properties of 53 nosocomial gentamicin resistant *E. faecalis* isolated from a single Dutch hospital (2000 - 2003)

MLST	Isolate	Time	*esp*	*cob*	*ccf*	*cpd*	*asI*	*cylM*	*cylB*	*cylA*	ERY	TET	LVX	STR	*5' flank*	*aph2.2*	*3'flank*
2	12	ICU	-	+	+	+	+	-	-	-	R	S	R	R	-	+	+
2	13	ICU	-	+	+	+	+	-	-	-	R	S	R	R	-	+	+
2	19	ICU	-	-	+	+	+	-	-	-	R	S	R	R	-	+	+
2	19	(ICU)	-	-	+	+	+	-	-	-	R	S	R	R	-	+	+
2	20	ICU	-	-	+	+	-	-	-	-	R	S	R	R	-	+	+
2	22	ICU	-	-	+	+	+	-	-	-	R	S	R	R	+	+	+
2	142	ADM	-	-	+	+	+	-	-	-	R	S	R	R	+	+	+
2	142	ICU	-	-	+	+	+	-	-	-	R	S	R	R	+	+	+
2	142	(ICU)	-	+	+	+	+	-	-	-	R	S	R	R	+	+	+
2	162	(ICU)	-	-	+	+	-	-	-	-	I	S	R	S	+	+	+
6	64	ICU	-	+	+	+	+	+	+	+	R	R	R	S	+	+	-
6	174	ICU	-	+	+	+	+	-	-	+	R	R	R	S	+	+	-
6	177	ADM	-	+	+	+	+	-	-	+	R	R	R	S	+	+	-
6	177	ICU	-	+	+	+	+	-	-	-	R	R	R	S	+	+	-
6	188	ADM	-	+	+	+	+	-	-	-	R	R	R	S	+	+	+
6	188	ICU	-	+	+	+	+	-	-	-	R	R	R	S	-	+	+
6	281	ICU	-	+	+	+	+	+	-	-	R	R	I	R	+	+	+
6	315	ICU	-	+	+	+	+	+	+	-	R	R	R	S	-	+	+
6	440	ICU	-	+	+	+	+	+	-	-	R	R	R	S	+	+	-
6	476	ICU	-	+	+	+	+	-	-	-	R	R	R	S	+	+	+
16	23	ICU	+	+	+	+	+	+	+	-	R	R	S	R	-	+	-
16	44	ADM	+	+	+	+	+	+	+	-	R	R	S	R	-	+	-
16	47	GW	+	+	+	+	+	+	+	+	R	R	S	R	-	+	-
16	47	(GW)	-	+	+	+	+	+	+	+	R	R	S	R	-	+	-
16	48	(GW)	+	+	+	+	+	+	+	+	R	R	S	R	-	+	-
16	94	ICU	+	+	+	+	+	+	+	-	R	R	S	R	-	+	-
16	106	ADM	-	+	+	+	+	-	-	-	R	R	S	R	-	+	+
16	127	(GW)	+	+	+	+	+	+	+	+	R	R	S	R	+	+	-
16	131	ICU	+	+	+	+	+	+	+	-	R	R	S	R	-	+	+
16	143	ADM	+	+	+	+	+	+	+	-	R	R	S	S	-	+	+
16	143	GW	+	+	+	+	+	+	+	-	I	R	S	S	-	+	-
16	182	ADM	+	+	+	+	+	+	+	-	R	R	S	R	-	+	-
16	182	ICU	+	+	+	+	+	+	+	-	R	R	S	R	-	+	-
16	189	ADM	+	+	+	+	+	+	+	-	R	R	S	R	-	+	-
16	189	GW	+	+	+	+	+	+	+	+	R	R	S	R	-	+	-
16	189	(GW)	-	+	-	+	+	+	+	-	R	R	S	R	-	+	-
16	207	ADM	-	+	+	+	+	+	+	-	R	R	S	S	-	+	-
16	346	(ICU)	+	+	+	+	+	+	+	-	R	R	S	S	-	+	-
16	404	ADM	+	+	-	+	+	+	-	-	R	R	S	R	-	+	-
16	404	ICU	+	+	-	+	+	+	+	-	R	R	S	R	-	+	-
16	404	(ICU)	+	+	-	+	+	+	+	-	R	R	S	R	-	+	-
16	437	ICU	-	+	+	+	+	-	-	-	R	R	S	R	-	+	-
159	181	ADM	+	+	+	+	+	-	-	-	S	S	R	S	-	+	+
159	181	GW+	+	+	+	+	+	-	-	-	S	S	R	S	-	+	+
159	181	(GW)	+	+	+	+	+	-	-	-	S	S	R	S	-	+	+
159	210	ICU	+	+	+	+	+	-	-	-	S	S	R	S	-	+	+
159	218	ADM	+	+	-	-	+	+	+	+	R	S	R	S	-	+	+
21	6	ICU	+	+	+	+	+	+	+	+	R	R	R	R	+	+	+
21	72	GW	+	+	+	+	-	-	-	-	R	R	S	R	-	+	-
23	69	GW	-	+	+	+	+	-	-	-	R	R	S	R	+	+	+
40	67	ICU	+	-	-	+	+	+	-	-	R	R	S	R	-	+	-
253	59	(ICU)	+	+	+	+	+	-	-	+	R	R	S	S	-	+	-
254	7	ICU	+	+	+	+	+	-	+	-	I	R	R	S	-	+	+

MLST = ST type; Time = time point when the respective *E. faecalis* strain was isolated. *esp* = enterococcal surface protein; *cob, ccf andcpd* = sex pheromones; *asI* = aggregation protein; *cylM*, *cylB*, *cylA* = cytolysin-associated genes *M*, *B* and *A*, respectively. All isolates were PCR negative for hyaluronidase (*hyl*) and gelatinase (*gelE*). ERY = erythromycin, TET = tetracycline, LVX = levofloxacin, STR = streptomycin. All isolates were sensitive to; ampicillin, nitrofurantoin, penicillin G, teicoplanin, vancomycin, and ciprofloxacin. All isolates were resistant to gentamicin, kanamicin, ciprofloxacin and cefuroxime. *aph2.2 = aac (6')-Ie-aph (2")-Ia, 5'flank* and *3'flank = IS256 on 5'* and *3'* end of *aac (6')-Ie-aph (2")-Ia* respectively.

Table 3b. Properties of 10 nosocomial gentamIcin sensitive *E. faecalis* isolated from a single Dutch hospital (2000-2003)

206	6	(ICU)	-	+	+	+	+	-	-	-	S	S	S	S	-	-	-
97	13	ADM	-	+	+	+	+	+	-	-	I	S	S	I	-	-	-
267	19	ADM	-	+	+	+	-	-	-	-	I	S	S	S	-	-	-
4	59	ADM	-	+	-	+	+	-	-	-	S	S	S	S	-	-	-
16	67	ADM	+	+	+	+	+	+	-	-	S	S	S	S	-	-	-
116	162	ADM	-	+	+	+	-	-	-	-	I	S	S	S	-	-	-
40	281	(ICU)	-	-	+	+	-	-	-	-	I	S	S	S	-	-	-
74	437	ADM	-	+	+	+	+	-	-	-	I	S	S	S	-	-	-
267	59	ICU	+	+	+	+	-	-	-	-	S	S	S	S	-	-	-
251	143	(GW)	-	-	+	+	-	-	-	-	S	S	S	S	-	-	-

MLST = ST type; Time = time point when the respective *E. faecalis* strain was isolated. *esp* = enterococcal surface protein; *cob, ccf andcpd* = sex pheromones; *asI* = aggregation protein; *cylM*, *cylB*, *cylA* = cytolysin-associated genes *M*, *B* and *A*, respectively. All isolates were PCR negative for hyaluronidase (*hyl*) and gelatinase (*gelE*). ERY = erythromycin, TET = tetracycline, LVX = levofloxacin, STR = Streptomycin, Q/D = quinupristin/dalfopristin, SXT = Cotrimoxazole, OFX = Orfloxacin. All isolates were sensitive, toteicoplanin, vancomycin and gentamicin, *aph2.2* = *aac (6')-Ie-aph (2")-Ia, 5'flank* and *3'flank* = *IS256 on 5'* and *3'* end of *aac (6')-Ie-aph (2")-Ia* respectively.

