Beta-lactamase Mediated Resistance in *Salmonella* spp. at a Tertiary hospital in KwaZulu-Natal

USHA GOVINDEN

Submitted in part fulfilment of the requirements for the degree of Doctor of Philosophy (Health Sciences) in the School of Pharmacy and Pharmacology in the Faculty of Health Sciences at the University of KwaZulu-Natal

Usha Govinden Student No. 9409195
School of Pharmacy and Pharmacology
University of kwaZulu-Natal

Supervisors Prof. SY Essack
Dean
Faculty of Health Science
University of KwaZulu-Natal

Prof AW Sturm
Dean
Nelson R Mandela School of Medicine
University of KwaZulu-Natal

Prof P Moodley
Department of Infection Control
University of KwaZulu-Natal

CONTENTS **PAGE**

ACKNOWLEDGEMENTS

I thank my supervisors Professor SY Essack, Professor AW Sturm and Professor P Moodley for their guidance and encouragement. I express my sincere appreciation and gratitude to Mrs Mocktar for technical assistance and Mr L Murugan and the staff of Pharmacy and Pharmacology for their support.
I am grateful to the National Research Foundation, Medicines Research Council and the University of KwaZulu-Natal for funding this study.
I thank my husband, my children, and my mum for their prayers, patience and love.

ABSTRACT

Extended spectrum β-lactamases (ESBLs) were characterized in *Salmonella* spp. isolates from a pediatric ward of a hospital in Durban. Forty one *Salmonella* spp. were subjected to serotyping, antibiotic susceptibility testing, E-Tests for ESBL detection, iso-electric focusing, polymerase chain reaction for detection of genes and sequencing. Isolates were screened for the presence of ^{bla}TEM, ^{bla}SHV, ^{bla}CTX-M, ^{bla}OXA , ^{bla}CMY, ^{bla}DHA and ^{bla}ACC genes. The most common serotype was *Salmonella* Typhimurium. Isolates were multi-drug resistant with 100% susceptibility only to meropenem and ciprofloxacin. Tazobactam was the most effective inhibitor. Forty-one percent of the isolates were resistant to ceftriaxone, thus limiting therapeutic options for *Salmonella* infections.TEM-1 was the most predominant β-lactamase found in 51% of isolates while SHV-12 found in 39 % was the most common ESBL. TEM-63 was evident in 29 %, TEM-116 in 10 % and TEM-131 was found in one isolate. The high ceftazidime MICs of isolates expressing only TEM-63 were indicative of R164S substitution which widens the binding cavity to accommodate the bulky side chains of oxyimino-aminothiazolyl cephalosporins. The identification of TEM-131 which differs from TEM-63 by 1 amino acid reiterates the evolutionary potential of the TEM-type β-lactamase. Other ESBLs identified included SHV-2, CTX-M-3, CTX-M-15 and CTX-M-37. CMY-2 and the OXA-1 β-lactamase were also detected. This is the first report of TEM-116, CTX-M-3, -15 and -37 in *Salmonella* spp. in South Africa. All isolates with nalidixic acid MICs > 48 µg/ml had the mutation D87N, or D87G in the QRDR of the *gyrA* gene. This study showed that *Salmonella* spp. may be multi-drug resistant with the propensity to harbour β-lactamases in unique combinations. The diversity of ESBLs and the co-expression of quinolone resistance suggests that their incidence in salmonellae needs to be monitored.

CHAPTER ONE - INTRODUCTION

1.1 *Salmonella* spp.

Salmonella organisms are Gram-negative bacilli that belong to the Enterobacteriaceae family. Microbial taxonomists consider almost all of the *Salmonella* that infect mammals and birds to be one species (*S. enterica*), based on iso-enzymes, rRNA sequences, and DNA hybridization. There are six subspecies of *S. enterica*, viz., *enterica* (1), *salamae* (2), *arizonae* (3a), *diarizonae* (3b), *houtenae* (4), and *indica* (6). Within these six subspecies are over 2000 serotypes of *Salmonella* determined by the carbohydrate structures and by flagella antigens. However, nearly all the serotypes that cause the disease in humans and domestic animals belong to subspecies *enterica*. Although closely related, there is enormous variation in the virulence and epidemiology of different serotypes of *S. enterica*. For instance, most serotypes cause only gastroenteritis, while specific serotypes cause enteric fever (Fierer and Guiney, 2001).

1.2 Salmonellae Infections

The World Health Organisation (WHO) in 2003 ranked infections as the leading global burden of disease and the leading cause of mortality in children (http://www.who.int/whr/2005/annexes-en.pdf Accesed 17 August 2008; Geddes *et al.*, 2007). Increasing antimicrobial resistance in non-typhoidal salmonellae (NTS) species has been a serious problem for public health worldwide. The high rate of resistance is hampering the use of conventional antibiotics, and there is growing resistance to newer antimicrobial agents (Su *et al.*, 2004). In developing countries NTS accounts for a steadily increasing proportion of human infections and represents about 20 - 30% of *Salmonella* serotypes, in particular multi-drug resistant *Salmonella enterica* serotype Typhimurium. The majority of extraintestinal NTS disease in African children occurs in infants and young children < 5 years old (Graham *et al.*, 2000; Wadula *et al.*, 2006). Outbreaks of NTS

predominantly present as a diarrheal disease acquired as food poisoning. In Africa the source and mode of transmission of *Salmonella* spp. is unclear and includes animals, animal products, water and infected humans. Young children may excrete the organism for up to 4 months. Outbreaks in hospitals and day-care centres support the existence of person-to-person spread which is associated with poor infection control and staff shortages. The clustering of patients in wards and the timing of presentation are associated with the spread of the organism (Wadula *et al.*, 2006).

1.3 Antibiotic Use and Resistance in *Salmonella* spp.

Although antibiotics are not usually recommended for *Salmonella* gastroenteritis, they are recommended for invasive *Salmonella* infection, e.g septicemia and meningitis which are common in infants, elderly and immunocompromised patients, particularly with human immunodeficiency virus (HIV). South Africa has the highest number of HIV-infected people in the world, with an estimated 5 million infected by the virus. Patients with HIV infection thus have an increased risk of invasive salmonellosis (Kruger *et al.*, 2004).

Up to a decade ago, conventional 1st-line antimicrobial agents such as ampicillin, chloramphenicol, and cotrimoxazole were the drugs of choice for the treatment of life-threatening *Salmonella* infections in many countries and, still remain the main therapeutic drugs of choice in most resource constrained African countries. However in the past two decades, isolation of *Salmonella* spp. multiply resistant to these agents has been reported. In 2007 the Enteric Diseases Reference Unit of the National Institute for Communicable Diseases in South Africa reported resistance to five or more antimicrobial agents in 33.8 % (n = 1502) of NTS isolates (Keddy, 2008). The prevalence of extraintestinal *Salmonella* infections caused by antibiotic resistant *Salmonella* spp. in several geographic areas of the world is increasing. In these cases, fluoroquinolones and the third generation cephalosporins are recommended as alternatives, with ciprofloxacin being the drug

of choice (Rotimi *et al.*, 2008). Widespread fluoroquinolone use in children has been discouraged because of the potential adverse effects on cartilage development. Therefore, extended-spectrum cephalosporins (especially cefotaxime or ceftriaxone), are the mainstay of treatment of serious infections due to NTS in children (Kruger *et al.*, 2004). Recently, reports of growing resistance to these agents have appeared in the literature from different parts of the world (Rotimi *et al.*, 2008).

The emergence of multi-drug resistant *Salmonella* is widespread in Africa (Graham *et al.*, 2000). In Zaire and Rwanda multi-drug resistant *Salmonella* Typhimurium were the predominant cause of bacteraemic illness in children, while in Kenya, this serotype was the main isolate in adults with salmonellae bacteraemia (Kariuki *et al.*, 2005). A marked increase in disease caused by *Salmonella* Typhimurium occurred in Kenya during the 1970s. Among Algerian infants *Salmonella* Typhimurium emerged as a dominant cause of salmonellosis from 1970 onwards (Graham *et al.*, 2000). Infection with multi-drug resistant NTS has been associated with an increased rate and duration of hospitalisation, a two-fold increased risk of death during a 2- year period after the infection, and an increased rate of invasive infection (Kariuki *et al* ., 2005).

1.4 β-lactam Antibiotics

ß-lactam antibiotics are the most frequently recommended class of antibiotics in the Standard Treatment Guideline (STG) and Essential Drugs List (EDL) devised by the South African Department of Health (National department of Health, 2006). Since the introduction of benzylpenicillin into clinical practice in 1940, several natural and synthetic ß-lactams have been described. Penicillin, penicillin derivatives, cephalosporins, cephamycins, carbapenems, monobactams and monocarbams are classified as ß-lactam antibiotics and are structurally related through the presence of a core β-lactam ring (Livermore and Williams, 1996; Williams, 1999). The physiological targets of β-lactam antibiotics are penicillin

binding proteins (PBPs) which are responsible for the synthesis and remodelling of the peptidoglycan which is a fundamental component of the cell wall of most bacteria and is of major importance in giving this layer its strength and rigidity. The amide group of the β-lactam ring is conformationally similar to the D-alanyl- D-alanine bond of peptidoglycan pentapeptide. Inhibition of transpeptidation results in the formation of a non functional peptidoglycan, causing bacteriolysis (Livermore and Williams, 1996; Matagne *et al.*, 1998).

ß-lactam resistance is becoming an increasing problem for clinicians worldwide, in both hospital and community settings. There are four major ways by which bacteria avoid the bactericidal effects of ß-lactams:

- production of ß-lactamases

β-lactamases hydrolyse the active β-lactam ring. The destruction of ß-lactams by ß-lactamases is the most common resistant mechanism in Gram-negative bacteria (Livermore, 1995).

- active efflux of the antibiotic

Efflux pumps expel the antibiotic before it can reach the target site hence inhibiting its action (Poole, 1994).

- decrease in the permeability of the antibiotic

The outer membrane in Gram-negative bacteria acts as an effective permeation barrier and retards the influx of antibiotic molecules into the bacterial cell. Altered porin profiles also retard the influx of the antibiotic (Nikaido and Vaara, 1985).

- alterations of penicillin-binding proteins (PBPs)

β-lactam antibiotics interfere with the biosynthesis of the bacterial cell wall by acting as analogues of the substrate for the PBPs that catalyse the synthesis of cross-linked peptidoglycan. A consequence of the alteration of PBPs is that the β-lactam antibiotic cannot bind to it and hence cannot inhibit it (Waxman and Strominger, 1983).

1.5 ß-lactamase Mediated Resistance

The first plasmid-encoded ß-lactamase that was able to destroy extended-spectrum ß-lactam antibiotics was described in Germany in 1983. It was related to the production of the variant of the SHV-1 (sulfhydryl variable) enzyme, a broad spectrum penicillinase found in *Klebsiella pneumoniae* (Canton *et al.*, 2006). SHV-1 differed from SHV-2 by replacement of glycine with serine at the 238 position. This mutation alone accounted for the extended spectrum properties of SHV-2. TEM-1 was first reported in 1965 from a patient in Greece, named Temoneira. This report was followed by the description in France of variants of TEM-1 and TEM-2 enzymes with hydrolytic properties similar to SHV-1 derivatives. TEM-1 is able to hydrolyse ampicillin at a greater rate than carbenicillin, oxacillin, or cephalothin, and has negligible activity against extended-spectrum cephalosporins. It is inhibited by clavulanic acid. TEM-2 has the same hydrolytic profile as TEM-1, but differs from TEM-1 by having a more active native promoter and by a difference in isoelectric point (5.6 compared to 5.4) (Paterson and Bonomo, 2005). The TEM and SHV derivatives were named as extended spectrum β-lactamases (ESBLs) in 1989 (Canton *et al.*, 2006). According to the structural classification and the function scheme, these ESBLs are generally class A enzymes of the 2be group, arising as a result of a few amino acid substitutions, from the common TEM and SHV-1β-lactamases (Bonnet *et al.*, 2000; Paterson and Bonomo, 2005). Changes at residue 164 are the most common changes observed in TEM variants. A reduction in the number of hydrogen bonds or the elimination of the electrostatic attraction weakens the linkage across the neck of the omega loop. This change allows more flexibility in the loop, which in turn opens more space for bulky ß–lactam substituents, thus increasing resistance to these ß–lactams (Knox 1995).

More than 150 TEM and over 90 SHV enzymes have been documented (http://www.lahey.org/Studies; Accessed 17 August 2008). ESBLs hydrolyse oxyimino-cephalosporins but are inhibited by clavulanic acid, are inactive against cephamycins and are often encoded by large plasmids that carry resistance

determinants to multiple antibiotics (Hopkins *et al.*, 2008; Mhand *et al.,* 1999). There are two major concerns with pathogens producing ESBLs, i.e., their capacity to cause therapeutic failures with cephalosporins and aztreonam when the isolate is susceptible in vitro, and their capacity for undetected, widespread dissemination (Hanson *et al.,* 2002).

Although reports of ESBLs associated with *Salmonella* spp. are not as many compared to those for other species in the family Enterobacteriaceae, the number of reported cases in this organism has been increasing (Mulvey *et al.*, 2003). ESBLs in salmonellae in Africa were first described in 1988 (Cardinale *et al.*, 2001). Salmonellae worldwide have been found to express a wide variety of ESBL- types including TEM, SHV, CTX-M, and PER enzymes. Additionally, *Salmonella* strains have been reported to produce plasmid-mediated AmpC-type ß-lactamases, the OXA- type class D ß-lactamase, and the plasmid-mediated Class A carbapenemase (Kruger *et al.*, 2004; Miriagou *et al.*, 2003).

A nosocomial outbreak of *Salmonella* infection in pediatric patients caused by *Salmonella enterica* serovar Isangi producing ESBLs was first reported from the Chris Hani Baragwanath Hospital, Johannesburg South Africa, in 2006 (Wadula *et al.*, 2006). Fortunately strains harbouring metallo-enzymes such as VIM-types or IMP types have not yet been reported for *Salmonella* spp. In the early 1990s, nosocomial epidemics due to TEM-type ESBL-producing *Salmonella* spp. occurred in Algeria. During the period 1984 -1990 extended-spectrum cephalosporin resistant NTS producing SHV-type ESBLs were frequently isolated in pediatric units of Tunisian hospitals, while hospital outbreaks in Tunisia over the period 1995 -2001 were caused by *Salmonella* strains producing SHV-2a. SHV-12- producing isolates of a novel serotype were isolated from human and poultry specimens in Senegal. Production of SHV-and TEM-type ESBLs is evident in NTS strains isolated in various European countries. There have been sporadic isolations of TEM-3, TEM-25- and SHV-2- producing strains in French hospitals. In some of these cases, the index strains had probably been introduced by patients

transferred from North African hospitals (Miriagou *et al.*, 2004). An ESBL study of 160 *Salmonella* spp. from 13 hospitals in South Africa conducted in 2004 reported that 15.6% of isolates produced TEM or SHV ESBLs (Kruger *et al.*, 2004). This study did not include the hospitals in Kwazulu-Natal.

The CTX-M ß-lactamases, a new family in class A ESBLs were characterized at the beginning of the 1990s with the first reports of the CTXM-1 enzyme from Germany (Bonnet *et al.*, 2000). CTX-M enzymes share extensive sequence similarity with the chromosomal ß-lactamases of *Klebsiella oxytoca.* They efficiently hydrolyse many newer broad-spectrum oxyimino-ß-lactams including cefotaxime, ceftriaxone, and aztreonam and are readily inhibited by tazobactam and clavulanate (Tzouvelekis *et al.*, 2000). There are over 70 CTX-M genes identified (http://www.lahey.org/Studies/; Accessed 17 August 2008) which are divided into five phylogenetic groups, (CTX-M-1, -2, -8, -9 and -25) based on their amino acid sequences. Ceftazidime hydrolyzing CTX-M-type ß -lactamases such as CTX-M-15, CTX-M-16 and CTX-M-19 were isolated in 2001 (Kimura *et al.*, 2007). CTX-M-type ESBLs display a level of resistance to cefotaxime and ceftriaxone significantly higher than to ceftazidime. The ceftazidime MICs for micro-organisms producing CTX-M-type ESBLs are usually within the susceptible range. Therefore the use of ceftazidime resistance as an indicator of ESBL production may miss ESBL-producing bacteria in the clinical microbiology laboratory (Rotimi *et al.*, 2008). A number of CTX-M mutants with increased ceftazidimase activity have been described. The mutations in these variants occur in two of the structural elements that de-limit the ß-lactam binding site, namely the terminal part of the B3 ß-strand and the omega loop. The Asp240Gly substitution in the terminal part of the B3 ß-strand is responsible for increased flexibility of the ß-strand, rendering the active site more accessible to the bulkier ceftazidime molecule, while the substitutions in the omega loop (at position 167) apparently modify the mode of interaction of ß-lactams with the binding site (Rossolini *et al.*, 2008).

Most CTX-M ß-lactamases reported in Africa (Kenya, Tanzania, Nigeria, Egypt) were from *K. pneumoniae* and *Escerichia coli* isolates. CTX-M-3 was found in a *Salmonella* isolate from the military hospital in Tunisia in 2001 and CTX-M-27 in isolates of *S. enterica* serotype Livingstone were the cause of a nosocomial outbreak in a neonatal ward in Tunisia in 2002 (Godet *et al.*, 2005). CTX-M enzymes are now endemic in many countries with both nosocomial and community emergence and some ESBL studies have identified CTX-M enzymes as the most prevalent ESBL. The epidemiology of CTX-M-producing strains is quite complex. Outbreaks of CTX-M clonal strains have been reported throughout the world (Abassi *et al.*, 2008).

PER-1, -2, and -3 comprise a highly clavulanate-sensitive family of ESBLs, with a different epidemiology from the TEM and SHV ESBLs. PER-1 was first identified in 1991 in a *Pseudomonas aeruginosa* isolate from a Turkish patient. PER-1 producing *Salmonella* Typhimurium strains were isolated from fatal nosocomial cases in 1992 at two hospitals in Istanbul (Vahaboglu *et al.*, 1996). PER-1 has also been detected in France, Italy, Belgium and Korea in *P. aeruginosa* and *Acinetobacter* spp. isolates (Paterson and Bonomo, 2005). PER-2 was first detected in *Salmonella* Typhimurium in Argentina, and is now reported to be the second most prevalent ESBL in that country. Recently PER-3 was discovered in an isolate of *Aeromonas punctata* in France (Moland *et al.*, 2008).

The presence of AmpC ß-lactamases in pathogens known not to have chromosomal *ampC* genes (like *Salmonella*) eventually led to the discovery of plasmid-borne AmpC enzymes such as ACT, ACC, DHA and CMY (Babic *et al.*, 2006). CMY (derived from *Citrobacter Freundii*) DHA (derived from *Morganella morganii)* and ACC-1 (derived from *Hafnia alvei*) have been found in *Salmonella* spp. (Miriagou *et al.*, 2004). CMY-2 is the most prevalent of the plasmid-mediated AmpC enzymes and the most widely distributed geographically. CMY-type ß-lactamases found in nosocomial enterobacteria, particularly in *K. pneumoniae,* and *Salmonella* spp. could have acquired the *ampC* gene from such microorganisms

(Miriagou *et al.*, 2002). CMY-2 confers resistance to various extended spectrum cephalosporins, including ceftriaxone, which is the antibiotic of choice for invasive *Salmonella* infections in children. The movement of the *ampC* gene on to plasmids and transmission to other organisms is of major concern (Hanson *et al.*, 2002). The expression of a plasmid-mediated CMY-2 ß-lactamase has been responsible for most ceftriaxone resistance in *Salmonella* spp. (Li *et al.*, 2005). In Africa the first report of the CMY-2 gene in *Salmonella* was from an Algerian clinical isolate of *S. enterica* serotype Senftenberg in 1997 (Koeck *et al.*, 1997), and in 2004 Kruger *et al.*, reported the CMY-2 gene in *Salmonella* Typhimurium and *S. enterica* serotype Schwarzengrund from South Africa.

Many organisms producing class C ß-lactamases may not be resistant to broad-spectrum cephalosporins when conventional Clinical laboratory standard institute breakpoints are used. Yet, adverse clinical outcomes in patients with infections caused by organisms producing plasmid-mediated class C ß-lactamases have been reported when these patients were treated with cephalosporins. It is imperative that *Salmonella* spp. producing plasmid-mediated class C ß-lactamases are detected and reported so that appropriate antimicrobial therapy and infection control measures can be initiated (Doi and Paterson, 2007).

KPC, SME, NMC-A and IMI comprise a small group of class A ß-lactamases (functional group 2f) with potent carbapenemase activities (Miriagou *et al.*, 2003). KPCs are capable of hydrolysing carbapenems, cephalosporins, and aztreonam, and they are inhibited by clavulanic acid and tazobactam (Cai *et al.*, 2008). The only carbapenemase reported in *Salmonella* spp. is of the KPC-type. KPC-producing *K. pneumoniae* strains have been found in hospitals in the USA and subsequently KPC-2 was found in a *Salmonella* serotype *Cubana* isolate also in a hospital in the USA. The emergence of *Salmonella* and *K. pneumoniae* strains producing plasmid-mediated KPC-type ß-lactamases in the USA further underlines the potential for exchange of resistance determinants between salmonellae and nosocomial enterobacteria (Miriagou *et al.*, 2004). The KPC-type reportedly confers

resistance to all ß-lactams with MICs of imipenem and meropenem reported as 16 and 8 mg/L respectively. These antibiotics are often the last therapeutic option used in cases of systemic infections in children due to ESBL-producing *Salmonella* (Arlet *et al.*, 2006). Other examples of non TEM, non SHV ESBLs such as GES, BES, SFO, TLA, IBC and VEB-1 have been described (Paterson and Bonomo, 2005) but not reported in *Salmonella* spp.

Historically the first characterised class D ß-lactamases were also referred to as oxacillinases because they commonly hydrolyse the isoxazolyl penicillin, oxacillin much faster than classical penicillins, i.e. benzylpenicillin. The designation OXA of the class D ß-lactamases, thus, refers to their preferred penicillin substrate. Most OXA-type ß-lactamases do not hydrolyse the extended-spectrum cephalosporins to a significant degree and are not regarded as ESBLs (Paterson and Bonomo, 2005). The first identified isolate expressing an OXA-type carbapenemase was the OXA-23 producing *A. baumannii* from Scotland. The isolate was recovered in 1985, before or at the time when imipenem was approved for general use. There has not been many reports of the OXA ß-lactamase in *Salmonella* spp. and the first, OXA-30, ß-lactamase was reported from an Australian pediatric *Salmonella* isolate in 2002. The substrate specificities of the OXA-type carbapenemases are diverse, but generally the enzymes hydrolyse penicillins (benzylpenicillin, ampicillin, piperacillin and ticarcillin) and the narrow spectrum cephalosporins, cephalothin and cephaloridine efficiently, while the extended-spectrum ß-lactams, ceftazidime, cefotaxime and aztreonam are not or very poorly hydrolysed. Most of the OXA-type carbapenemases have low hydrolytic activities against imipenem and especially against meropenem. Generally, class D ß-lactamases are inhibited less efficiently by clavulanate than the majority of the other group 2 ß-lactamases to which the class D enzymes belong. All OXA-type carbapenemases are inhibited more efficiently by tazobactam than by clavulanate. Most of the OXA-type carbapenemases confer only reduced susceptibility to the carbapenems, but unless secondary resistance mechanisms, such as altered permeability, reduced affinity of PBPs for carbapenems or increased influx are involved, the clinical

detection of organisms producing these enzymes remains difficult. The chromosomal location of many of the OXA-type carbapenemase encoding genes has contributed to the slow spread of these genes (Rasmussen and Hoiby, 2006).

1.6 Quinolone Resistance

The quinolones target bacterial type II topoisomerases, DNA gyrase and topoisomerase IV, which play important roles in DNA replication, chromosome segregation and DNA compaction. DNA gyrase is composed of two GyrA and two GyrB subunits and Topoisomerase IV is composed of two ParC and two ParE subunits (Okumura *et al.*, 2008). Qnr-type plasmid-mediated quinolone resistance determinants belong to the pentapeptide-repeat family of proteins and protects DNA gyrase from quinolone inhibition. Three major groups of Qnr determinants, Qnr A, Qnr B, and Qnr S have been identified worldwide in various members of the family Enterobacteriaceae (Wu *et al.*, 2008).

In *Salmonella* spp. as in other Enterobacteriaceae, a single point mutation in the quinolone resistance-determining region (QRDR) of the *gyrA* gene can mediate resistance to nalidixic acid and reduced susceptibility to fluoroquinolones such as ciprofloxacin. The most frequent point mutations in *Salmonella* spp. associated with resistance to quinolones occur in the *gyrA* gene resulting in substitutions at the Ser-83 position, often to Tyr, Phe, or Ala, and Asp-87 substitutions to Asn, Gly or Tyr. Substitutions in ParC are not as frequent as those found in GyrA. Changes in GyrB and ParE are rarely found in *Salmonella* spp. Although target gene mutations and efflux pumps are two mechanisms most commonly associated with fluoroquinolone resistance in bacteria, the additive or synergistic contribution of the two mechanisms in emerging fluoroquinolone resistance is not clear in *Salmonella* spp. There is evidence of strains with no mutation in the QRDR, but with a lack of the OmpF porin, which showed decreased susceptibility to fluoroquinolones (Fabrega *et al.*, 2008).