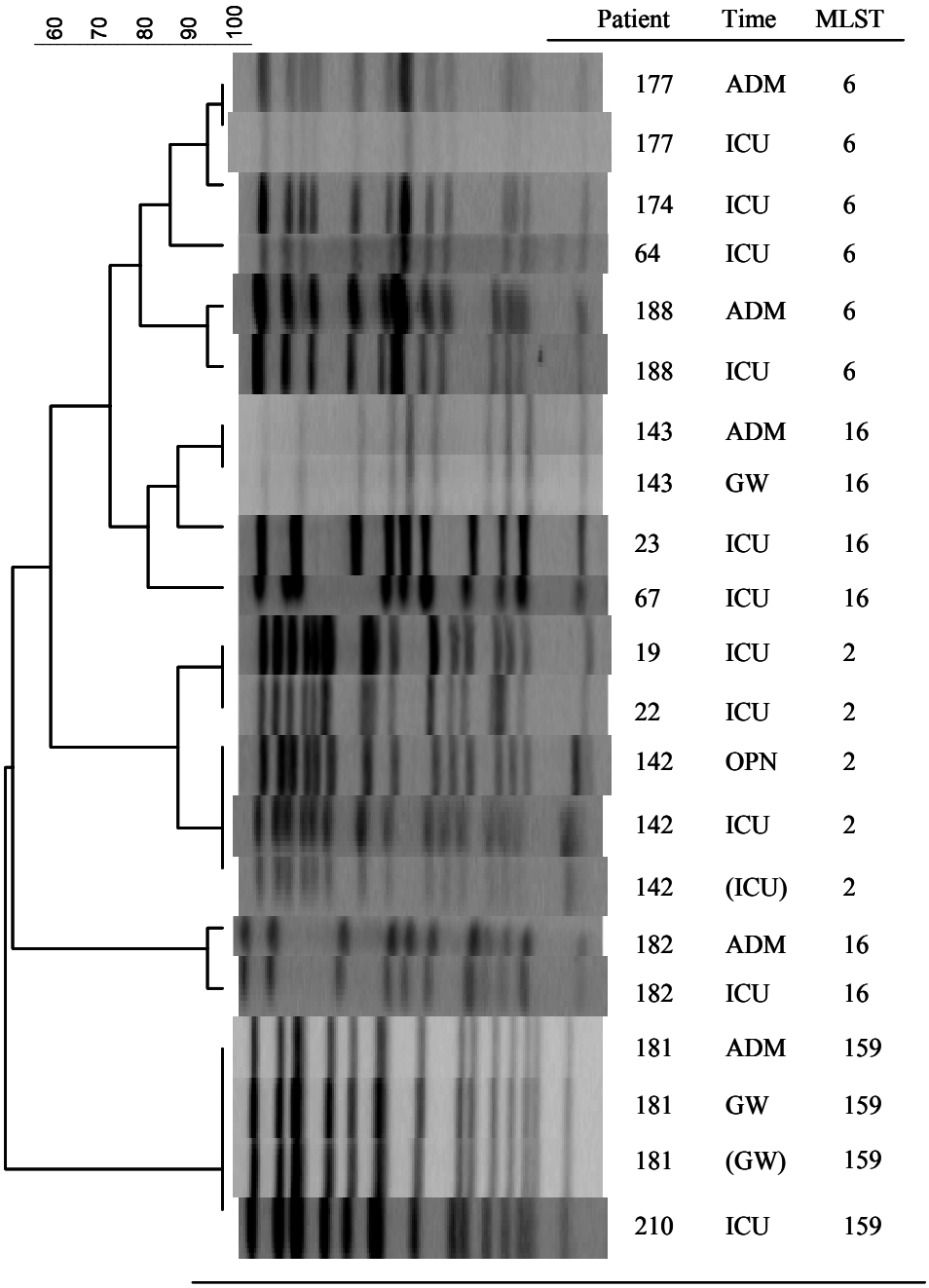

Figure 1. PFGE patterns of 21 high-level gentamicin resistant isolates obtained from 13 patients. Cluster analysis was performed using the method of DICE with UPGMA with band tolerances set to 1.5%. Time = time point when the respective *E. faecalis* strain was isolated (see text for details). MLST = multi locus-sequence genotype

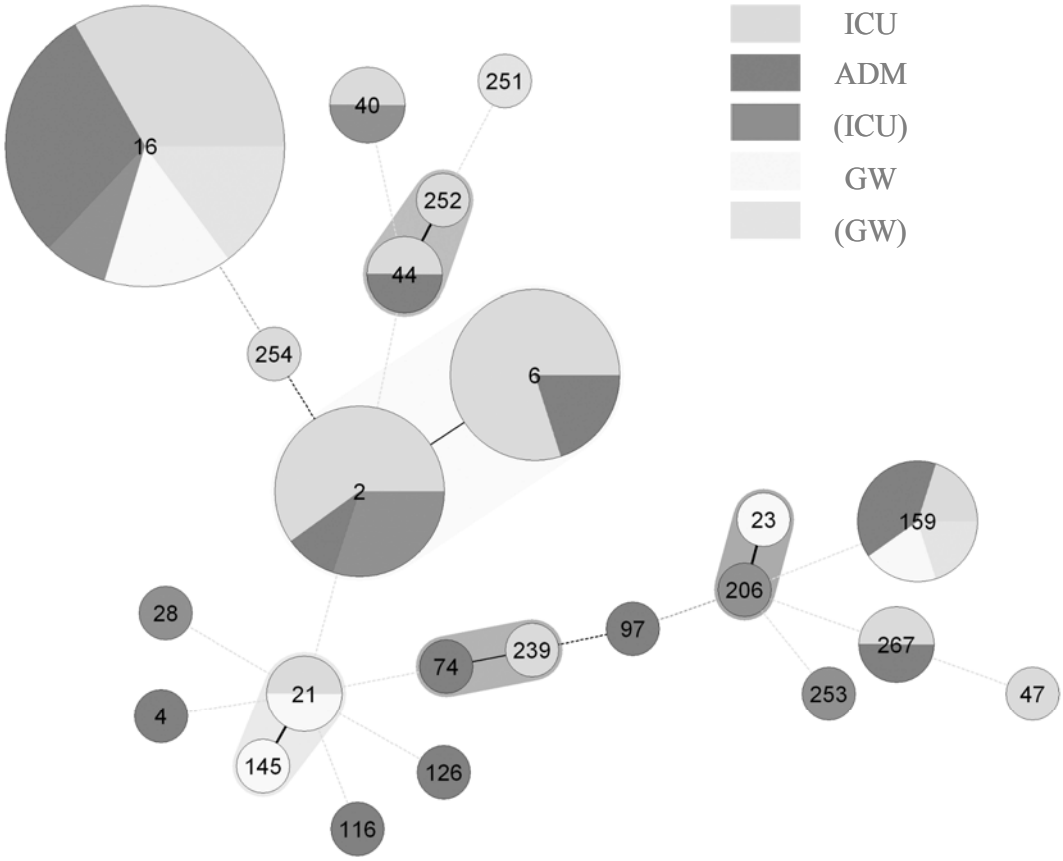

Figure 2. eburst minimum spanning tree showing clustering of 53 high-level gentamicin reistant and 10 gentamicin susceptible *Enterococcuc faecalis* MLST genotypes isolated from patients during admission to a Dutch hospital. ADM = isolated at time of admission; ICU = isolated during stay on ICU ward; GW = isolated during an exclusive stay on a general ward; (ICU) = isolated 1 – 3 months after discharge from hospital (previous ICU patient); (GW) = isolated 1 – 3 months after discharge from hospital (previous general ward patient). Numbers in circles represent MLST types. Single-locus variants are connected by thick short solid lines; double-locus variants by thin solid lines, STs which differ in three loci are connected by black dotted lines , and gray dotted lines connect STs that differ in more than three loci. ST distribution is indicated by pie charts.

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Chapter 10

Summary and concluding remarks

Nederlandse samenvatting

The growing problem of antimicrobial resistance involves practically all types of pathogens and remains a significant public health concern worldwide [1, 2]. Further, one of the most alarming trends is the development of antimicrobial resistance for virtually all currently available antimicrobial drugs, sometimes even combined in a single organism. Indeed, both the Infectious Diseases Society of America and the European Society of Clinical Microbiology and Infectious Diseases have highlighted their concerns regarding the large percentage of clinical isolates that are resistant to most antimicrobial agents [3, 4]. In particular, the bacterial pathogens *Staphylococcus aureus, Enterococcus* species, *Escherichia coli, Acinetobacter* species, *Pseudomonas* species, *Streptococcus pneumoniae* and *Klebsiella* species are currently most associated with this problem [5-9]. This thesis provides further data on the clinical relevance and importance of antimicrobial resistance in six bacterial species, including several of the ones mentioned above.

Resistance to β-lactam antibiotics

β-lactam antibiotics are a broad class of antibiotics that include penicillins and their derivatives including cephalosporins, monobactams, and carbapenems. They are one of the most important groups of antimicrobial agents currently used in the treatment of infectious diseases caused by both Gram-negative and Gram-positive bacteria. However, the development of resistance to these agents (by both Gram-negative and Gram-positive bacterial pathogens) has created great concern among health care professionals.

The main mechanism of β-lactam resistance, particularly amongst Gram-negative bacteria, is the production of β-lactamases, hydrolytic enzymes whose activity causes disruption of the amide bond of the four-membered β-lactam ring, leading to hydrolysis of the β-lactam ring, and inactivation of the β-lactam antibiotic. In fact, there are several types of β-lactamases known that confer resistance to β-lactam antibiotics. A few clinically relevant examples of these are discussed in the following section.

The BRO- β-lactamase

Moraxella catarrhalis has been recognized as an important human pathogen, because it can cause upper respiratory tract infections (e.g. acute otitis media) in children and lower respiratory tract infections (e.g. chronic obstructive pulmonary disease, COPD) in adults with pre-existing and chronic pulmonary disease. Although *M. catarrhalis* strains are generally susceptible to most antibiotics used for the treatment of respiratory infections, they tend to be resistant to penicillin because of the widespread production of BRO β-lactamase, a unique β-lactamase first described in Sweden in 1976 [10, 11]. Subsequently, two types of BRO, named BRO-1 and BRO-2, coded by the *bro-1* and *bro-2* genes respectively, have been reported. The two BRO enzymes are distinguishable by their level of β-lactamase production (BRO-1> BRO-2) and different iso-electric focusing patterns (resulting from a single amino-acid substitution). Further, the *bro-2* gene also has a 21 bp deletion in its promoter region compared to *bro-1* [12]. Despite some initial confusion, it is now accepted that the *bro* gene is located on the bacterial chromosome [13], and that it may originally have been acquired from an unknown Gram-positive bacterium, due to the fact that there is no significant sequence similarity between *bro* and the β-lactamase genes of other Gram-negative bacteria, and that the gene possesses an LPXTG signal sequence motif, which is characteristic for Gram-positive bacteria [14, 15].