Resistance to nalidixic acid has been suggested to be an indicator of low level fluoroquinolone resistance (Rodriguez-Avial *et al.*, 2005). Although resistance to fluoroquinolones remains rare in *Salmonella* spp, reduced susceptibility is increasing worldwide and it has been suggested that fluoroquinolone-susceptible strains that test resistant to nalidixic acid may be associated with clinical failure or delayed response in fluoroquinolone-treated patients with extraintestinal salmonellosis (Cattoir *et al.*, 2007; CLSI, 2008). Detection of *Salmonella* spp. isolates showing decreased susceptibility to fluoroquinolones has become important as a result of the increasing prevalence of these strains and their association with treatment failure (Aznar *et al.*, 2007). The increasing quinolone resistance in *Salmonella* spp. may have serious clinical consequences. Although antimicrobial treatment is commonly not needed in gastroenteritis caused by NTS, effective therapy is necessary in invasive infection. If such an infection is caused by a *Salmonella* strain with reduced fluoroquinolone susceptibility, treatment with a fluoroquinolone may not be a safe alternative (Hakenen *et al.*, 2006).

A better understanding of the biology and epidemiology of resistant *Salmonella* isolates is needed to combat the emergence and spread, and to determine appropriate empirical therapy of infections caused by these organisms (Hanson *et al.*, 2002). Limited research of ß-lactamase mediated resistance on *Salmonella* spp. in South Africa motivated this study.

1. 7. Aim

To characterise resistance to ß-lactamase mediated resistance in putative ESBL positive *Salmonella* isolates collected at a tertiary hospital in KwaZulu-Natal.

1.8 Objectives

1.8.1 To verify the identity of the *Salmonella* bacterial strains by serotyping.
1.8.2 To determine the antibiogram conferred by the organisms using the disc diffusion and minimum inhibitory concentration methods
1.8.3 To verify the production of ESBLs using the E-Test.
1.8.4 To determine the pI value of β-lactamases produced by isoelectric focusing.
1.8.5 To detect the presence of β-lactamase genes by polymerase chain reaction.
1.8.6 To identify the genes detected by DNA sequencing.
Peripheral investigation
1.8.7 To detect mutations in the quinolone resistance determining region and to search for the *qnrA* gene.

CHAPTER TWO - PAPERS

2.1 Published Papers

- Govinden, U., Mocktar, C., Moodley, P., Sturm AW and Essack, S.Y. 2008. *Characterisation of ESBLs in Salmonella spp. at a tertiary hospital in Durban South Africa.* Diagnostic Microbiology and Infectious Disease, **62: 86-91**.

- Govinden U, Mocktar C, Moodley P., Sturm A W and Essack S.Y. 2007. *Geographical evolution of the CTX-M β-lactamase – an update.* African Journal of Biotechnology, **6 : 831-839.**

- Govinden, U., Mocktar, C., Moodley, P., Sturm A.W. and Essack, S.Y. 2006. *CTX-M-37 in Salmonella enterica serotype Isangi from Durban South Africa.* International Journal of Antimicrobial Agents, **28: 288-291.**

2.2 Submitted Paper

- Govinden, U., Mocktar, C., Moodley, P., Sturm AW and Essack, S.Y. *Detection of mutations in the gyrA of clinical Salmonella spp.* African Journal of Biotechnology. Manuscript number AJB-08-682.

ELSEVIER

International Journal of Antimicrobial Agents 28 (2006) 288–291

INTERNATIONAL JOURNAL OF Antimicrobial Agents

www.ischemo.org

CTX-M-37 in *Salmonella enterica* serotype Isangi from Durban, South Africa

U. Govinden [a,*], C. Mocktar [a], P. Moodley [b], A.W. Sturm [b], S.Y. Essack [a]

[a] *School of Pharmacy and Pharmacology, University of KwaZulu-Natal, Durban 4000, South Africa*
[b] *Medical Microbiology, University of KwaZulu-Natal, Durban 4000, South Africa*

Received 7 March 2006; accepted 19 May 2006

Abstract

β-Lactamase-mediated resistance was investigated in 59 putative extended-spectrum β-lactamase (ESBL)-positive *Salmonella* spp. from the paediatric ward of a tertiary hospital in Durban, South Africa. Three *Salmonella enterica* serotype Isangi cultured from stool samples were multidrug resistant, with susceptibility only to meropenem, piperacillin/tazobactam and cefoxitin. Isoelectric focusing revealed β-lactamases with isoelectric points of pI 5.8, 6.8 and 7.2. Sequencing identified β-lactamases CTX-M-37 and TEM-1. To our knowledge, this is the first report of CTX-M-37 from *S. enterica* serotype Isangi in South Africa.

Keywords: *Salmonella enterica* serotype Isangi; CTX-M-37; Multidrug resistant

1. Introduction

Resistance to expanded-spectrum β-lactam antibiotics in Enterobacteriaceae is often due to the presence of extended-spectrum β-lactamases (ESBLs), which are plasmid-mediated bacterial enzymes found in enteric Gram-negative organisms [1]. Although reports of ESBLs associated with *Salmonella* spp. are relatively rare compared with those for other species in the Enterobacteriaceae family, the number of reported cases in this organism has been increasing in recent years. *Salmonella* have been found to express a wide variety of ESBL types, including TEM, SHV, PER, OXA and CTX-M enzymes [2].

CTX-M-type β-lactamases are encoded by transferable plasmids and are found in various enterobacteria, mostly *Salmonella typhimurium*, *Escherichia coli*, *Klebsiella pneumoniae* and *Proteus mirabilis* [3]. This novel family of plasmid-mediated ESBLs has been classified in Ambler class A and in group 2be of the Bush, Jacoby and Medeiros classification [4].

CTX-M β-lactamases were characterised at the beginning of the 1990s in the first reports of the MEN-1 (CTX-M-1) β-lactamase. CTX-M-producing strains have since been reported over a wide geographic area [5]. In some countries, CTX-M-type enzymes are the most frequently isolated ESBLs from Gram-negative strains. CTX-M enzymes have been involved in several outbreaks, although isolation of CTX-M-producing strains remains sporadic [4]. Between January 2002 and March 2004, CTX-M-type ESBLs were reported for the first time in the UK, the USA, Italy, Turkey Bulgaria and Romania [6].

Approximately 40 CTX-M β-lactamases have been described and are divided into five major groups on the basis of similarities in amino acid sequences [7]. According to Woodford et al., CTX-M-37 can be categorised into the CTX-M-1 group. In South Africa, CTX-M-2- and CTX-M-3-type enzymes have been detected in *K. pneumoniae* [8]. In 2001, Kariuki et al. [9] reported a novel CTX-M-12, also from a *K. pneumoniae* isolate, in Kenya, but there has been no report of CTX-M-type

* Corresponding author. Tel.: +27 31 260 7413; fax: +27 31 260 7792.
E-mail address: Govindenu@ukzn.ac.za (U. Govinden).

0924-8579/$ – see front matter © 2006 Published by Elsevier B.V. and the International Society of Chemotherapy.
doi:10.1016/j.ijantimicag.2006.05.028

enzymes in *Salmonella* spp. in South Africa. We report the first cefotaximase CTX-M-37 from a multidrug-resistant (MDR) *Salmonella enterica* serotype Isangi isolated in South Africa.

2. Materials and methods

2.1. Bacterial strains

Salmonella isolates were cultured from stool samples received from the paediatric ward of a tertiary hospital in Durban in 2001. β-Lactamase characterisation was undertaken for 59 ESBL-positive *Salmonella* isolates received by the Antimicrobial Research Unit of the School of Pharmacy and Pharmacology at the University of KwaZulu-Natal. The identity of the strains as *Salmonella* spp. was confirmed using the API 20E system (bioMeriéux sa, Marcy l'Etoile, France). Serotyping of the isolates was performed by the hospital laboratory using the slide agglutination method on the basis of lipopolysaccharide (O) and flagellar (H) antigens and commercially available antisera, according to the Kauffman–White scheme for *Salmonella* serotyping [10]. *Escherichia coli* NCTC 50192 served as a source of plasmid markers.

2.2. Susceptibility testing and ESBL detection

Susceptibility testing was undertaken using the National Committee for Clinical Laboratory Standards disk diffusion test. Minimum inhibitory concentrations (MICs) were extrapolated by the BIOMIC® automated reading system and software (Giles Scientific, New York, NY), using the following antibiotics: ampicillin, amoxicillin/clavulanic acid, ampicillin/sulbactam, piperacillin, carbenicillin, ticarcillin, ceftazidime, cefalothin, ceftriaxone, cefepime, cefuroxime (parenteral), cefotaxime, cefoxitin, meropenem, aztreonam and piperacillin/tazobactam. ESBL production was confirmed by the Etest method according to the manufacturer's guidelines (AB Biodisk, Solna, Sweden).

2.3. Plasmid analysis

Plasmid DNA was extracted by the method of Kado and Liu [11]. Samples were analysed by electrophoresis in 1× TBE buffer at 120 V for 1 h on 0.8% agarose gels. Plasmid size was estimated by comparison with plasmids from *E. coli* NCTC 50192.

2.4. Isoelectric focusing (IEF) of β-lactamases

Bacterial cells were broken by the freeze–thaw method [12] and IEF of crude extracts was performed on polyacrylamide gels containing ampholines with an isoelectric point range of pI 3.5–9.5. β-Lactamase bands were visualised with nitrocefin (Oxoid Ltd., Basingstoke, UK).

2.5. PCR detection of bla_{TEM}, bla_{SHV} and bla_{CTX-M} genes

Bacterial DNA was prepared by suspending a few fresh colonies from an overnight Mueller–Hinton agar culture in 50 μL of sterile distilled water and heating the cells at 95 °C for 5 min. Polymerase chain reaction (PCR) amplification was then performed in a Gene Amp PCR System (Applied Biosystems, Foster City, CA). All the primers used are described in Table 1. PCR for amplification of bla_{TEM} and bla_{SHV} genes was carried out as described by Essack et al. [13]. The amplification mixture for the detection of bla_{CTX-M} genes was prepared in a final volume of 50 μL containing 2 μL of the template DNA, 10 pmol of primer, 25 μL of master mix (Applied Biosystems) and water. The PCR programme consisted of an initial denaturation step at 94 °C for 3 min, followed by 25 cycles of DNA denaturation at 94 °C for 30 s, primer annealing at 54 °C for 1 min and primer extension at 72 °C for 2 min and a final extension step at 72 °C for 7 min. Aliquots (5 μL) of PCR product were analysed by gel electrophoresis with 2% agarose. Negative controls comprised PCR mixtures with the addition of water instead of template DNA. Gels were stained with ethidium bromide at 10 μg/mL and photographed with ultraviolet illumination. A 1000 bp DNA ladder (Fermentas, Lithuania; purchased from Inqaba Biotechnical Industries) was used as a size marker.

2.6. Sequencing

The primers used for DNA sequencing are shown in Table 1. Sequencing of the amplified products was performed with the BigDye version 3.1 dye terminator cycle sequencer from Applied Biosystems.

2.7. Nucleotide sequence accession number

Sequences were analysed using the BLAST 2.0 (Basic Local Alignment Search Tool) software available on the website of the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST/; accessed 6 September 2005). The nucleotide and amino acid sequences of CTX-M-37 have been deposited in GenBank and assigned accession number AY935578.

3. Results

Serotyping confirmed isolates 509, 541 and 640 to be *S. enterica* serotype Isangi. The MIC data are shown in Table 2.

All three isolates were ESBL-positive. Isolates 509, 541 and 640 exhibited considerable resistance to carbenicillin, piperacillin, ticarcillin, cefotaxime, ceftazidime, cefalothin, ceftriaxone, cefuroxime and cefepime. Resistance was also noted with aztreonam, amoxicillin/clavulanic acid (MICs > 64 μg/mL), ampicillin and ampicillin/sulbactam (MIC > 48 μg/mL). Susceptibility to piperacillin/tazobactam, cefox-

Table 1
Primers used in this study

Primer	Function	Sequence (5′–3′)	Nucleotide positions
TEM 1 (F)	Amp	ATGAGTATTCAACATTTCCGTG	1–22
TEM 2 (R)	Amp/seq	TTCTGTGACTGGTGAGTACT	324–305
TEM 3 (R)	Seq	GAGTAAGTAGTTCGCCAGTT	595–576
TEM 4 (R)	Seq	TTACCAATGCTTAATCAGTGAG	861–840
SHV 1 (F)	Amp/seq	ATGCGTTATATTCGCCTGTG	1–20
SHV 2 (R)	Amp/seq	GTTAGCGTTGCCAGTGCTCG	865–846
SHV 3 (R)	Seq	CCGTTTCCCAGCGGTCAAGG	601–582
CTX-M-3A	Amp/seq	GGTTAAAAAATCACTGCG	3–20
CTX-M-1B	Amp/seq	CCGTTTCCGCTATTACAA	950–933
CTX-MF	Amp/seq	TTTGCGATGTGCAGTACCAGTAA	205–227
CTX-MR	Amp/seq	CGATATCGTTGGTGGTGCCATA	748–727

F, forward primer; R, reverse primer; Amp, amplification; Seq, sequencing.

Table 2
β-Lactam minimum inhibitory concentrations (MICs) for strains 509, 541 and 640

β-Lactam	MIC (μg/mL)		
	509	541	640
Carbenicillin	>512	>512	>512
Piperacillin	>512	>512	>512
Ticarcillin	>256	>256	>256
Cefotaxime	>256	>256	>256
Ceftazidime	>128	>128	>128
Cefalothin	>128	>128	>128
Ceftriaxone	>128	>128	>128
Cefuroxime	>96	>96	>96
Cefepime	>96	64	>96
Aztreonam	>64	>64	>64
Amoxicillin/clavulanic acid	>64	>64	>64
Ampicillin/sulbactam	>48	>48	>48
Ampicillin	>48	>48	>48
Piperacillin/tazobactam	8	6	10
Cefoxitin	6	4	6
Meropenem	1	0.5	1

itin and meropenem was exhibited by all three isolates. Plasmids of 63.8 kb and 148.5 kb were detected. β-Lactamases with pI values of 5.8, 6.8 and 7.2 were observed for isolates 509 and 640, whilst isolate 541 contained only two isoelectric points at pI 5.8 and 7.2. PCR amplification revealed that all three isolates harboured bla_{TEM} and $bla_{CTX\text{-}M}$ β-lactamase genes. No PCR products were obtained with primers specific for bla_{SHV} genes. Sequence data for each amplicon identified the genes as $bla_{TEM\text{-}1}$ and $bla_{CTX\text{-}M\text{-}37}$. The pI of 5.8 can be ascribed to the TEM-1 β-lactamase and the pI of 7.2 could account for the CTX-M-37 β-lactamase. The pI of 6.8, although suggestive of a TEM-type β-lactamase, needs further investigation.

4. Discussion

Resistance of *Salmonella* to expanded-spectrum cephalosporin antibiotics is of significant public health importance. Since 2000, the Enteric Diseases Reference Unit of the National Institute for Communicable Diseases in South Africa has noted increasing numbers of non-typhoidal *Salmonella* isolates, particularly *S. enterica* serotype Typhimurium and *S. enterica* serotype Isangi, with positive screening tests for ESBLs [2]. An outbreak of typhoid in KwaZulu-Natal owing to MDR resistant *S. typhimurium* was first reported in South Africa in 1992. There was a high mortality rate during this outbreak and only two children were successfully treated with the third-generation cephalosporins ceftriaxone and cefotaxime [14].

Isolates 509, 541 and 640 were ESBL-positive and resistant to multiple antibiotics, being susceptible only to meropenem, cefoxitin and piperacillin/tazobactam. Susceptibility to piperacillin/tazobactam is consistent with most class A ESBLs, as CTX-M enzymes exhibit greater susceptibilities to β-lactamase inhibitors [7]. The MIC of cefotaxime (>256 μg/mL) in all three isolates was higher than that for ceftazidime (>128 μg/mL). In contrast to TEM- and SHV-type cefotaxime-hydrolysing ESBLs, CTX-M enzymes are much more active against cefotaxime than against ceftazidime. The amino acid residues critical for their extended spectrum of activity are distinct from those of TEM- and SHV-1-derived ESBLs [2].

Amino acid substitutions in TEM- and SHV-type β-lactamases are known to result in the development of their extended spectrum of activity. In the case of SHV-2, the G238S substitution results in enlargement of the omega loop, giving larger molecule substrates such as the oxyimino-cephalosporins access to the active site. Similar events have occurred for the CTX-M enzymes, most notably amino acid substitution D240G in CTX-M-15, CTX-M-16, CTX-M-25, CTX-M-27, CTX-M-28, CTX-M-29, CTX-M-30 and CTX-M-32, which results in greater hydrolysis of ceftazidime [6,15]. Poirel et al. [16] have recently reported that CTX-M-19, a Pro167Ser mutant, could hydrolyse ceftazidime efficiently. Although the MIC of ceftazidime for isolates 509, 541 and 640 is >128 μg/mL, there was no amino acid substitution detected at Asp240 or Pro167. However, all characteristic substitutions assumed to be implicated in cephalosporin hydrolysis such as Cys-69, Ala-205, Ser-237, Thr-244 and Arg-276 in the CTX-M-type β-lactamases [17] are present

in CTX-M-37, which could contribute to the high MIC of ceftazidime and other cephalosporins.

The Asn104, Asn132, Ser237 and Asp240 residues present in all three isolates establish hydrogen bonds with the amide and aminothiazole groups of the acylamide cefotaxime chain. This unusual acyl intermediate of CTX-M enzymes in complex with cefotaxime may therefore be involved in the activities of the oxymino-cephalosporinases by fixing cefotaxime tightly in the binding site [7].

The intensive use of broad-spectrum cephalosporins such as cefotaxime could account for the emergence of the CTX-M enzyme. Mutants of CTX-M enzymes harbouring improved catalytic efficacies against ceftazidime have recently been observed, suggesting that the enzymes are evolving as a result of ceftazidime selection pressure [6]. The continued use of ceftazidime in South Africa could contribute to the evolutionary potential of the CTX-M enzyme. Screening by molecular methods will be necessary to determine the prevalence of CTX-M-producing strains in our hospitals.

5. Conclusion

Although the first CTX-M enzymes were characterised from strains isolated in 1989, their significant expansion started only in 1995 [7]. CTX-M-37 (GenBank accession number AY649755) was first reported in 2004 in Mangolia from an *Enterobacter cloacae* clinical isolate (http://www.ncbi.nlm.nih.gov; accessed 6 September 2005). To our knowledge, this is the first report of CTX-M-37 from *S. enterica* serotype Isangi in South Africa.

Acknowledgments

This study was funded by research grants from the MRC, NRF and the University of KwaZulu-Natal.

References

[1] Bradford PA, Yang Y, Sahm D, et al. CTX-M-5, a novel cefotaxime-hydrolyzing β-lactamase from an outbreak of *Salmonella typhimurium* in Latvia. Antimicrob Agents Chemother 1998;42:1980–4.

[2] Kruger T, Szabo D, Keddy KH, et al. Infections with nontyphoidal *Salmonella* species producing TEM-63 or a novel TEM enzyme, TEM-131, in South Africa. Antimicrob Agents Chemother 2004;48:4263–70.

[3] Tzouvelekis LS, Tzelepi E, Tassios PT, et al. CTX-M-type beta-lactamases: an emerging group of extended-spectrum enzymes. Int J Antimicrob Agents 2000;14:137–42.

[4] Eckert C, Gautier V, Saladin M, et al. Dissemination of CTX-M-type β-lactamases among clinical isolates of Enterobacteriaceae in Paris, France. Antimicrob Agents Chemother 2004;48:1249–55.

[5] Bonnet R, Sampaio JLM, Labia R, et al. A novel CTX-M beta-lactamase (CTX-M-8) in cefotaxime-resistant Enterobacteriaceae isolated in Brazil. Antimicrob Agents Chemother 2000;44:1936–42.

[6] Munday CJ, Boyd DA, Brenwald N, et al. Molecular and kinetic comparison of the novel extended spectrum beta-lactamases CTX-M-25 and CTX-M-26. Antimicrob Agents Chemother 2004;48:4829–34.

[7] Bonnet R. Growing group of extended-spectrum β-lactamases: the CTX-M enzymes. Antimicrob Agents Chemother 2004;48:1–14.

[8] Paterson DL, Hujer KM, Hujer AM, et al. Extended-spectrum β-lactamases in *Klebsiella pneumoniae* bloodstream isolates from seven countries: dominance and widespread prevalence of SHV- and CTX-M-type β-lactamases. Antimicrob Agents Chemother 2003;47:3554–60.

[9] Kariuki S, Corkill JE, Revathi G, et al. Molecular characterization of a novel plasmid-encoded cefotaximase (CTX-M-12) found in clinical *Klebsiella pneumoniae* isolates from Kenya. Antimicrob Agents Chemother 2000;45:2141–3.

[10] Kauffman F. Serologic diagnosis of *Salmonella* species. Copenhagen, Denmark: Munksgaard; 1972.

[11] Kado CI, Liu ST. Rapid procedure for detection and isolation of large and small plasmids. J Bacteriol 1981;145:1365–73.

[12] Livermore DM, Williams JD. Mode of action and mechanisms of bacterial resistance. In: Lorian V, editor. Antibiotics in laboratory medicine, 4th ed. Baltimore, MD: Williams & Wilkins Co.; 1996. p. 502–78.

[13] Essack SY, Hall LMC, Pillay DG, et al. Complexity and diversity of *Klebsiella pneumoniae* strains with extended-spectrum β-lactamases isolated in 1994 and 1996 at a teaching hospital in Durban, South Africa. Antimicrob Agents Chemother 2001;45:88–95.

[14] Coovadia YM, Gathiram V, Bhamjee A, et al. An outbreak of multiresistant *Salmonella typhi* in South Africa. Q J Med 1992;82:91–100.

[15] Woodford N, Ward ME, Kaufmann ME, et al. Community and hospital spread of *Escherichia coli* producing CTX-M extended-spectrum beta-lactamases in the UK. J Antimicrob Chemother 2004;54:735–43.

[16] Poirel L, Naas T, LeThomas I, et al. CTX-M-type extended spectrum beta-lactamase that hydrolyzes ceftazidime through a single amino acid substitution in the omega loop. Antimicrob Agents Chemother 2001;45:3355–61.

[17] Sabate M, Tarrago R, Navarro F, et al. Cloning and sequence of the gene encoding a novel cefotaxime-hydrolyzing beta-lactamase (CTX-M-9) from *Escherichia coli* in Spain. Antimicrob Agents Chemother 2000;44:1970–3.

African Journal of Biotechnology Vol. 6 (7), pp. 831-839, 2 April 2007
Available online at http://www.academicjournals.org/AJB
ISSN 1684–5315 © 2007 Academic Journals

Review

Geographical evolution of the CTX-M β-lactamase – an update

Govinden, U.[1*], Mocktar, C.[1], Moodley, P.[2], Sturm, A. W.[2] and Essack S.Y.[1]

[1]School of Pharmacy and Pharmacology, University of Kwazulu Natal, Durban, 4000.
[2]Nelson R Mandela, School of Medicine, Department of Medical Microbiology, University of Kwazulu Natal, Durban, 4000.

Accepted 23 January, 2007

The CTX-M- type extended spectrum β-lactamases (ESBLs) that preferentially hydrolyze cefotaxime are emerging globally and comprise of more than 50 enzymes. The emergence of novel CTX-M β-lactamases in several countries is noted as opposed to the transfer of established CTX-M genes from one country to another, suggestive of a *de novo* dissemination of CTX-M genes.

Key words: CTX-M β-lactamase, geographic evolution, epidemiology.

INTRODUCTION

Extended spectrum β-lactamases (ESBLs) are molecular class A or D β-lactamases , which are able to hydrolyze oxymino cephalosporins at a rate equal to or higher than 10% of that for benzylpenicillin, have an active-site serine, and are generally inhibited by β-lactamase inhibitors such as clavulanic acid, sulbactam or tazobactam. ESBLs are mostly encoded by large plasmids (up to 100 kb and even more) that are transferable from strain to strain and between bacterial species (Stürenburg and Mack, 2003). Hence, ESBLs are an increasingly important cause of resistance to multiple β-lactam drugs in gram-negative bacteria (Livermore and Hawkey, 2005) 'Classical' ESBLs such as TEM (Temoniera) and SHV (sulfhydryl variable) have evolved from the widespread plasmid encoded enzyme families (Stürenburg and Mack, 2003).

Within a few years after its first isolation from an *Escherichia coli* isolate from a Greek patient named Temoniera, the TEM-1 β-lactamase was found worldwide and production of this enzyme is now the most commonly encountered mechanism of resistance to the β-lactam group of drugs in gram-negative bacilli. The first TEM variant with increased activity against extended-spectrum cephalosporins was TEM-3, which was reported in 1987. Since then there has been a rapid increase in the number and variety of extended-spectrum TEM variants (Stürenburg and Mack, 2003). More than 150 TEM derivatives have currently been documented (http:// www.lahey.org/Studies; last assessed 1 December 2006).