Since 1976, there has been a rapid rise in the prevalence of worldwide clinical β-lactamase resistant *M. catarrhalis* isolates (which now approaches 90–99% of all clinical

isolates), considered to be the fastest dissemination rate of any known bacterial β-lactamase gene [15].

In **Chapter 2**, we show that more than 90% of a selection of global *M. catarrhalis* isolates appear to be β-lactamase and *bro* gene positive. With respect to geographical region, increased *bro-1* β-lactam MICs were observed in *M. catarrhalis* isolates originating from the Far East, and especially Japan, possibly due to differences in geographical antibiotic prescribing policies. Additionally, the prevalence of *bro* positivity between the two-age groups (children <5 years and adults > 20 years) was significantly different, which again may be due to differences in antibiotic prescribing policies (i.e. differences in age group specific prescription). Further, no clonal isolates, linked to a geographical region (including Japan and/or the Far East) were found (data not shown). Previous studies have also reported a lack of genotypic linkage between *M. catarrhalis* genotype and *bro* type, with at least one study reporting ampicillin MIC variation in apparently identical *M. catarrhalis* isolates cultured from an otitis media patient in Japan [16]. Further, a recent global survey of 195 *M. catarrhalis* isolates revealed no association between *M. catarrhalis* genotypes in child or adult age groups [17]. However, recent research has identified that mechanisms not associated with the *bro* gene per se may be involved in β-lactamase resistance, which may possibly have an effect on the results we obtained from the Far East and between children or adults [18]. Further research in this field is necessary.

The results from our study are worrying, because at the clinical level, β-lactamase production by *M. catarrhalis* has been reported to indirectly benefit other bacterial species present within mixed bacterial infections (e.g. *S. pneumoniae* and *H. influenzae*), as production of BRO β-lactamase may allow β-lactam susceptible bacterial isolates to evade antibiotic therapy, possibly facilitating treatment failure [19, 20].

Interestingly, recent studies have indicated an increase in MIC levels to both cefuroxime and cefaclor, as well as an overall decrease in susceptibility to cephems [21-26], though in our study, this effect was observed for Far East isolates only. Based on past experience, with respect to the rapid dissemination of penicillin resistance in *M. catarrhalis*, it seems logical that the global monitoring of these antibiotic resistances in this bacterial species should continue.

Extended spectrum β-lactamases

TEM and SHV-type extended-spectrum β-lactamases (ESBLs) were the most prevalent ESBLs in clinical isolates until the 1990s. However, since then CTX-M ESBL enzymes have started becoming increasingly prevalent in both clinical and nosocomial environments. Initially, clinical problems associated with ESBL-positive bacterial isolates tended to be associated with nosocomial outbreaks of. *K. pneumoniae*, mostly in intensive care settings [27]. However, community acquired *E. coli* has now become the main pathogen associated with ESBL-type carriage and infection [28, 29], with recent studies stressing the importance of the influx of community-acquired CTX-M-positive bacterial isolates into the hospital environment [30]. Further, in contrast to infection caused by ESBL-producing *K. pneumoniae*, approximately one-half of infections caused by ESBL-producing *E. coli* affects non-hospitalized patients [31].

The presence of ESBL-carrying bacteria, including CTX-M types, has now been documented worldwide including in various South American countries such as Argentina, Brazil, Uruguay, Peru [32-37], and India [38, 39]. In fact, the prevalence of ESBL producing *Klebsiella* species and *E. coli* from South America is reported to be amongst the highest in the world, estimated to be in the range of approximately 45-52% and 8-18%, respectively [40], with reports of high ESBL prevalence in this region dating back to the late 1980s [37].

These statistics match the tendency towards higher infection rates by ESBL producing bacteria in countries that are less heavily industrialized. For example, the prevalence of ESBL-carrying bacteria in Sweden is approximately 3%, but >25% in Turkey and Portugal, and >30% in South America [40]. Based upon the data currently available on the prevalence of ESBL types, it appears that CTX-M is now the most predominant ESBL reported worldwide [41], including in the South American continent.

In **Chapter 3,** we show the predominance of CTX-M-2 and CTX-M-56 carriage (though with no genotypic clonality), in ESBL producing isolates of the enterobacteriaceae *K. pneumoniaea* and *E. coli* from Paraguay. The prevalence of the CTX-M-2 group has been previously reported to be high in South America, with CTX-M-2 group enzymes predominating in this continent [34]. Therefore, this study reiterates the current importance of CTX-M-2 (as well as CTX-M-56) ESBL enzymes in South America, and indicates that urgent action is required to combat the prevalence and spread of ESBL-carrying enterobacteriaceae in Paraguay, as well as in South America as a whole.

Similar to many other countries, ESBL carrying enterobacteriaceae in India are becoming a major problem. For example, a study from North India reported the prevalence of ESBL as 63.6% in *E. coli* and 66.7% in *K. pneumoniae* isolates in 2003 [42]. From India, in the past, TEM and SHV type ESBLs were predominant clinical *K. pneumoniae* and *E. coli* isolates. However, in the recent past CTX-M type ESBLs were reported in several parts of India, particularly the comparatively new variant CTX-M-15, which is now the most common CTX-M type reported worldwide (reported in Portugal, Korea, Western Africa, Bulgaria, Canada, France, Italy, Japan, Poland, Romania, Russia and Turkey [34, 37, 43-48]). Indeed, this particular CTX-M ESBL was first identified in enterobacterial isolates from India in 1999 [49]. The rapid dissemination of the CTX-M-15 gene in enterobacterial isolates is enhanced by its plasmid location, and likely, by the insertion sequence IS*Ecp1* that may act as a transposition platform [49].

In **chapter 5**, we found a very high incidence of ESBL-gene carriage among *K. pneumoniae* isolates obtained from an NICU and other wards from a single tertiary hospital in India, with the most notable result being the presence of CTX-M-15 in 92% of isolates (36/39). The isolates in this study were found to possess a range of genotypes, and were not clonal in nature, with no relationship between clustering and type of infection, or antibiotic resistance profile. This study emphasises the major role that CTX-M-15 plays in facilitating antimicrobial resistance in Indian hospitals.

Extended-spectrum β-lactam and fluoroquinolone resistance in *Salmonella* species

In addition to *K. pneumoniae* and *E. coli,* antimicrobial resistance in other enterobacterial species such as non-typhoidal salmonellae has become a major problem worldwide. Non-typhoidal salmonellae are one of the principal pathogens implicated in food-borne gastroenteritis worldwide, with treatment failures due to the development of resistance to extended-spectrum β-lactams and/or fluoroquinolones in non-typhoidal *Salmonella* species being well documented [50-52]. In India, non-typhoidal salmonellae constitute approximately 20% of the *Salmonella* serovars currently circulating [53], and dissemination of isolates resistant to extended-spectrum β-lactamases is frequently observed.

In **chapter 6**, we observed 52% of non-typhoidal salmonellae isolates harbouring ESBL genes and exhibiting an ESBL phenotype. Though several different serovars were identified, the great majority of ESBL producers belonged to the *Salmonella* Agona serovar, which also represented the most abundant serovar isolated in this study in different hospitals. Further, multidrug-resistant *S.* Agona has been responsible for at least two hospital outbreaks in paediatric wards in Brazil [54, 55]. In both cases, the strains were found to harbour large

plasmids that conferred resistance to multiple antibiotics. At the current moment in time, there are very few studies that have investigated antibiotic resistance in non-typhoidal salmonellae [56], though, from our results, it appears that *S.* Agona may also play an important role in non-typhoidal salmonellae ESBL-mediated resistance in India. More importantly, of particular concern is the fact that two isolates of *S.* Agona recovered in this study possessed both ESBL and fluoroquinolone resistance genes, potentially seriously limiting antimicrobial treatment options, and possibly providing a reservoir for the spread of this combination of antibiotic resistances to other pathogenic bacteria. Additionally, 48% of the non-typhoidal *Salmonella* isolates in this study were found to possess the SHV-12 ESBL phenotype and gene. SHV-12 is currently one of the most common non-CTX-M ESBLs circulating and has been identified in many Gram-negative species, including *Salmonella* species [57, 58]. The ESBL enzymes that tend to be responsible for cephalosporin resistance in the genus *Salmonella* are TEM and SHV β-lactamase derivatives, though CTX-M type ESBLs have recently (though infrequently) been reported. In a study by Rotimi *et al*., 3.4% of *Salmonella s*pp. were found to be CTX-M-15 positive [59]. The presence of ESBL and fluoroquinolone antibiotic resistance in non-typhoidal *Salmonella* isolates within resource poor countries is jeopardizing classical antibiotic therapy. Continued surveillance of circulating ESBL producing, as well as fluoroquinolone resistant, salmonellae is therefore warranted in India.

Vancomycin resistance in *Enteroccus faecium*

Enterococci are an important group of bacteria especially involved in several types of nosocomial infections such as urinary tract infections, bacteremia, intra-abdominal infections, skin, soft tissue infections and endocarditis. Enterococci are found in both the "healthy" environment and hospital settings, frequently expressing glycopeptide/high-level aminoglycoside resistance. The intrinsic low-level resistance of enterococci to many commonly used antimicrobial agents e.g. β-lactams and glycopeptides may have allowed them to survive in hospital environments and successfully acquire other antibiotic resistance genes involved in, for example high-level resistance to glycopeptides (vancomycin and teicoplanin in *E. faecium*), and high-level aminoglycosides (gentamicin resistance in *E. faecalis*).

In enterococci, seven different vancomycin resistance types have so far been described, namely VanA, VanB, VanC, VanD, VanE, VanG and VanL [60]. Though, vancomycin resistance is usually more predominant in *E. faecium* than *E. faecalis*, with VanA type being the most important from the clinical point of view. This is because it is the most frequently found type of vancomycin resistance in clinical enterococcal isolates, characterized by both high-level and inducible resistance to vancomycin and teicoplanin. Vancomycin resistant *E. faecium* has been reported worldwide, having been promoted via the extensive use of vancomycin in hospitals, as well as the animal growth promoter avoparcin (an analogue of vancomycin). In *E. faecium,* VanA type resistance has been shown to be directly facilitated by the carriage of transposon Tn*1546* [61], a transposon widely disseminated in humans, animals and the environment [60]. In fact, significant diversity has now been identified within Tn*1546*-related elements since it was first described by Arthur *et al.* in *Enterococcus faecium* BM4147 in1993 [61]. This variation, in the form of point mutations, insertion sequence (IS) elements, and deletions, has been reported in several epidemiological studies [62-65]. Based on these studies, Schouten *et al.* (2001) proposed an evolutionary scheme comprising 2 lineages in Tn*1546,* based on the nature of these mutations and insertion sequences [66].

In **chapter 7,** we describe two extra lineages associated with *vanA* positive *E. faecium* isolates from hospitals in Saudi Arabia. In the study, we found that the majority of polymorphic events in Tn*1546* involved the insertion of IS*1216V* within the intergenic regions of either *vanS* and *vanH,* or *vanX* and *vanY* genes, along with other less frequent insertions of IS*1485* and IS*1251*. IS*1216V* insertions are one of the most common events in Tn*1546*-bearing vancomycin-resistant enterococci worldwide. The IS*1485* insertion was first described in enterococcus species by Cheng *et al*. in 1999 [67]. However, the "Saudi isolate insertion events" were new to this study.

In **chapter 8,** we reported on a novel Tn*1546* variant containing an IS*Efa5* insertion in *vanA E. faecium* isolates obtained from hospitals in Paraguay. This IS element has only previously been described in Brazilian *E. faecium* isolates, where the IS*Efa5* was exclusively inserted in the intergenic region of *vanX* and *vanY* [64]. In our Paraguayan isolates the IS*Efa5* insertion was found between *vanS* and *vanH* genes. Currently, the IS*Efa5* insert has been described only in South America, and it is interesting to speculate whether this particular event is limited to South American *vanA E. faecium* isolates only. Our results provide further evidence for the evolving nature of Tn*1546* in *vanA E. faecium* isolates. Additionally, surveillance of *vanA* type *E. faecium* and the diversity of Tn*1546* resistances is important because vancomycin resistant *S. aureus* isolates have been shown to carry Tn*1546* including the insertion of IS*1251* elements [68], and Tn*1546* surveillance will help to trace the dissemination of Tn*1546* from *E. faecium* to *S. aureus* allowing predictions to be made regarding the mechanisms on future spread of this important antimicrobial resistance element.

Finally, in recent years, a clonal complex of *vanA* type *E. faecium* isolates (CC17) has been identified that is particularly associated with global nosocomial-infections [69] (though there is some evidence to suggest that this grouping may be influenced by the high rates of recombination associated with the *E. faecium* species [70]). Our results from Saudi Arabia and Paraguay (countries not previously reporting studies on *vanA* resistance in *E. faecium*) support the high prevalence of "CC17" clonal complex isolates in global nosocomial settings, as more than 90% of *vanA* positive *E. faecium* isolates from Saudi Arabia and from Paraguay belonged to CC17.

High-level aminoglycoside resistance in *Enterococcus faecalis*

As mentioned above, the intrinsic low-level resistance of enterococci to many commonly used antimicrobial agents may have helped them to survive in hospital environments and successfully acquire several different antibiotic resistance genes. *E. faecalis* accounts for approximately 75% of all enterococcal infections [71], and this species is generally resistant to many commonly used antimicrobial agents, including aminoglycosides, aztreonam, cephalosporins, clindamycin, the semi-synthetic penicillins, nafcillin and oxacillin, and trimethoprim-sulfamethoxazole. High-level resistance to aminoglycosides (e.g. gentamicin) is one possible cause of treatment failure in nosocomial infections caused by *E. faecalis*, as aminoglycosides are usually administered along with other groups of antimicrobial agents to generate a synergistic effect and help ensure successful antimicrobial therapy. Aminoglycoside resistance in *E. faecalis* is conferred by genes that encode various aminoglycoside-modifying enzymes (AMEs), e.g. *aac (6')-Ie-aph (2")-Ia*, *aph (2")-Ib*, *aph (2")-Ic*, and *aph (2")-Id*. These AMEs are becoming widespread in the hospital environment, resulting in bacterial pathogens that possess high-level resistance to aminoglycosides, especially with respect to the high-level gentamicin resistant (HLGR) *E. faecalis*. *E. faecalis* readily acquire transposon Tn*4001,* a transposon that possess a bifunctional enzyme, 6'-acetyltransferase-2"-phospho-transferase encoded by the fused *aac6-aph2* gene [72-74]. Further, an association between the emergence of HLGR strains and the

use of other non-aminoglycoside antimicrobial agents, such as glycopeptides, cephalosporins, quinolones and carbapenems, is now well documented [75-77].

In **chapter 9**, results obtained from HLGR positive *E. faecalis* isolates cultured from Erasmus MC showed that all of the HLGR isolates were resistant to ciprofloxacin and cefuroxime. Previous studies have reported that 2 clonal complexes (CC2 and CC9) and the single ST16 of *E. faecalis* are especially associated with high-level gentamicin resistance in the nosocomial setting, with the results from our study being comparable, further verifying the importance of CC2 and ST-16 genotypes in nosocomial infection [78-80]. Of the major ST types circulating in Erasmus MC, ST-2 isolates tended to be tetracycline susceptible but resistant to levofloxacin and streptomycin, whilst ST-6 isolates tended to be resistant to tetracycline and levofloxacin but susceptible to streptomycin. Further, ST-16 isolates tended to be susceptible to levofloxacin. The investigation of resistance and susceptibility profiles for these antibiotics in the above ST types is important, because these antibiotics are vital in the treatment of *E. faecalis* mediated nosocomial infections. Though all of the HLGR isolates isolated at the Erasmus MC were susceptible to ampicillin, the latest NETHMAP report of 2009 [81], concerning the consumption of antimicrobial agents and antimicrobial resistance among medically important bacteria in The Netherlands, showed that there has been an increase in the number of β-lactamase positive *E. faecalis* isolates in Dutch hospitals. Though not yet a problem at Erasmus MC, this issue may become a problem in the future and needs to be closely monitored.

Research has shown that the majority of HLGR *E. faecalis* isolates possess a plasmid that contains the transposon Tn*4001* [80, 82]. Casetta *et al.* [83] suggested that Tn*4001* may be stabilized when incorporated into the *E. faecalis* chromosome, with a loss of flanking IS*256* insertion sequences being associated with carriage on plasmids. Interestingly, all of the major HLGR *E. faecalis* ST types in our study, possessed deletion events in one or more of the Tn*4001* IS*256* flanking sequences. This may suggest that plasmids play an important role in facilitating high-level gentamicin resistance within Erasmus MC. However, further research is necessary to determine whether a single plasmid type or multiple plasmid types are involved in the transmission of high-level gentamicin resistance between bacterial species within Erasmus MC or in other hospitals where HLGR *E. faecalis* is found.

Concluding remarks

Antimicrobial resistance is a continuing and increasing threat to both global public health and global healthcare systems, meaning that the control of antimicrobial resistance is a major issue faced by society today. International surveillance and epidemiological studies of antimicrobial resistance are continuing to show increasing resistance against the commonly used antimicrobial agents. Further, the fact that globalization is booming, means that it is important to understand international patterns of resistance spread, as global travel and the concomitant spread of antibiotic resistant microorganisms is becoming much easier. Additionally, selective pressure facilitated by the inappropriate and widespread use of antibiotics is a major contributor to the problem of antibiotic resistance and should be addressed both at the national level (via governmental legislation) and at the local level (via hospital prescription guidelines).

The evolution shown by the different “platforms” involved in antimicrobial resistance has significantly reduced the effectiveness of policies adapted for tackling antimicrobial resistance. In this respect, another important aspect of antibiotic resistance is to understand the mechanisms involved in the spread of antimicrobial resistance on a global scale. This may be achieved via the setting up of active surveillance programs, as surveillance programs

provide important information on antimicrobial resistance trends, allowing predictions to be made.