The SHV family of β-lactamases appears to have been derived from *Klebsiella* spp. SHV-1, is universally found in *Klebsiella pneumoniae.* In 1983, three strains of *K. pneumoniae* and one strain of *Serratia marcescens* isolated in West Germany were able to transfer resistance to cefotaxime as well as to the newer cephalosporins. This new plasmidic β-lactamase, called SHV-2, was derived from a point mutation in SHV-1. This mutation, at position 238 from glycine to serine resulted in an enhanced affinity of the SHV-1 β-lactamase for oxyimino cephalosporins, with a significant rise in the MIC of cefotaxime and a more limited rise in the MIC of ceftazidime. Subsequently, a number of ESBL variants containing amino acid changes have been reported (Stürenburg and Mack, 2003). There are now over 90 SHV-type enzymes (http://www.lahey.org/Studies; last accessed 1 December 2006).

In 1989, non-TEM, non-SHV, ESBL-producing bacteria expressing a higher level of resistance to cefotaxime than to ceftazidime was described in *E. coli* isolates from Germany. Owing to the high activity against cefotaxime, these new members of the ESBL family were named CTX-M β-lactamases (Rasmussen and Hoiby, 2004). The CTX-M family comprises more than 50 enzymes from various countries as described in Table 1 and can be

*Corresponding author. E-mail: Govindenu@ukzn.ac.za. Tel: + 2731-2608251; Fax + 2731-2607792.

subclassified by amino acid sequence similarities. A phylogenic study reveals five major groups of CTX-M enzymes with the members of each group sharing >94% identity, whereas ≤ 90 % identity is observed between the members belonging to distinct groups (Bonnet, 2004). The five major groups are clusters of CTX-M-1,-2,-8, -9 and -25 (http://www.lahey.org/Studies/, last accessed 1 December 2006).

As a group, the CTX-M-type ß-lactamases are closest in amino acid identity to the chromosomal cephalosporinases of *Kluyvera georgiana*, *Kluyvera cryorescens*, and *Kluyvera ascorbata* (Paterson et al., 2003). The natural CTX-M β-lactamases of *K. ascorbata*, designated KLUA β-lactamases, are clustered in the CTX-M-2 group. The KLUA-2 β-lactamase of *K. ascorbata* strain IP15.79 is identical to the CTX-M-5 β-lactamase characterized in a *Salmonella enterica* serovar Typhimurium strain. The natural ß-lactamase KLUG-1 of *K. georgiana* strain CUETM4246-74 clusters with the CTX-M-8 β-lactamase. These relationships of amino acid sequences between the natural β-lactamase of *Kluyvera* strains and the CTX-M β-lactamase suggest that the natural ß-lactamases of *K. ascorbata* and *K. georgiana* are the progenitors of the CTX-M-2 and CTX-M-8 groups, respectively. A natural CTX-M β-lactamase has also been characterized from *K. cryocrescens*. This ß-lactamase, designated KLUC-1, shares only 85 to 86% identity with the most closely related β-lactamases, which belong to the ß-lactamases of the CTX-M-1 group, although an enzyme identitical to CTX-M-3 was isolated from a strain of *K. ascorbata*. The CTX-M-9 group is related to enzymes from *Kluyvera spp.* isolated from Guyana, which were identical to CTX-M-14 (Bonnet, 2004; Pitout et al., 2005a).

Different genetic elements are associated with *bla* CTX-M genes. IS*ECP1*-like insertion sequences are most frequently reported. This insertion sequence element has been found to be associated with four out of the five *bla* CTX-M clusters (CTX-M-1,-2,-9 and -25 clusters) (Lartigue et al., 2006). Many CTX-M genes are located near or within transposons, or within mobile gene cassettes, which could permit rapid dissemination, thus CTX-M-producing strains have a growing distribution and prevalence. South America, Mediterranean and Eastern European countries as well as East Asia account for most reported isolates (Hopkins et al., 2006).

EPIDEMIOLOGY OF CTX-M B-LACTAMASES

America

In Argentina, a nationwide replacement of cefotaxime with ceftriaxone in 1990 co-incided with severe infections, diagnosed with meningitis, septicaemia, and enteritis. The infections emerged in August 1990 and were caused by multiresistant strains of *Salmonella typhimurium* which were resistant to cefotaxime but susceptible to ceftazidime, owing to the production of CTX-M-2 (Rasmussen and Hoiby, 2004). ESBLs were assessed by Patterson et al. (2003) in a collection of 455 isolates of *K. pneumoniae* from 12 hospitals in 7 countries between 1996 and 1997. Of the 18 ESBL positive isolates from Argentina, 11 produced the CTX-M-2 type β-lactamase. An ESBL study of 427 enterobacterial strains identified CTX-M-2 in 19 isolates and CTX-M-31 (a variant of CTX-M-2) in 2 isolates (Quinteros et al., 2003). An ESBL study of 18 Enterobacteriacae strains collected in 1996 and 1997 from hospitals in Brazil identified CTX-M-8 in *Citrobacter amalonaticus* and CTX-M-2 in *Proteus mirabilis* (Bonnet et al., 2000). CTX-M-9 and CTX-M-16 (differing from CTX-M-9 by only 1 amino acid substitution) were observed in 2 of 3 *E. coli* strains from hospitals in Brazil in 1996 (Bonnet et al., 2001). During the period 2000 to 2002 CTX-M-14 ß-lactamases were responsible for a community-wide outbreak in the Calgary Health Region of Canada (Pitout et al., 2005b). In 2004 Abdalhamid et al. reported the presence of CTX-M-30 in *Citrobacter freundii* from 4 different patients in Canada. In 2004 in Colombia, 7 *K. pneumoniae* isolates that were collected from three different hospitals were positive for the CTX-M-1 group and CTX-M-12 was identified in 1 isolate (Villegas et al., 2004). In 2004 Liebana et al. reported CTX-M-15 that was characterized in 2002 from a paediatric patient with a *S. enterica* serotype Infantis infection in Honduras.

Asia

In Japan in 1986, Matsumoto et al. discovered a non-TEM, non-SHV ESBL, in a cefotaxime-resistant *E. coli* strain isolated from the faecal flora of a laboratory dog which was used for pharmacokinetic studies of ß-lactam antibiotics (Bonnet, 2004). A few years later, Ishii et al. (1995) reported on a CTX-M-1-related enzyme, designated Toho-1 (CTX-M-44), which was produced by a cefotaxime-resistant *E. coli* strain isolated from the urine of a patient in 1993 in Japan. In 1998, Ma et al. also reported a cefotaxime resistant *E. coli* isolate containing Toho-2 (CTX-M-45). Surveys of ESBL-producing Enterobacteriaceae in Japan showed that the CTX-M-2 and CTX-M-3 enzymes predominate. At least three outbreaks involving CTX-M enzymes have occurred in Japan, implicating clonal *E. coli* spread (Bonnet, 2004). The molecular types of CTX-M- ß-lactamases in Japan were investigated in 1397 gram-negative bacilli collected between 2001 and 2003. 317 isolates were positive for CTX-M-type ß-lactamases. The investigation revealed that gram-negative nosocomial bacilli producing the CTX-M-1, -2 or -9 group of enzymes had already been dispersed in various clinical settings in Japan, although strains that produce TEM-or SHV-derived ESBLs are infrequently found (Shibata et al., 2006).

The first cefotaximase-producing (CTX-M-3) in a non-clinical *S. enterica* serovar Senftenberg in Japan was reported in 2004. In this study 58 clinical and non-clinical

Table 1. CTX-M-type β-lactamases [a].

β-Lactamase	Organism	Country	Year of Genbank submission	GenBank Nucleotide accession number
CTX-M-1	*E. coli*	Germany	1995	X92506
CTX-M-2	*S. typhimurium*	Argentina	1995	X92507
CTX-M-3	*C. freundii*	Poland	1996	Y10278
CTX-M-4	*S. typhimurium*	Greece	1997	Y14156
CTX-M-5	*S. typhimurium*	Latvia	1997	U95364
CTX-M-6	*S. typhimurium*	Greece	1998	AJ005044
CTX-M-7	*S. typhimurium*	Greece	1998	AJ005045
CTX-M-8	*C. amalonaticus*	Brazil	1999	AF189721
CTX-M-9	*E. coli*	Spain	1999	AF174129
CTX-M-10	*E. coli*	Spain	2000	AF255298
CTX-M-11	*K. pneumoniae*	China	2000	AY005110
CTX-M-12	*K. pneumoniae*	Africa	2000	AF305837
CTX-M-13	*K. pneumoniae*	China	2000	AF252623
CTX-M-14*	*E. coli*	China	2000	AF252622
CTX-M-15	*E. coli*	India	2001	AY044436
CTX-M-16	*E. coli*	Brazil	2001	AY029068
CTX-M-17	*K. pneumoniae*	France	2001	AY033516
CTX-M-18*	*K. pneumoniae*	France	2000	AF325133
CTX-M-19	*K. pneumoniae*	France	2000	AF325134
CTX-M-20	*P. mirabilis*	France	2001	AJ416344
CTX-M-21	*E. coli*	France	2001	AJ416346
CTX-M-22	*K. pneumoniae*	China	2002	AY080894
CTX-M-23	*E. coli*	Germany	2002	AF488377
CTX-M-24	*K. pneumoniae*	China	2002	AY143430
CTX-M-25	*E. coli*	Canada	2002	AF518567
CTX-M-26	*K. pneumoniae*	United Kingdom	2002	AY157676
CTX-M-27	*E. coli*	France	2002	AY156923
CTX-M-28	*E. coli*	France	2003	AJ549244
CTX-M-29	*E. coli*	China	2003	AY267213
CTX-M-30	*C. freundii*	Canada	2003	AY292654
CTX-M-31	*Providencia spp.*	Argentina	2003	AJ567481
CTX-M-32	*E. coli*	Spain	2003	AJ557142
CTX-M-33	*E. coli*	Greece	2003	AY238472
CTX-M-34	*E. coli*	Spain	2003	AY515297
CTX-M-35	*k. oxytoca*	Japan	2004	AB176534
CTX-M-36	*E. coli*	Japan	2004	AB177384
CTX-M-37	*E. cloacae*	Mongolia	2004	AY649755
CTX-M-38	*K. pneumoniae*	China	2004	AY822595
CTX-M-39	*E. coli*	Israel	2005	AY954516
CTX-M-40	*E. coli*	United Kingdom	2004	AY750914
CTX-M-41	*P. mirbilis*	Israel	2005	DQO23162
CTX-M-42	*E. coli*	Russia	2005	DQO61159
CTX-M-43	*A. baumanni*	Bolivia	2005	DQ102702
CTX-M-44 (Toho-1)	*E. coli*	Japan	1994	D37830
CTX-M-45 (Toho-2)	*E. coli*	Japan	1996	D89862

Table 1. Contd.

CTX-M-46	*K. pneumoniae*	China	2004	AY847147
CTX-M-47	*E. coli*	China	2004	AY847143
CTX-M-48	*K. pneumoniae*	China	2004	AY847144
CTX-M-49	*K. pneumoniae*	China	2004	AY847145
CTX-M-50	*K. pneumoniae*	China	2004	AY847146
CTX-M-51	*E. coli*	Spain	2005	DQ211987
CTX-M-52	*K. pneumoniae*	China	2005	DQ223685
CTX-M-53	*S. enterica*	France	2005	DQ268764
CTX-M-54	*K. pneumoniae*	Korea	2005	DQ303459
CTX-M-55	*E. coli*	China	2005	DQ343292
CTX-M-56		Not released		
CTX-M-57	*S. enterica*	United Kingdom	2006	DQ810789
CTX-M-58		Not released		
UOE-1	*E. coli*	Japan	2000	AY013478

*Amino acid sequences of CTX-M-14 and CTX-M-18 are identical.
[a]Data adapted from Lahey (http://www.lahey.org/Studies/ ; last accessed 1 December 2006) and Genbank (http://www.ncbi.nlm.nih.; last accessed 1 December 2006).

isolates of various *Salmonella* serovars were screened for ESBL production. Only 1 strain of *S. enterica* serovar Senftenberg was isolated from river water in Hiroshima in 1999 and displayed an ESBL phenotype (Ahmed et al., 2004).

In China, CTX-M-3,-9,-13 and-14 type enzymes have been reported from *E. coli*, *K. pneumoniae*, and *Enterobacter cloacae* strains. At the Huashan Hospital in China, CTX-M enzymes were the second most frequent ESBLs after SHV enzymes in *K. pneumoniae* (8 of 80) and *E. coli* (13 of 58) strains in 1999 (Bonnet, 2004). Molecular characterization of 57 ESBL strains in a study in 2005 in China revealed that the majority of the strains (94.7%) were CTX-M type, with a predominance of CTX-M-14 and -3 types (Pottumarthy et al., 2005). An ESBL study in the Anhui province in China identified 54 CTX-M positive *E. coli* and *K. pneumoniae* isolates. The isolates contained CTX-M-14 type ß-lactamase with one to three point mutations occurring in eight isolates. The enzymes were designated CTX-M-46,-47,-48,-49 and -50 (Li and Li, 2005; http://www.lahey.org/Studies/; last accessed 1 December 2006).