Finally, the following steps would be extremely helpful in tackling antimicrobial resistance:

- A reduction in the misuse of antimicrobial agents by designing and implementing standard and strict prescription practices.
- Development of novel antibiotics (10 x '20). [Www.idsociety.org].
- Using novel antimicrobials selectively, i.e. only when other therapies have failed.
- The development of rapid, inexpensive, and accurate (point-of-care) diagnostic methods to allow rapid decision-making regarding accurate antimicrobial therapy.
- The improvement of public and healthcare worker education.
- The improvement of hygiene in hospitals and in the community.
- A ban on the non-therapeutic use of antimicrobial agents in food and cattle.
- A reduction in antimicrobial usage via alternative approaches, such as vaccines, phages, and probiotics.
- Development of better alternative methods for anti-infection therapy circumventing chemotherapy and thereby preventing the development of resistance.

However, it remains to be seen whether governments, hospitals and individuals are able (or willing) to implement these strategies. Only a combined and global effort will ultimately limit the spread of antimicrobial resistance and the damaging consequences of a lack of action.

Nederlandse samenvatting

Het groeiende probleem van antimicrobiële resistentie is te detecteren in allerlei ziekteverwekkers en veroorzaakt wereldwijd een significante belasting van de zorginstellingen in de gezondheidszorg [1, 2]. Eén van de meest alarmerende tendensen is de ontwikkeling van antimicrobiële resistentie voor vrijwel alle nu verkrijgbare antibiotica. Zowel de Amerikaanse vereniging voor infectieziekten als de Europese vereniging van Klinische Microbiologie en Besmettelijke Ziekten, hebben allebei hun zorg geuit ten aanzien van het grote percentage klinische isolaten dat bestand is geworden tegen de meeste antibiotica [3, 4]. Momenteel zijn de *Stafylokokken*, *Enterokokken*, *Escherchia coli*, *Acinetobacter*, *Pseudomonas*, *Streptokok pneumoniae* en *Klebsiella* soorten die het meest geassocieerd worden met dit probleem [5-9]. Dit proefschrift bevat studies die data verlenen en de klinische relevantie weergeven van 6 verschillende bacteriële soorten die antibiotica resistent zijn, met inbegrip van verscheidene van de hierboven vermelde soorten.

De weerstand tegen β-lactam antibiotica

β-lactam antibiotica is een brede klasse van antibiotica's en omvatten penicillines en hun derivaten met inbegrip van, cephalosporins, monobactams, en carbapenems. Zij zijn één van de belangrijkste groepen antibiotica die momenteel in de behandeling van besmettelijke ziekten worden gebruikt, zowel veroorzaakt door Gram negatieve als Gram positieve bacteriën. De ontwikkeling van resistentie tegen deze antibiotica (door zowel Grampositieve als Gramnegatieve bacteriële ziekteverwekkers) heeft tot grote zorg geleid onder gezondheidszorgpersoneel.

Het belangrijkste mechanisme van β-lactam weerstand is de productie van β-lactamases. Dat zijn hydrolytische enzymen die verstoring van de amideband teweeg brengen in de β-lactam ring. Deze verstoring leidt tot het openen van de β-lactam ring wat inactivering van het β-lactam antibioticum veroorzaakt. In feite, zijn er verscheidene bekende soorten β-lactamases die resistentie tegen β-lactam antibiotica genereren. Een paar klinisch relevante voorbeelden hiervan worden besproken in de volgende sectie.

BRO- β-lactamase

Moraxella catarrhalis wordt gezien als een belangrijke menselijke ziekteverwekker, omdat het besmettingen veroorzaakt in de bovenste delen van de luchtwegen in kinderen (bijvoorbeeld oorontsteking) en onderste delen van de luchtwegen bij volwassenen (b.v. chronische obstructieve longziekte, COPD) die al reeds een bestaande of chronische longziekte doormaken. Hoewel *M. catarrhalis* over het algemeen vatbaar is voor de antibiotica die gebruikt worden voor luchtweginfectie, neigen zij steeds vaker resistent te worden tegen penicilline wegens de steeds wijder verspreide productie van BRO β-lactamases in deze bacterie. BRO β-lactamase is een unieke lactamase die voor het eerst in Zweden in 1976 wordt beschreven [10, 11]. Latere studies melden dat er twee soorten BRO zijn, *bro-1* en *bro-2*, gecodeerd door *bro-1* en *bro-2* genen. De twee enzymen BRO zijn te onderscheiden door hun niveau van β-lactamase productie (BRO-1> BRO-2). Daarnaast hebben de twee BRO lactamases verschillende iso-electrische patronen (als gevolg van één enkele aminozuur substitutie). Verder, heeft gen *bro-2* ook een 21 bp deletie in zijn promotorgebied in vergelijking met de promotor van *bro-1* [12].

Ondanks wetenschappelijke discussies, is het nu duidelijk geworden dat het bro gen in het bacteriële genoom ligt [13]. Dit gen is zeer waarschijnlijk verkregen via een tot nu toe onbekende Gram positieve bacterie, omdat er tot nu toe geen significante vergelijkenissen

zijn gevonden tussen het bro gen en verschillende andere β-lactamase genen die aanwezig zijn in Gram negatieve bacteriën. Daarnaast bevat het bro gen een LPXTG signaal motief voor uitscheiding van eiwitten karakteristiek voor Gram positieve bacteriën [14, 15].

Sinds 1976 is er een snelle wereldwijde groei van *M. catarrhalis* stammen die klinische β-lactamase resistentie bezitten. Op dit moment is 90-99% van alle klinische isolaten resistent en is bro gecodeerde lactamase daarmee de snelst verspreidde resistentie ten opzichte van elke ander bekende bacteriële β-lactamase [15].

In Hoofdstuk 2, tonen wij aan dat meer dan 90% van een wereldwijde *M. catarrhalis* isolaten collectie, β-lactamase en bro gen positief zijn. Met betrekking tot geografische gebieden, werden verhoogde bro-1 β-lactam MICs waargenomen in *M. catarrhalis* isolaten die komen uit het Verre Oosten, en vooral Japan. Deze waarneming kan misschien verklaard worden door een ander antibioticum beleid in deze gebieden. Daarnaast, bleek de aanwezigheid van het bro gen tussen de twee leeftijdsgroepen (kinderen < 5 jaren en volwassenen > 20 jaar) significant te verschillen. Dit is zeer waarschijnlijk opnieuw toe te schrijven aan verschillen in het antibioticum beleid (d.w.z. verschillen in antibiotica verstrekking in deze leeftijdsgroepen in geografische gebieden). Verder werden er geen clonale isolaten gevonden, met betrekking tot een geografisch gebied (met inbegrip van Japan en/of het Verre Oosten) (resultaten niet weergegeven). Dit is in overeenstemming met eerdere studies die ook geen genotypische (clonale link) tussen M. catarrhalis genotype en bro type vonden. Er is echter wel 1 Japanse studie die ampenicilline MIC variatie vond in *M. catarrhalis* stammen, geïsoleerd uit patiënten met een oorontsteking, die ogenschijnlijk genotypisch identiek leken te zijn aan elkaar [16]. Verder, blijkt uit een recent overzicht van 195 *M. catarrhalis* wereldwijd geïsoleerde stammen dat ook hier geen associatie was tussen *M. catarrhalis* genotypen gevonden in kinderen of volwassen. De stammen collectie is dus heterogeen [17]. Niettemin heeft zeer recent onderzoek ook aangetoond dat er mechanismen zijn die niet verbonden zijn aan het bro gen, maar wel invloed hebben op β-lactamase resistentie. Dit kan van effect zijn op de resultaten die wij hebben verkregen met stammen uit het Verre Oosten en het significante verschil van aanwezigheid van het bro gen tussen kinderen en volwassenen [18]. Verder onderzoek op dit gebied is dus noodzakelijk.

De resultaten van onze studie zijn erg verontrustend, omdat op klinisch niveau, β-lactamase productie door *M. catarrhalis* indirect invloed heeft op aan andere bacteriële soorten. Bijvoorbeeld een *M. catarrhalis* menginfectie met een *Streptokok pneumoniae* of *Haemophilus influenzae* bacterie. Met een dergelijke menginfectie kan de productie van BRO β–lactamase, β-lactam gevoelige bacteriën laten ontsnappen aan deze antibiotica therapie. Hierdoor heeft een behandeling van zo'n infectie een verhoogd risico op mislukking [19, 20].

Interessant is daarnaast dat recente studies een verhoging van de MIC niveaus in *M. catarrhalis* vonden voor antibiotica cefuroxime en cefaclor. Een algemene daling van gevoeligheid tegen cephems werd ook geconstateerd [21-26]. In onze studie werd dit effect alleen waargenomen voor de stammen die uit het Verre Oosten zijn geïsoleerd. Gebaseerd op ervaringen uit het verleden, met betrekking tot de snelle verspreiding van penicilline resistentie in *M. catarrhalis*, lijkt het niet meer dan logisch dat het wereldwijd monitoren op resistentie door moet gaan.

Breed spectrum β -lactamases

TEM en SHV-type breed spectrum β-lactamases (ESBLs) waren de meest voorkomende ESBL klinische isolaten uit de jaren '90. Sindsdien is er een toename van de enzymen CTX-M ESBL geconstateerd in ziekenhuis omgevingen. Eerst werden de klinische problemen gerelateerd aan ESBL-positieve *K. pneumoniae* op de intensieve zorg afdelingen in ziekenhuizen [27]. Ondertussen is nu *E. coli* de belangrijkste pathogeen geworden die

geassocieerd is met ESBL dragerschap en infectie [28, 29]. Recente studies benadrukken het probleem van de toevloed via de gemeenschapverworvene CTX-M-positieve bacteriële isolaten naar het ziekenhuismilieu [30]. In tegenstelling tot besmetting die door ESBL *K. pneumoniae* veroorzaakt worden, vind ongeveer de helft van besmettingen die door ESBL *E. coli* worden veroorzaakt plaats buiten het ziekenhuis [31].

De aanwezigheid van ESBL bevattende bacteriën met daarnaast de CTX-M types worden nu wereldwijd gedocumenteerd, met inbegrip diverse Zuid-Amerikaanse landen zoals Argentinië, Brazilië, Uruguay, Peru [32-37], en India [38, 39]. In feite, is de aanwezigheid van ESBL producerende *Klebsiella* en *E. coli* soorten geïsoleerd in Zuid-Amerika een van de hoogste in de wereld. De omvang van positieve isolaten wordt geschat op 45-52% en 8-18% [40]. Vroegere publicaties geven aan dat ESBL aanwezigheid in dit gebied ook al hoog was in de jaren '80 voor ESBLs [37]. Deze statistieken markeren de tendens naar hogere infectiegraden door ESBL producerende bacteriën in landen die minder zwaar geïndustrialiseerd zijn. Bijvoorbeeld, is de aanwezigheid van ESBL positieve bacteriën in Zweden ongeveer 3%, maar > 25% in Turkije en Portugal, en > 30% in Zuid-Amerika [40]. Gebaseerd op de huidige gegevens over de aanwezigheid van ESBL, blijkt het dat CTX-M nu de meest overheersende ESBL type is [41], inclusief het Zuid-Amerikaanse continent.

In Hoofdstuk 3, tonen wij de overheersende aanwezigheid van CTX-M-2 en CTX-M-56 (hoewel zonder genotypische clonality) aan in ESBL producerend isolaten van *Enterobacteriaceae*, *K. pneumoniaea* en *E.coli* soorten geïsoleerd in Paraguay. De hoge aanwezigheid van de CTX-M-2 groep is eerder gerapporteerd in Zuid-Amerika [34]. Deze studie bevestigt het huidige probleem van CTX-M-2 (evenals CTX-M-56) ESBL enzymen in Zuid-Amerika, en wijst erop dat er dringend actie ondernomen moet worden om het overwicht en de verspreiding van deze types onder controle te krijgen. Ook in India, lijken ESBL positieve bacteriën een belangrijk probleem aan het worden. Als voorbeeld, toonde een studie uit 2003 aan, uitgevoerd in Noord-India, dat de aanwezigheid van ESBL in 63.6% in *E. coli* en 66.7% in *K. pneumoniae* kon worden aangetoond [42]. TEM en SHV was het overheersende type ESBLs aanwezig in klinische *K. pneumoniae* en *E. coli* isolaten in India. Recent is in verschillende delen van India het CTX-M type ESBLs waargenomen, in het bijzonder een betrekkelijk nieuwe variant CTX-M-15, die nu wereldwijd het meest gemelde type is (gerapporteerd in Portugal, Korea, Westelijk Afrika, Bulgarije, Canada, Frankrijk, Italië, Japan, Polen, Roemenië, Rusland en Turkije [34, 37, 43-48]). Dit bepaalde type CTX-M ESBL werd als eerste geïdentificeerd in *Enterobacteriele* isolaten vanuit India in 1999 [49]. De snelle verspreiding van dit CTX-M gen in *Enterobacteriele* isolaten wordt veroorzaakt door de plaats van dit gen op een plasmide met daarbij waarschijnlijk geholpen in de verspreiding door de insertie sequentie ISEcp1 die dienst kan doen als transpoase [49].

In hoofdstuk 5, vonden wij een zeer hoge aanwezigheid van ESBL dragerschap onder *K. pneumoniae* isolaten verkregen uit NICU en andere afdelingen van één enkel tertiair ziekenhuis in India, met het opmerkelijke resultaat dat CTX-M-15 in 92% van de isolaten aanwezig was (36/39). Genotypisch werd in deze studie aangetoond dat deze stammen heterogeen zijn. Er werd geen verband gevonden tussen groeper en type van besmetting, of antibiotisch resistentie. Deze studie benadrukt dus de belangrijke rol die CTX-M-15 kan spelen in het vergemakkelijken van het verkrijgen van antibiotica resistentie in de Indische ziekenhuizen.

Wijdverbreide β-lactam en flouroquinolone resistentie in de soorten van *Salmonella's*

Naast *K. pneumoniae* en *E. coli*, is antibiotica resistentie in andere Enterobacteriële soorten zoals niet typhoide *Salmonella's* wereldwijd een belangrijk probleem geworden. De niet typhoide *Salmonella's* zijn één van de belangrijkste ziekteverwekkers die geïsoleerd

worden na voedsel gerelateerde infecties. De huidige behandelingsbelemmeringen voor deze infecties zijn op dit moment toe te schrijven aan de ontwikkeling van resistentie tegen breed spectrum van β-lactams en/of fluoroquinolones bij de niet typhoide soorten van *Salmonella* [50-52]. In India, vormen de niet typhoide *Salmonella's* ongeveer 20% van het totaal aantal geïsoleerde *Salmonella's serovars* [53], en verspreiding van isolaten resistent tegen breed spectrum β-lactamases wordt steeds vaker geobserveerd.

In hoofdstuk 6, namen wij waar dat 52% van de niet typhoide *Salmonella's* ESBL genen bevatten, daarnaast werd bij deze stammen ook het ESBL fenotype waargenomen. Hoewel verscheidene verschillende serovars werden geïdentificeerd, behoorde de grote meerderheid van de ESBLs tot de *Salmonella's serovar Agona*. Serovar Agona representeert het type *Salmonella* dat ook het meest uit de verschillende ziekenhuizen werd geïsoleerd. Verder blijkt dat de Multiresistente *S. Agona*, ten minste verantwoordelijk blijkt te zijn voor twee ziekenhuisuitbraken op kindergeneeskunde afdelingen in Brazilië [54, 55]. In beide gevallen werden grote plasmiden gedetecteerd die resistentie tegen veelvoudige antibiotica verleenden. Op dit moment zijn er zeer weinig studies die antibiotische resistentie in niet typhoide *Salmonella's* hebben bestudeerd [56]. Uit onze resultaten blijkt dat *S. Agona* ook een belangrijke bijdrage lijkt te leveren in de niet typhoide *Salmonella* ESBL-gereguleerde resistentie in India. Wat nog belangrijker en een bron van zorg is, is het feit dat twee S. Agona isolaten in deze studie genen bevatten van zowel ESBL als van fluoroquinolone resistentie. Dit dragerschap kan potentieel ernstige infectie behandelingsopties beperken, en daarnaast als een reservoir dienen voor de verdere verspreiding van deze combinatie resistentie genen onder andere pathogene bacteriën. Bovendien bleek in deze studie 48% van de niet typhoide isolaten van *Salmonella's* het SHV-12 ESBL gen te bezitten met daarnaast het corresponderend fenotype. SHV-12 is momenteel één van de meest voorkomende niet CTX-M ESBLs en wordt in vele Gram negatieve soorten geïdentificeerd, met inbegrip van *Salmonella* [57, 58]. De ESBL enzymen die verantwoordelijk lijken te zijn voor cephalosporin resistentie in de soort *Salmonella* zijn TEM en SHV β-lactamase derivaten, hoewel CTX-M type ESBLs onlangs ook (niet in grote aantallen) werd gerapporteerd.
In een studie door Rotimi et al., bleek 3.4% van de *Salmonella's* positief te zijn voor CTX-M-15 [59]. De aanwezigheid van ESBL en fluoroquinolone resistentie in niet typhoide *Salmonella's* brengt in de arme landen de klassieke antibiotische therapie in gevaar. Het voortdurende toezicht op het doorgeven van ESBL, evenals fluoroquinolone resistentie aanwezig in *Salmonella* is daarom ook in India noodzakelijk.

Vancomycin resistentie in *Enteroccus faecium*

Enterococci is een belangrijke groep bacteriën vooral betrokken bij verscheidene soorten ziekenhuis besmettingen zoals urineweg infecties, bacteremia, introabdominale infecties, huid, zachte weefselbesmettingen en endocarditis. Enterococci worden gevonden in zowel de samenleving als in het ziekenhuis gevonden en hebben vaak een hoge glycopeptides/aminoglycosides resistentie. De intrinsiek gevonden lage resistentie van Enterococci tegen veel algemeen gebruikte anti-microbiotica bv. β-lactams en glycopeptides kunnen eraan hebben bijgedragen dat deze bacterie met succes kan overleven in een ziekenhuis omgevingen. De al aanwezige lage resistentie tegen vele antibiotica heeft er zeer waarschijnlijk toe bij gedragen dat andere antibiotisch resistentie genen werden verworven, bijvoorbeeld resistentie op hoog niveau tegen glycopeptides (vancomycin en teicoplanin in *E. faecium*), en aminoglycosides (gentamicin weerstand in *E. faecalis*).
In Enterococcen zijn tot dusver zeven verschillende vancomycine resistentie types beschreven, namelijk VanA, VanB, VanC, VanD, Vin, VanG en VanL [60]. Vancomycin resistentie gewoonlijk meer aanwezig is in *E. faecium* dan in *E. faecalis*, waarbij VanA het

belangrijkst gen is vanuit het klinische oogpunt gezien. VanA wordt namelijk het vaakst gevonden bij resistenties in klinische Enterococcen isolaten, karakteristiek met hun hoge en induceerbare resistentie tegen vancomycine en teicoplanine. Vancomycine resistentie in *E. faecium* wordt wereldwijd gemeld, en wordt geïnduceerd door wijdverbreid gebruik van vancomycine in de ziekenhuizen, evenals het dierlijke avoparcin een groeipromotor die analoog is aan vancomycine). In *E. faecium*, wordt VanA resistentie gefaciliteerd door transposon Tn*1546* [61]. Dit is een transposon die wijdverspreid voorkomt in mensen, dieren en het leefmilieu [60]. In feite, is de significante diversiteit gerelateerd aan het Tn*1546* transposon, die voor het eerst werd beschreven door Arthur *et al*., gevonden in *Enterococcus faecium* BM4147 in 1993 [61]. De variaties in dit transposon gevonden in de vorm van puntmutaties, insertie sequentie elementen (IS) en deleties, zijn ook beschreven in verschillende andere epidemiologische studies [62-65]. Gebaseerd op deze studies, stelde Schouten et a., (2001) een evolutie schema voor bestaand uit 2 lijnen gebaseerd op de aard van deze mutaties en insertie sequenties in dit Transposon [66].