In Taiwan, at the National Cheng Kung University Hospital, a study of ESBL-producing *K. pneumoniae* strains conducted in 1999 revealed predominance (57.9%) of unrelated CTX-M-3-producing strains. Another survey performed in 24 hospitals between 1998 and 2000 showed inter- and intra-hospital clonal dissemination of CTX-M-3-producing (28 of 50) and CTX-M-14-producing (22 of 50) *K. pneumoniae* strains (Bonnet, 2004). In 2003 Paterson et al., reported the presence of a CTX-M-3 type ß-lactamase in a single *K. pneumoniae* isolate from a hospital in Taiwan. During September 2000 to December 2001 88 ESBL Enterobacteriaceae isolates from the Chang Gung children's hospital in Taiwan produced CTX-M-3 in 52 isolates. This was the most prevalent ESBL. CTX-M-3 was also the most common type of ESBL produced by *E. coli* and *K. pneumoniae* (Wu et al., 2003). Wu et al. also identified CTX-M -3 in 22 out of 34 *S. marcescens* clinical isolates from a medical centre in Taiwan. A study reported in 2006 from seven medical centres in Taiwan, described CTX-M-type ß-lactamases as one of the most prevalent ESBLs. CTX-M- 3, -9, -14, -15, -17, -19, and -38 were identified in this study (Yan et al., 2006).

In different parts of Korea, the CTX-M-14 enzyme was also observed in *K. pneumoniae* and *E. coli* strains between 1995 and 1996 and in a *Shigella sonnei* strain isolated during an outbreak of gastroenteritis in 2000 (Bonnet, 2004). CTX-M-3, -9, -14 and -15 were detected in 41 out of 603 isolates of Enterobacteriaceae collected in 2003 from three university hospitals in Korea (Kim, 2005). In 2004 a nosocomial outbreak of paediatric gastroenteritis in Korea was caused by CTX-M-14 type ESBL producing strains of *S. enterica* serovar London. The isolates had pulsed-field gel electrophoresis patterns identical to those of the previously isolated antimicrobial susceptible strains from community-acquired gastroenteritis, suggesting the susceptible clone acquired the resistance (Yong et al., 2005). A novel ceftazidime-hydrolyzing CTX-M mutant, CTX-M-54, produced by a *K. pneumoniae* isolate in Korea was reported by Bae et al. (2006). CTX-M-14 and a variant designated CTX-M-17 have commonly been observed in *E. coli* and *K. pneumoniae* strains since 1996 in Ho Chi Minh City, Vietnam (Bonnet, 2004).

CTX-M-37 (Genbank accession number AY 649755) was first reported in 2004 in Mongolia from *E. cloacae* cli-

nical isolate (http://www.ncbi.nlm.nih. last accessed 1 December 2006). Five Enterobacteriaceae strains producing CTX-M-2, -9, -11 and -15 were reported in 2004 in Singapore (Koh et al., 2004). In India, CTX-M-15 was reported in 2001 from six non-clonally related members of the family Enterobacteriaceae (Karim et al., 2001).

Europe and Middle East

At a Hospital in Warsaw, Poland, during a 4-month period between 1996 and 1997, the majority (27 of 35) of ESBL-producing strains of the family Enterobacteriaceae expressed a CTX-M-3-like enzyme. A 4-month survey performed in seven Polish hospitals in 1998 revealed the predominance of an SHV -ESBL (60.4%) and similar frequencies of TEM and CTX-M ESBLs (20.8 and 18.8%, respectively). A wider survey undertaken between 1998 and 2000 in 15 hospitals in 10 different cities of Poland revealed the countrywide dissemination of the CTX-M-3 enzyme. This great inter- and intra-hospital outbreak was due to the clonal spread of a few strains and more particularly to the dissemination of a CTX-M-3-encoding plasmid in *E. coli*, *K. pneumoniae*, *Klebsiella oxytoca*, *C. freundii*, *S. marcescens*, *E. cloacae*, and *Morganella morganii*. *S. enterica* serovar Typhimurium strains harboring a distinct CTX-M-3-encoding plasmid have also been reported. CTX-M-15, a variant of CTX-M-3 previously described in India, has also been observed in Poland as well as in Bulgaria, Romania, and Turkey (Bonnet, 2004). The CTX-M-2 type ß-lactamase in a single *K. pneumoniae* isolate from a hospital in Turkey was reported by Paterson et al. (2003). A large outbreak of *Salmonella* gastroenteritis that involved 4000 children in Latvia in 1990 was still ongoing when reported in 1998. The majority of cases were associated with *S. typhimurium* strains producing CTX-M β-lactamases. CTX-M-5 was found in one of these strains (Tzouvelekis, 2000).

A small outbreak involving CTX-M-4-producing *S. enterica* serovar Typhimurium strains occurred in Russia in 1996. The strain involved has been observed in Greece and Hungary. Clonal spread of CTX-M-producing *S. enterica* serovar Typhimurium strains in at least three European countries was detected. The enzymes implicated (CTX-M-4,-6, and -7) were variants of CTX-M-2, like the CTX-M-5 observed in the Latvian strain. CTX-M-3-producing *E. coli* strains unrelated to those reported in Poland were also isolated in Greece (Bonnet, 2004). An outbreak of *K. pneumoniae* producing CTX-M-3-type β-lactamases occurred in Novosibirsk in the period 1997 to 1998. The outbreak was caused by a proliferation of 2 major clones. Between 1997 and 1998, nosocomial isolates of *E. coli* and *K. pneumoniae* were collected from 28 Russian hospitals with CTX-M-1 β-lactamase being the most prevalent (Rasmussen and Hoiby, 2004).

The CTX-M enzyme was first characterized in Western Europe in two *E. coli* strains isolated in 1989 in Germany and in France from an Italian patient. In 2004 Stürenburg et al. reported the presence of CTX-M-23 from *E. coli* and *K. pneumoniae* strains which were isolated from a 46-year-old man in Germany during treatment of postoperative peritonitis with ceftazidime (Stürenburg et al., 2004). Since 1989, 11 different CTX-M enzymes have been reported in France from sporadic *E. coli* (CTX-M-1,-2,-9,-14, -18, -19, - 21 and -27), *P. mirabilis* (CTX-M-1,-2 and -20), and *E. cloacae* (CTX-M-1 and -3) isolates (Bonnet, 2004). However, in 2006 Eckert et al. (2006) reported 7 CTX-M-type β-lactamases (CTX-M-1, -2, -3, -9, -14, -15 and -24) among 28 strains of Enterobacteriaceae that were collected from five different hospitals in Paris, France. The emergence and spread of three clonally related virulent isolates of CTX-M-15-producing *E. coli* in a French geriatric hospital was reported in 2004 (Leflon-Guibout et al., 2004).

Paterson et al. (2003) identified 1 CTX-M-2 type ß-lactamase from a *K. pneumoniae* isolate in Belgium in 2003. Three hundred and sixty Enterobacteriaceae and non fermenting gram-negative bacilli isolated during one week in 2004 at 5 hospitals in Netherlands were evaluated for the presence of ESBLs. CTX-M-1,-2,-9 and -15 were found among 18 isolates (Naiemi et al., 2006). At a Hospital in Madrid, Spain, the investigation of ESBL-producing *Enterobacter* strains from 1989 to 2000 showed the persistence of CTX-M-10 over a 12-year period in unrelated isolates. At a Hospital in Barcelona, Spain, the majority (6 out of 10) of ESBL-producing Enterobacteriaceae isolated between 1994 and 1996 produced CTX-M-9 enzymes. In the same area, a CTX-M-9-like enzyme was also observed in three *S. enterica* serovar Virchow strains isolated between 1997 and 1998. In the northwest area of Spain, 50% of ESBL-producing strains of the family Enterobacteriaceae isolated in 2001 produced the CTX-M-14 enzyme (Bonnet, 2004). Four *S. enterica* serovar Virchow strains resistant to broad-spectrum cephalosporins were isolated from patients with gastroenteritis in 1997 and 1998 in Murcia and Barcelona, Spain. The isolates expressed a CTX-M-9 type β-lactamase (Simarro et al., 2000). In 2003 Pagani et al. reported the detection of CTX-M-1,-2 and -15 in 12 out of 232 ESBL producers from a Spanish hospital in Northern Italy. The most prevalent CTX-M ESBLs [CTX-M-9 (27.3%) and CTX-M-14 (20.5%)] were found in *E. coli*, in a nationwide study of *E. coli* (n = 170) and *K. pneumoniae* (n = 70) producing ESBLs in Spanish hospitals in 2005, whilst CTX-M-10 was found in only 3 *K. pneumoniae* isolates (Hernandez et al., 2005). In 2005 a large outbreak was caused by CTX-M-1- producing multiresistant *K. pneumoniae* in a Spanish intensive care unit (51 patients) (Mena et al., 2006). The prevalence and types of genes encoding ESBLs in 642 clinical isolates of Enterobacteriaceae, *Pseudomonas aeruginosa*, and *Acinetobacter* spp. were assessed in Bolivia in 2004. 106 out of the 150 putative ESBL producing isolates con-

tained CTX-M-2, and 32 isolates contained CTX-M-43 (Celenza et al., 2006). A study in 2005 reported the detection of CTX-M-1-producing clinical isolates of *C. amalonaticus* and *M. morgani* from an area of Northern Italy where CTX-M producers were found to be widespread in *E. coli*. This study showed that the CTX-M-1 β-lactamase was possibly acquired by these unusual hosts *in vivo*, after co-infection with *E. coli* strains carrying conjugative plasmids bearing the *bla* CTX-M-1 gene (Mugnaioloi et al., 2005). The first description of CTX-M-15 producing *K. pneumoniae* in Portugal was reported in 2005 (Conceicao et al., 2005).

The first outbreak caused by *K. pneumoniae* producing CTX-M-26 was recorded in Birmingham England in 2001 (Livermore and Hawkey, 2005). The United Kingdom (UK) has experienced a sudden rise in ESBL rates, largely due to the appearance and spread of *E. coli* producing CTX-M-15 type β-lactamase. The first significant outbreak of CTX-M producers in the UK occurred in 2001 involving *K. pneumoniae* with CTX-M-26 at one site, but by 2003, cloned and diverse *E. coli* with CTX-M-15 were widespread (Livermore and Hawkey, 2005). CTX-M-40 from an *E. coli* strain was reported in 2006 from the UK. The strain had been isolated from an immunocompromised hospital patient in 1998, which had initially been treated with piperacillin/tazobacam and netilmicin but did not respond clinically until piperacillin/tazobacam was replaced with meropenem (Hopkins et al., 2006).

Among 149 ESBL producing Enterobacteriaceae isolates collected from patients in Austria from 1998 to 2004, 38 *E. coli* isolates and 11 *Klebsiella* spp. were CTX-M producers. The proportion of CTX-M producers (group 1 and group 9) among all ESBL positive isolates rose from 0% in 1998 to 58% in 2004. One *E. coli* isolate was identical to the UK epidemic CTX-M-15- producing strain, although no epidemiological link with the UK was apparent (Eisner et al., 2006). Between January 2001 and April 2005 a large collection of human and animal isolates of *Salmonella* spp. was collected in Ireland to determine the prevalence of ESBLs. Seven ESBL producing isolates were detected. Two isolates produced CTX-M-15 and one isolate produced CTX-M-14 (Morris et al., 2006).

In 2005 a study was reported on the extent of ESBL producing Enterobacteriaceae at hospital and community level in Lebanon. Out of a total of 72 strains, 83% expressed the CTX-M-15 β-lactamase (Moubareck et al., 2005). The molecular epidemiology of ESBL producing *E. coli* isolates (n = 20) was investigated in a tertiary care-teaching hospital in Tel Aviv, Israel in 2005. 15 isolates exhibited CTX-M-2, and 3 isolates exhibited a new CTX-M-39 β-lactamase (Chmelnitsky et al., 2005). ESBL production was demonstrated in five independent, multi-drug-resistant isolates of enteroaggregative *E. coli* (EAEC) from the United Arab Emirates, representing 11.3% of the EAEC isolates recovered during 1 year. All five isolates carried the *bla*CTX-M-15. This is the first detailed description and characterization of ESBL production in EAEC and also the first report of CTX-M-producing organisms encountered on the Arabian Peninsula (Sonnevend et al., 2006).