In hoofdstuk 7, beschrijven wij twee extra lijnen geassocieerd met VanA positieve *E. faecium* isolaten, geïsoleerd vanuit de ziekenhuizen in Saudi-Arabië. In deze studie, vonden wij dat de meerderheid van de polymorfe veranderingen in Tn*1546* veroorzaakt werden door de insertie van IS*1216V* aanwezig binnen een DNA stretch van de genen *vanS, vanH*, of *vanX* en vanY genen, samen met ander minder frequente gevonden inserties van IS*1485* en IS*1251*. Insertie van IS*1216V* is één van de meest voorkomende gebeurtenissen in transposon Tn*1546* gevonden in vancomycin resistente Enterococcen. De insertie IS*1485* werd voor het eerst beschreven in de Enterococcus soorten door Cheng *et al*. in 1999 [67], de gevonden insertie aanwezig in een isolaat uit Saudi-Arabië was een nieuwe bevinding.

In hoofdstuk 8, beschrijven wij een nieuwe variant van Tn*1546* die een nieuwe insertie genaamd IS*Efa5* bevat, geïsoleerd uit een VanA postieve *E. faeciums* aanwezig in ziekenhuizen in Paraguay. Dit IS element is slechts alleen eerder beschreven in een Braziliaanse *E. faecium* isolaat, waar IS*Efa5* uitsluitend in een DNA stretch gebied van VanX en VanY [64] werd gevonden. In onze Paraguayanse isolaten werd de insertie van IS*Efa5* gevonden tussen de VanS en VanH genen. Momenteel, is de insertie IS*Efa5* alleen beschreven in Zuid-Amerika. Het is dus interessant om te speculeren of deze insertie alleen beperkt is tot Zuid-Amerikaanse VanA *E. faecium* isolaten. Onze studie levert daarnaast meer bewijs over de evolutie van Tn*1546* in VanA *E. faecium* positieve isolaten. Daarnaast is het belangrijk om de diversiteit van Tn1546 VanA *E. faecium* te monitoren, omdat er voor vancomycin resistente *S. aureus* isolaten is aangetoond dat deze ook drager zijn van Tn*1546* met inbegrip van het IS*1251* element [68]. Het monitoren van de verspreiding van Tn*1546* van *E. faecium* en overdracht van *S. aureus* Tn*1546* aan *E. faecium* zal helpen om voorspellingen te kunnen doen over de verspreiding, van dit belangrijke resistentie element, nu en in de toekomst.

Tot slot, melden we dat de laatste jaren een clonale vorm van het VanA type *Enterococcus faecium* isolaten (CC17) is geïdentificeerd. Dit type is geassocieerd met ziekenhuisinfecties [69]. Voor dit type is er bewijsmateriaal die de suggestie wekt dat deze groepering door een hoge recombinatie frequentie, geassocieerd aan de *E. faecium* soorten, wordt bewerkstelligd [70]. Onze resultaten verkregen met stammen uit Saudi-Arabië en Paraguay, landen die geen eerder studies hebben gerapporteerd over VanA resistentie in *E. faecium*, ondersteunen de hoge aanwezigheid van het CC17 klonale isolaten in ziekenhuizen. Meer dan 90% van de VanA positieve *E. faecium* VanA isolaten van Saudi-Arabië en Paraguay behoord tot het klonale complex CC17.

De aminoglycosideresistentie is op hoog niveau in *Enterococcus faecalis*

Zoals hierboven vermeld, de al aanwezige lage resistentie van Enterococcen tegen veel algemeen gebruikte antibiotica helpen deze bacterie om in ziekenhuizen te overleven. En verwerft met succes nu ook verschillende andere antibiotica resistentie genen. *E. faecalis* veroorzaakt 75% van alle Enterococcoide besmettingen [71]. Deze soort is over het algemeen bestand tegen vele algemeen gebruikte antibiotica, met inbegrip van aminoglycosides, aztreonam, cephalosporins, clindamycin, de halfsynthetische penicilline, nafcillin en oxacillin, en het trimethoprim -trimethoprim-sulfamethoxazole. Resistentie is op hoog niveau tegen aminoglycosides (b.v. gentamicin) is een mogelijke oorzaak van de behandeling-belemmeringen in ziekenhuis besmettingen die door *E. faecalis* worden veroorzaakt. Aminoglycosides worden gewoonlijk samen met andere groepen antibiotica's gegeven om een synergetisch effect te produceren om hiermee een succesvol antibiotica therapie te verzekeren. De weerstand van Aminoglycoside resistentie in *E. faecalis* wordt geregeld door genen die diverse aminoglycoside-modificerende enzymen (AMEs) coderen, b.v. aac (6') - d.w.z. -d.w.z.-aph (2") - Ia, aph (2") - Ib, aph (2") - Ic, en aph (2")-Id. Deze AMEs wordt wijdverspreid in ziekenhuizen gevonden. Dit resulteert in bacteriële ziekteverwekkers die zeer resistent zijn tegen aminoglycosides, vooral met betrekking tot gentamicin resistentie in E. *faecalis*. *E. faecalis* verwerft daarnaast het transposon Tn*4001* gemakkelijk, een transposon die codeert voor een bifunctional enzyme, 6'-acetyltransferase-2'-phosphotransferase, die door het gen aac6-aph2 wordt gecodeerd [72-74]. Verder, is er een associatie tussen de aanwezigheid van HLGR stammen en het gebruik van andere niet-aminoglycoside antibiotica, zoals glycopeptides, cephalosporins, quinolones en carbapenems, nu goed gedocumenteerd [75-77].

In hoofdstuk 9, worden de resultaten getoond die uit de HLGR positieve *E. faecalis* isolaten werden verkregen, gekweekt in het Erasmus MC. De HLGR positieve *E. faecalis* stammen bleken allemaal bestand te zijn tegen ciprofloxacin en cefuroxime. Eerdere studies hebben gerapporteerd dat 2 klonale types (CC2 en CC9) en de enige ST16 type van *E. faecalis* kloon sterk geassocieerd worden met een hoge mate van gentamicin resistentie. Deze resistentie wordt voornamelijk gevonden in ziekenhuizen, waarbij onze studie vergelijkbare resultaten laat zien. Onze studie bevestigt daarmee de associatie van deze klonale stammen CC2/CC9 en ST16 genotypen in ziekenhuis infecties [78-80]. Van de belangrijkste ST types die in Erasmus MC circuleren, blijkt ST-2 vatbaar te zijn voor tetracycline, maar daarnaast resistent te zijn tegen levofloxacin en streptomycine. Terwijl ST6 resistent blijkt te zijn tegen tetracycline en levofloxacin, maar gevoelig is voor streptomycine. Verder blijken ST16 isolaten vatbaar te zijn voor levofloxacin. Het onderzoek van resistentie en gevoeligheidsprofielen voor deze antibiotica in de bovengenoemde ST types is belangrijk, omdat deze antibiotica in de behandeling van *E. faecalis* essentieel zijn. Alhoewel alle HLGR isolaten die in het Erasmus MC worden geïsoleerd gevoelig blijken te zijn voor ampicilline, blijkt uit het recentste Nethmap- rapport van 2009 [81], betreffende het gebruik van antibiotica en resistentie onder medisch belangrijke bacteriën in Nederland, een toename te zijn van het aantal van β-lactamase positieve *E. faecalis* isolaten in de Nederlandse ziekenhuizen. Niettemin is dit bij het Erasmus MC nog niet het geval, om te voorkomen dat dit ook hier in de toekomst een probleem wordt, moet men dit goed in de gaten houden.

Onderzoek heeft aangetoond dat de meerderheid van HLGR *E. faecalis* isolaten een plasmide bezitten die een transposon Tn4001 bevat [80, 82]. Casetta et a.l [83], vond dat Tn4001 wordt gestabiliseerd wanneer deze wordt opgenomen in het *E. faecalis* chromosoom, waarbij het verlies van flankerende sequenties van IS256 geassocieerd zijn met de aanwezigheid op plasmiden. Interessant, alle belangrijke HLGR *E. faecalis* ST types in onze studie, bezaten één of meer deleties van de flankerende Tn4001 IS256 sequenties. Dit kan er op wijzen dat plasmiden een belangrijke rol spelen in het verkrijgen van gentamicin resistentie binnen het Erasmus MC. Verder onderzoek is noodzakelijk om te bepalen of één

enkel plasmide type of meerdere plasmide types betrokken zijn in de transmissie van gentamicin resistentie tussen bacteriële soorten binnen Erasmus MC of in andere ziekenhuizen waar HLGR *E. faecalis* geïsoleerd worden.