Australia

The CTX-M-3 type ß-lactamase from a nosocomial *K. pneumoniae* isolate was reported for the first time in Australia by Paterson et al. in 2003.

Africa

The first report from Africa of a CTX-M-type β-lactamase (CTX-M-12) was from Kenya in 2001.This study involved nine *K. pneumoniae* isolates from new born babies at Kenyatta National Hospital in Nairobi, Kenya (Kariuki et al., 2001). CTX-M-15 reported in 2005 in Africa, was found in 5 *E. coli* isolates and 1 *K. pneumoniae* isolate out of 19 ESBL producing isolates in Tanzania. In this study only 1 *Salmonella* isolate was also ESBL positive, but did not produce the CTX-M enzyme (Blomberg et al., 2005). In 2005 Gangoue-Pieboji et al. reported the emergence of CTX-M-15 in three isolates of *K. pneumoniae* and *E. coli* derived from patients with urinary tract infections acquired during hospitalization in Cameroon. In Nigeria in 2005 ESBLs were characterized from 30 selected multi-drug resistant *K. pneumoniae* strains isolated from patients with community acquired urinary tract infections from Southwest Nigeria. The 30 strains produced multiple β-lactamases with 57% producing CTX-M β-lactamase. Only 2 CTX-M type genes were sequenced and were found to produce CTX-M-15 (Soge et al., 2006). The presence of β-lactamases with an extended spectrum of activity in 46 clinical *E. coli* isolates in Egypt was reported in 2006. 28 out of 46 strains produced CTX-M β-lactamases. CTX-M-14,-15 and -27 were found, with CTX-M-15 (25 out of the 28 strains) being the most prevalent (Al- Agamy et al., 2006). In 2001 a *Salmonella* isolate from the military hospital in Tunisia was found to produce a CTX-M-3 ESBL. CTX-M-27 production in 16 isolates of *S. enterica* serotype Livingstone were the cause of a nosocomial outbreak in the neonatal ward of Farhat Hached Hospital, in Tunisia in 2002 (Godet et al., 2005). In 2003 Paterson et al. identified a CTX-M-2-type and a CTX-M-3-type β-lactamases in 2 separate *K. pneumoniae* isolates from South Africa. This was the first report of CTX-M–type β-lactamases in South Africa. CTXM-37 was also reported in 2006 in Durban, South Africa from three *S. enterica* serotype Isangi strains. This study involved 59 putative ESBL *Salmonella* strains from a tertiary hospital in Durban (Govinden et al., 2006). A further study in 2006 of ESBL positive *Salmonella* sp. revealed the presence of a CTX-M-38 enzyme (Genbank accession number DQ864700). A study in Central African Republic was conducted between 2003 and 2005 to determine the fre-

quency of ESBLs and to characterize β-lactamases in 450 Enterobacteriaceae isolates at the Institut Pasteur de Bangui. Of the 4% of ESBL producing strains, CTX-M-15 was present in 10 *E. coli* and 1 *K. pneumoniae* isolate, whilst CTX-M-3 was present in only 1 *E. aerogenes* isolate (Frank et al., 2006). Four sequential ESBL-producing isolates of *K. pneumoniae* were detected during routine culture and susceptibility tests in the Ampath service laboratory in Cape Town, South Africa. The first and fourth isolates were susceptible to ertapenem, whereas the second and third were resistant. All 4 isolates belonged to the same strain and produced a group 1 CTX-M enzyme (Elliott et al., 2006).

BIOCHEMICAL EVOLUTION

Most CTX-M enzymes exhibit a much greater hydrolytic efficiency against cefotaxime than against ceftazidime. In the cefotaxime intermediate structure with Toho-1, residues Pro167, Asn170, Ser237, Asp240, and Arg274 are surrounded the bulky side chain of cefotaxime. In addition both side oxygens of Asp240 interact with the amino group in the aminothiazole ring, which may be involved in the binding of cefotaxime (Shimamura et al., 2002). In the CTX-M ESBLs, unlike those of TEM- and SHV-, increased activity against the bulky third generation cephalosporins, especially ceftazidime appears to occur not from gross enlargement of the active site, but from increased flexibility of the ß3 strand and possibly other regions. This increased flexibility is correlated with higher ceftazidimase activity and lower stability (Chen et al., 2005).

The presence of Lys and Arg residues at position 240 are known to increase the enzymatic activities of the TEM and SHV ESBLs against ceftazidime. The Lys and Arg residues are positively charged and can form an electrostatic bond with the carboxylic acid group on oxyimino substituents of ceftazidime. Neutral residue Gly240 is not able to form electrostatic interactions with ß-lactams but could favor the accommodation of the oxyimino-ceftazidime side chain (Bonnet, 2004). Residue Gly240 is present in the ESBLs PER, VEB-1, and BES-1, which have hydrolytic activity against ceftazidime (Delmas et al., 2006).

Amino acid positions 240 and 167 seem to be involved in the evolution of CTX-M enzymes. CTX-M-15,-16,-27 and -32, which derive from CTX-M-3, -14,-9 and -1, respectively, by a Gly240Asp substitution, has greater catalytic efficiencies against ceftazidime (Bonnet, 2004). Munday et al. (2004) reported that CTX-M-25, which also has an Asp240Gly substitution, resulted in good enzymatic affinity towards ceftazidime, whilst CTX-M-26 which lacks the Asp240Gly substitution showed almost no activity towards ceftazidime. Comparison of the amino acid structures of other CTX-M enzymes available in the GenBank database reveals a glycine molecule at position 240 for CTX-M-28,-29,-33, -41 and -43, suggesting that these enzymes may also have ceftazidimase activity. To confirm the importance of Asp240-Gly substitution in the hydrolysis of ceftazidime, Cartelle et al. (2004) replaced the Gly240 with Asp in CTX-M-32 by using site directed mutagenesis. A lower MIC and lower catalytic efficiency was detected with the CTX-M-32 mutant. However site directed mutagenesis studies of CTX-M-9 by Aumeran and colleagues demonstrated that a substitution at position 240 of Asp240Lys (instead of Asp240Gly) was similar to mutations that promote ceftazidime activity found in the TEM and SHV ESBLs but did not result in increased hydrolysis of cetazidime for this enzyme (Munday et al., 2004). A random mutagenesis technique was used by Delmas et al. (2006) to predict the evolutionary potential of CTX-M-9 towards the acquisition of improved catalytic activity against ceftazidime. The mutants conferred 1- to 128- fold higher MICs of ceftazidime than the parental enzyme CTX-M-9. In addition to other mutants the substitutions Asp240Gly and Pro167Ser were noted. The kinetic constants of the three most active mutants revealed two distinct ways of improving catalytic efficiency against ceftazidime also suggesting that the CTX-M enzymes harbouring the substitution Asp240Gly are the most probable phylum for new mutants conferring the highest level of resistance to β-lactams (Delmas et al., 2006).

CTX-M-19, which derives from CTX-M-18 by a Pro167Ser substitution, is able to hydrolyze ceftazidime more efficiently than cefotaxime (Poirell et al., 2001). In laboratory-derived mutants of TEM-1, PSE-4 and BPS-1, a very similar mutation, Pro167Ser has been shown to be closely associated with ceftazidime resistance. CTX-M-23 with a Pro167Thr substitution is also associated with a higher level of resistance to ceftazidime than to cefotaxime. Even though residue 167 is not a direct part of the catalytic mechanism, this position seems to have a direct influence on substrate specificity (Stürenburg et al., 2004).

CONCLUSION

The widespread use of ceftriaxone and/or cefotaxime has been proposed as a reason for the emergence of CTX-M enzymes. The increased frequency of isolation and reporting of CTX-M ESBLs is alarming and is likely to represent only the tip of the iceberg for the underdeveloped continents where technology for the analysis of ESBL enzymes is scarce. The loss of the oxyimino-cephalosporins for treatment of infections represents a serious problem that seems to reach unprecedented levels globally (Munday et al., 2004). CTX-M enzymes are now endemic in many countries with both nosocomial and community emergence. The diversity of the CTX-M enzymes is noted especially in the Far East, Eastern Europe and Western Europe and some ESBL studies have identified CTX-M enzymes as the most prevalent

ESBL. The emergence of novel CTX-M β-lactamases in several countries is noted as opposed to the transfer of established CTX-M genes from one country to another, suggestive of a de novo dissemination of CTX-M genes. Despite many publications on ESBL enzymes, insight in the quantitative global distribution is lacking. A co-ordinated study to obtain this information is urgently needed.

REFERENCES

Abdalhamid B, Pitout DD, Moland ES, Hanson ND (2004). Community disease caused by *Citrobacter freundii* producing a novel CTX-M β-lactamase, CTX-M-30 in Canada. Antimicrob. Agents Chemother. 48: 4435-4437.

Ahmed AM, Nakano H, Shimamoto T (2004). The first characterization of extended-spectrum β-lactamase producing *Salmonella* in Japan. J. Antimicrob. Chemother. 57: 283-284.

Al-Agamy MH, Ashour ME, Wiegand I (2006). First description of CTX-M β-lactamase-producing clinical *Escherichia coli* isolates from Egypt. Int. J. Antimicrob. Agents 27: 545-548.

Bae IK, Lee BH, Hwang HY, Jeong SH, Hong SG, Chang CL, Kwak HS, Kim HJ, Youn H (2006). A novel ceftazidime-hydrolysing extended-spectrum β-lactamase, CTX-M-54, with a single amino acid substitution at position 167 in the omega loop. J. Antimicrob. Chemother. 58: 315-319.

Bonnet R, Sampaio JLM, Labia R, De Champs C, Sirot D, Chanal C, Sirot R (2000). A novel CTX-M β-lactamase (CTX-M-8) in cefotaxime-resistant Enterobacteriaceae isolated in Brazil. Antimicrob. Agents Chemother. 44: 1936-1942.

Bonnet R, Dutour C, Sampaio JLM, Chanal C, Sirot D, Labia R, De Champs C, Sirot J (2001). Novel cefotaximase (CTX-M-16) with increased catalytic efficiency due to substitution Asp-240→Gly. Antimicrob. Agents Chemother. 45: 2269-2275.

Bonnet R (2004). Growing group of extended-spectrum β-lactamases: the CTX-M enzymes. Antimicrob. Agents Chemother. 48: 1-14.

Blomberg B, Jureen R, Manji KP, Tamin BS, Mwakagile DSM, Urassa WK, Fataki M, Msangi V, Tellevik MG, Maselle SY, Langeland N (2005). High rate of fatal cases of paediatric septicemia caused by gram-negative bacteria with extended-spectrum β-lactamases in Dar es Salaam, Tanzania. J. Clin. Microbiol. 43: 745-749.

Cartelle M, Thomas MD, Molina F, Moure R, Villaneuva R, Bou G (2004). High-level resistance to ceftazidime conferred by a novel enzyme, CTX-M-32, derived from CTX-M-1 through a single Asp240Gly substitution. Antimicrob. Agents Chemother. 48: 2308-2313.

Celenza G, Pellegrini C, Cacammo M, Segatore B, Amicosante G, Perilli P (2006). Spread of *bla* $_{CTX-M-type}$ and *bla*$_{PER-2}$ β-lactamase genes in clinical isolates from Bolivian hospitals. J. Antimicrob. Chemother. 57: 975-978.

Chen Y, Delmas J, Sirot J, Shoichet B, Bonnet R (2005). Atomic resolution structures of CTX-M β-lactamases: extended spectrum activities from increased mobility and decreased stability. J. Mol. Biol. 348: 349-362.

Chmelnitsky I, Carmeli Y, Leavitt A, Schwaber MJ, Navon-Venezia S (2005). CTX-M-2 and a new CTX-M-39 enzyme are the major extended-spectrum β-lactamases in multiple *Escherichia coli* clones isolated in Tel Aviv, Israel. Antimicrob. Agents Chemother. 49: 4745-4750.

Conceicao T, Brizio A, Duarte A, Lito LM, Cristino JM, Salgado MJ (2005). First description of CTX-M-15 producing *Klebsiella pneumoniae* in Portugal. Antimicrob. Agents Chemother. 49: 477-478.

Delmas J, Robin F, Carvalho F, Mongaret C, Bonnet R (2006). Prediction of the evolution of ceftazidime resistance in extended-spectrum β-lactamase CTX-M-9. Antimicrob. Agents Chemother. 50: 731-738.

Eckert C, Gautier V, Arlet G (2006). DNA sequence analysis of the genetic environment of various *bla*$_{CTX-M}$ genes. J. Antimicrob. Chemother. 57: 14-23.

Eisener A, Fagan EJ, Feierl G, Kessler HH, Marth E, Livermore DM, Woodford N (2006). Emergence of Enterobacteriaceae isolates producing CTX-M extended-spectrum β-lactamase in Austria. Antimicrob. Agents Chemother. 40: 785-787.