Conclusie en opmerkingen

Antibiotica resistentie is een toenemende en voortdurende bedreiging voor zowel globale volksgezondheid als globale gezondheidszorgsystemen. Controle op antibiotica resistentie is daarom een belangrijke kwestie die voor de maatschappij van vandaag onder ogen moet worden gezien. Internationaal toezicht en epidemiologische studies tonen aan dat antibiotica resistentie een stijgende trend laat zien tegen de algemeen gebruikte antibiotica. Verder, betekent het feit dat de globalisering een hoge vlucht neemt, het belangrijk is om de internationale patronen van antibiotica resistentie te begrijpen en te volgen. Het internationale reizen draagt bij aan het feit dat antibiotica resistentie van micro-organismen steeds makkelijker wordt verkregen.

Bovendien, is de selectieve druk sterk door het vele ongepaste en algemene gebruik van antibiotica. Dit is een ander belangrijke bijdrager aan het probleem van antibiotica resistentie en zou zowel op het nationale niveau (via de regeringswetgeving) en op het plaatselijke niveau (via de richtlijnen van het ziekenhuisvoorschrift) moeten worden aangepakt.

Het ontstaan van verschillende „platforms“ die betrokken zijn bij het monitoren van antibiotica resistentie hebben duidelijk de doeltreffendheid van het antibiotica beleid aangetast. Een ander belangrijk aspect van antibiotica resistentie is het begrijpen van de mechanismen die betrokken zijn bij de verspreiding van resistentie op internationaal niveau. Dit kan via het opzetten van actieve toezichtprogramma's worden bewerkstelligd, aangezien de toezichtprogramma's belangrijke informatie kunnen verstrekken over de tendensen die er zijn in het ontstaan van antibiotica resistentie. Deze informatie zou kunnen worden gebruikt om toekomstige voorspellingen te kunnen doen over de verspreiding van antibiotica resistentie.

Tot slot zouden de volgende stappen in het aanpakken van antibiotica resistentie uiterst nuttig zijn:

- Een vermindering van het misbruik van antibiotica door standaard en strikte voorschriftpraktijken te ontwerpen en uit te voeren.
- Ontwikkeling van nieuwe antibiotica’s (10 x '20). [www.idsociety.org].
- Nieuwe antibiotica’s selectief gebruiken, d.w.z. slechts wanneer andere therapie geen effect meer heeft.
- De ontwikkeling van snelle, goedkope, en nauwkeurige diagnostische methodes om snelle en nauwkeurige antibiotica therapie toe te staan.
- De verbetering van scholing van burgers en gezondheidsmedewerkers t.a.v. antibiotica gebruik.
- De verbetering van de hygiëne in ziekenhuizen en in de samenleving.
- Een verbod op het niet-geneeskundige gebruik van antibiotica in de voedselindustrie en veehouderij
- Een vermindering van antibiotica gebruik via alternatieve benaderingen, zoals vaccins, bacteriofagen, en Probiotica.
- Ontwikkeling van betere alternatieve methodes voor anti-infectie therapie om hiermee de chemotherapy te omzeilen, zodat resistentie voorkomen kan worden.

Op dit moment valt het te bezien of overheden, ziekenhuizen en individuen deze strategieën kunnen en willen toepassen. Slechts een gezamenlijke en internationale aanpak zal uiteindelijk alleen de verspreiding van antibiotica resistentie kunnen voorkomen.

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Curriculum Vitae

Mushtaq Khan was born in Pulwama, Kashmir, India on 31st October 1972 and completed his secondary education at M. M. Higher secondary school Pulwama in 1992. Subsequently, finished the Bachelor's degree in Pharmacy from Oxford college of Pharmacy Bangalore (Bangalore University) India in 2000. Following to the pharmacy degree, worked in the pharmaceutical industry in sales and marketing for 4 years in India. Afterwards, joined the Masters degree course in "Molecular Life Sciences" in 2005 at HAN-University, Nijmegen, The Netherlands. After successfully finishing the Masters degree, eventually joined the department of Medical Microbiology and Infectious Diseases at the Erasmus University Medical Centre, Rotterdam, The Netherlands, in 2007, researching into the antimicrobial resistance spread and the role of mobile genetic elements in few clinically important bacteria under the supervision of Dr. John. P Hays.

List Of Publications

1) **Mushtaq A. Khan**, Martin van der Wal, David J. Farrell, Luke Cossins, Alex van Belkum, Alwaleed Alaidan and John P. Hays. Analysis of VanA vancomycin-resistant *Enterococcus faecium* isolates from Saudi Arabian hospitals reveals the presence of clonal cluster 17 and two new Tn*1546* lineage types. *J Antimicrob Chemother* 2008; 62: 279–283.

2) **M. A. Khan**, J. B. Northwood, R. G. J. Loor, A. T. R. Tholen, E. Riera, M. Falcon, Paraguayan Antimicrobial Network, A. van Belkum, M. van Westreenen and J. P. Hays. High prevalence of ST-78 infection-associated vancomycin-resistant *Enterococcus faecium* from hospitals in Asunción, Paraguay. *Clin Microbiol Infect* 2009 Jul 20. [Epub ahead of print].

3) **Mushtaq A. Khan**, Nicole Lemmens, Esteban Riera, Tamara Blonk, Janet Goedhart, Alex Van Belkum, Wil Goessens, John P. Hays and Mireille Van Westreenen. Dominance of CTX-M-2 and CTX-M-56 among extended-spectrum β-lactamases produced by *Klebsiella pneumoniae* and *Escherichia coli* isolated in hospitals in Paraguay. *J Antimicrob Chemother*. 2009 Dec; 64(6): 1330-2.

4) **Mushtaq A. Khan**, John Blackman Northwood, Foster Levy, Suzanne J. C. Verhaegh, David J. Farrell, Alex Van Belkum and John P. Hays. *bro* β-lactamase and antibiotic resistances in a global cross-sectional study of *Moraxella catarrhalis* from children and adults. *J Antimicrob Chemother*. 2010 Jan; 65(1): 91-7.

5) Godfred A. Menezes, **Mushtaq A. Khan** and John P. Hays. Important methodological considerations with respect to the differentiation of CTX-M-15 and CTX-M-28 extended-spectrum β-lactamases. *Indian Journal of Medical Microbiology*. *In press*.

6) Mohamudha R. Parveen, **Mushtaq A. Khan**, Godfred A. Menezes, Belgode N. Harish, Subhash C. Parija and John P. Hays. Molecular characterization of extended-spectrum β-lactamase producing *Klebsiella pneumoniae* isolated from blood cultures in Pondicherry, India. *Submitted.*

7) Godfred. A. Menezes, **Mushtaq. A. Khan**, Belgode. N. Harish, Subhash. C. Parija, Wil. Goessens and John. P. Hays. Molecular characterization of antimicrobial resistance in non-typhoidal salmonellae from India. *Submitted.*

8) **Mushtaq. A. Khan**, Saswati Sinha, Hubert P. Endtz, John Blackman Northwood, Alex van Belkum and John. P. Hays. Molecular epidemiology of high-level aminoglycoside resistant *Enterococcus faecalis* patients during and after hospitalisation. *Submitted*.

PhD Portfolio

Summary of PhD training and teaching

PhD student: Mushtaq Ahmad Khan Department: Medical Microbiology and Infectious Diseases, ErasmusMC, Rotterdam, The Netherlands Research School: Post-graduate Molecular Medicine	PhD period: 2007-2010 Promotor(s): Prof.dr.dr. A. van Belkum Supervisor: Dr.J.P. Hays

PhD training	**Year**
General courses	
- The Workshop on Phylogeny and Genetics in Microbiology and Virology	2008
- The Workshop on Basic data analysis on gene expression arrays I	2008
- Biomedical English Writing and Communication	2009
International Scietific Presentations	
- DRESP2 Scientific meeting, Budapest, Hungary (oral presentation)	2007
- DRESP2 Scientific meeting, Catania, Italy (oral presentation)	2007
- DRESP2 Scientific meeting, Stockholm, Sweden (oral presentation)	2008
- 19th European Congress of Clinical Microbiology and Infectious Diseases, Helsinki, Finland	2009
National Scietific Presentations	
- 1st International Workshop on Haemophilus Influenzae and Moraxella Catarrhalis (HinMax 2008), Rotterdam, The Netherlands (oral presentation)	2008
- The 2nd Symposium and Workshop on Molecular Microbiology of Infectious Diseases, ErasmusMC, Rotterdam, The Netherlands (oral presentation)	2008
- 11th ANNUAL Molecular Medicine Day	2007
- 12th ANNUAL Molecular Medicine Day (poster presentation)	2008
- 13th ANNUAL Molecular Medicine Day (poster presentation)	2009
- Scientific meeting Nederlandse Vereniging Voor Microbiologie (NVVM) Arnhem, The Netherlands	2007
- Scientific meeting Nederlandse Vereniging Voor Microbiologie (NVVM) Arnhem, The Netherlands (poster presentation)	2008
- Scientific meeting Nederlandse Vereniging Voor Microbiologie (NVVM) Arnhem, The Netherlands (poster presentation)	2009