Elliot E, Brink AJ, Greune JV, Els Z, Woodford N, Turton J, Waner M, Livermore D (2006). *In vivo* development of ertapenem resistance in a patient with pneumoniae caused by *Klebsiella pneumoniae* with an extended-spectrum β-lactamase. Clin. Infect. Dis. 42: 95-98.

Frank T, Arlet G, Gautier V, Talarmin A, Bercion R (2006). Extended-spectrum β-lactamase-producing Enterobacteriaceae in Central African Republic. Emerg. Infect. Dis. 12: 863-864.

Godet OB, Salem YB, Fabre L, Demartin M, Grimont PAD, Mzoughi R, Weill FX (2005). Nosocomial outbreak caused by *Salmonella enterica* serotype Livingstone producing CTX-M-27 in a neonatal unit in Sousse, Tunisia. J. Clin. Microbiol. 43: 1037-1044.

Govinden U, Mocktar C, Moodley P, Sturm AW, Essack SY (2006). CTX-M-37 in *Salmonella enterica* serotype Isangi from Durban, South Africa. Int. J. Antimicrob. Agents 28: 288-291.

Hernandez JR, Martinez L, Canton R, Coque TM, Pascual A and the Spanish group for nosocomial infections (2005). Nationwide study of *Escherichia coli* and *Klebsiella pneumoniae* producing extended-spectrum β-lactamases in Spain. Antimicrob. Agents Chemother. 49: 2122-2125.

Hopkins KL, Graham AD, Threlfall EJ (2006). Novel plasmid-mediated CTX-M-8 subgroup extended-spectrum β-lactamase (CTX-M-40) isolated in the UK. Int. J. Antimicrob. Agents 27: 570-575.

Ishii Y, Ohno A, Taguchi H, Imajo S, Ishiguro M, Matsuzawa H (1995).Cloning and sequence of the gene encoding a cefotaxime-hydrolyzing class A beta-lactamase isolated from *Escherichia coli* Antimicrob. Agents Chemother. 39: 2269-2275.

Kim J, Lim JM, Jeong YS, Seol SY (2005). Occurrence of CTX-M-3, CTX-M-15, CTX-M-14, and CTX-M-9 Extended-Spectrum ß-Lactamases in Enterobacteriaceae Clinical Isolates in Korea. Antimicrob. Agents Chemother. 49: 1572-1575.

Karim A, Poirel A, Nagarajan S, Nordman P (2001). Plasmid-mediated extended-spectrum β-lactamase (CTX-M-3 like) from India and gene association with insertion sequence IS*ECP1*. FEMS Microbiol. Lett. 201: 237-241.

Kariuki S, Corkill JE, Revathi G, Musoke R, Hart CA (2001) Molecular characterization of a novel plasmid-encoded cefotaximase (CTX-M-12) found in clinical *Klebsiella pneumoniae* isolates from Kenya. Antimicrob. Agents Chemother. 45: 2141-2143.

Kariuki S, Revathi G, Kariuki N, Muyodi J, Mwituria J, Munyalo A, Kagendo D, Murungi L, Hart CA (2005). Increasing prevalence of multidrug-resistant non-typhoidal salmonellae, Kenya, 1994-2003. Int. J. Antimicrob. Agents 25: 38-43.

Kim J, Lin YM, Jeong YS, Seol SY (2005). Occurrence of CTX-M-3, CTX-M-15, CTX-M-14 and CTX-M-9 extended-spectrum β-lactamases in Enterobacteriaceae clinical isolates in Korea, Antimicrob. Agents Chemother. 49: 1572-1575.

Koh TH, Wang GCY, Sng LH, Yi Z, Koh TY (2004). CTX-M and plasmid-mediated AmpC-producing Enterobacteriaceae, Singapore. Emerg. Infect. Dis. 10: 1172-1173.

Lartigue MF, Poirel L, Aubert D, Nordmann P (2006). In Vitro Analysis of IS*Ecp1B*-Mediated mobilization of naturally occurring ß-Lactamase gene *bla*$_{CTX-M}$ of *Kluyvera ascorbata*. Antimicrob. Agents Chemother. 50: 1282-1286.

Leflon-Guibout V, Jurand C, Bonacorsi S, Espinasse F, Guelfi MC, Duportail F, Heym B, Bingen E, Chanoin MHN (2004). Emergence and spread of three clonally related virulent isolates of CTX-M-15-producing *Escherichia coli* with variable resistance to aminoglycosides and tetracycline in a French geriatric hospital. Antimicrob. Agents Chemother. 48: 3736-3742.

Liebana E, Batchelor M, Torres C, Brinas L, Lagos LA, Abdalhamid B, Hanson ND, Martinez-Urtaza JM (2004). Pediatric infection due to multiresistant *Salmonella enterica* serotype Infantis in Honduras. J. Clin. Microbiol. 42: 4885-4888.

Livermore DM, Hawkey M (2005). CTX-M: changing the face of ESBLs in the UK. J. Antimicrob. Chemother. 56:451-454.

Li H, Li JB (2005). Detection of five novel CTX-M-type extended-spectrum β-lactamases with one to three CTX-M-14 point mutations in isolates from Hefei, Anhui Province, China. J. Clin. Microbiol. 43:

4301-4302
Ma L, Ishii Y, Ishiguro M, Matsuzawa H, Yamaguchi K (1998). Cloning and sequencing of the gene encoding Toho-2, a class A β-lactamase preferentially inhibited by tazobactam. Antimicrob. Agents Chemother. 42: 1181-1186.
Mena A, Plasencia V, Gracia L, Ignacio J, Albert AS, Borrell N, Perez JL, Oliver A (2006). Characterization of a large outbreak by CTX-M-1-producing *Klebsiella pneumoniae* and mechanisms leading to *in vivo* carbapenem resistance development. J. Clin. Microbiol. 44: 2831-2837.
Miriagou V, Tassios PT, Legakis NJ, Tzouvelekis LS (2004). Expanded-spectrum cephalosporin resistace in non-typhoidal *Salmonella*. Int. J. Antimicrob. Agents 23: 547-555.
Morris D, Whelan M, Corbett-Feeney G, Cormican M, Hawkey P, Li X, Doran G (2006) First report of extended-spectrum β-lactamase-producing *Salmonella enterica* isolates in Ireland. Antimicrob. Agents Chemother. 50: 1608-1609.
Mugnaioli C, Luzzaro F, De Luca F, Brigante G, Amicosante G, Rossolini GM (2005). Dissemination of CTX-M-type extended-spectrum β-lactamase genes to unusual hosts. J. Clin. Microbiol. 42: 4183-4185.
Munday CJ, Boyd DA, Brenwald N, Miller M, Andrews JM, Wise R, Mulvey MR, Hawkey PM (2004). Molecular and kinetic comparison of the novel extended-spectrum β-lactamases CTX-M-25 and CTX-M-26. Antimicrob. Agents Chemother. 48: 4829-4834.
Moubareck C, Daoud Z, Hakime NI, Hamze M, Mangeney N, Matta H, Mokhbat E, Raymond R, Sarkis DK, Populaire FD (2005). Countrywide spread of community- and hospital-acquired extended-spectrum β-lactamase (CTX-M-15) producing Enterobactericeae in Lebanon. J. Clin. Microbiol. 43: 3309-3313.
Naiemi NA, Bart A, De Jong MD, Vandenbroucke-Gruls C.M, Rietra PJGM, Depets-Ossenkopp YJ, Wever PC, Spanjaard L, Bos AJ, Dium B. (2006). Widely distributed and predominant CTX-M extended-spectrum β-lactamases in Amsterdam, the Netherlands. J. Clin. Microbiol. 43: 3012-3014.
Pagani L, Dell' Amico E, Migliavacca R, D'Andrea MM, Giacobone E, Amicosante G, Romero E, Rossolini GM (2003). Multiple CTX-M-type extended-spectrum β-lactamases in nosocomial isolates of Enterobacteriaceae from a hospital in northern Italy. J. Clin. Microbiol. 41: 4264-4269.
Paterson DL, Hujer KM, Hujer AM, Yeiser B, Bonomo MD, Rice LB, Bonomo RA and the international *Klebsiella* study group (2003). Extended-spectrum β-lactamases in *Klebsiella pneumoniae* bloodstream isolates from seven countries. Dominance and widespread prevalence of SHV- and CTX-M-type β-lactamases. Antimicrob. Agents Chemother. 47: 3554-3564.
Gangoué-Piéboji J, Bedenic B, Shiro SK, Randegger C, Adiogo D, Ngassam P, Ndumbe P, Hachler H (2005). Extended-spectrum β-lactamase producing Enterobacteriaceae in Yaounde Cameroon. J. Clin. Microbiol. 43: 3273-3277.
Pitout JDD, Nordman P, Laupland KB, Poirel L (2005 a). Emergence of Enterobacteriaceae producing extended-spectrum β-lactamases in the community. J. Antimicrob. Chemother. 56: 52-59.
Pitout JDD, Gregson DB, Church DL, Elsayed S, Laupland KB (2005b). Community-wide outbreaks of clonally related CTX-M-14 β-lactamase-producing *Escherichia coli* strains in the Calgary health region. J. Clin. Microbiol. 43: 2844-2849.
Poirel L, Naas T, Thomas I, Karim A, Bingen E, Nordman P (2001). CTX-M-type extended-spectrum β-lactamase that hydrolyzes ceftazidime through a single amino acid substitution in the omega loop. Antimicrob. Agents Chemother. 45: 3355-3361.
Pottumarthy S, Yu Y, Sader HS, Jones RN, Chen M (2005). Susceptibility testing accuracy of a CTX-M-type extended-spectrum β-lactamase organism-producing population of Enterobacteriaceae: intermethod analysis for 9 β-lactams. Diag. Microbiol. Infect. Dis. 53: 131-141.
Quinteros M, Radice M, Gardella N, Rodriguez M, Costa N, Korbenfeld D, Couto E, Gutkind G, and the microbiology study group (2003). Extended-spectrum β-lactamases in Enterobacteriaceae in Buenos Aires, Argentina, public hospitals. Antimicrob. Agents Chemother. 47: 2864-2867.
Rasmussen JW, Hoiby N (2004). Cefotaximases (CTX-M-ases), an expanding family of extended-spectrum β-lactamases. Can. J. Microbiol. 50: 137-165
Shibata N, Kurokawa H, Doi Y, Yagi T, Yamane K, Wachino JI, Suzuki S, Kimura K, Ishikawa S, Kato H, Ozawa Y, Shibayama K, Kai K, Konda T, Arakawa Y (2006). PCR classification of CTX-M-type β-lactamase genes identified in clinically isolated gram-negative bacilli in Japan. Antimicrob. Agents Chemother. 50: 791-795.
Shimamura T, Ibuka A, Fushinobu S, Wakagi T, Ishiguro M, Ishii Y, Matsuzawa H (2002). Acyl-intermediate structures of the extended-spectrum class A β-lactamase, Toho-1 in complex with cefotaxime, cephalothin and benzylpenicillin. J. Biol. Chem. 277: 46601-46608.
Simarro E, Navarro F, Ruiz J, Miro E, Gomez J, Mirelis B (2000). *Salmonella enterica* serovar Virchow with CTX-M-like β-lactamase in Spain. J. Clin. Microbiol. 38: 4676-4678.
Soge OO, Queenan AM, Ojo KK, Adeniyi BA, Roberts MC (2006). CTX-M-15 extended spectrum β-lactamase from Nigerian *Klebsiella pneumoniae*. J. Animicrob. Chemother. 57: 24-30.
Sonnevend A, Dhaheri KA, Mag T, Herpay M, Kolodziejek J, Noworthy N, Usmani A, Sheikh FA, Pai T (2006). CTX-M-15-producing multidrug-resistant enteroaggregative *Escherichia coli* in the United Arab Emirates. Clin. Microbiol. Infect. 12: 582.
Stürenburg E, Mack D (2003). Extended-spectrum β-lactamases: implications for the clinical microbioloby laboratory, therapy and infection control. J. Infect. 47: 273-295
Stürenburg E, Kuhn A, Mack D, Laufs R (2004). A novel extended-spectrum β-lactamase CTX-M-23 with a P167T substitution in the active-site omega loop associated with ceftazidime resistance. J. Antimicrob. Chemother. 54:406-409.
Tzouvelekis LS, Tzelepi E, Tassios PT, Legakis NJ (2000). CTX-M-type β-lactamases: an emerging group of extended -spectrum enzymes. Int. J. Antimicrob. Agents 14: 137-142.
Villegas MV, Correa A, Perez F, Zuluaga T, Radice M, Gutkind G, Casellas JM, Ayala J, Lolans K, Quinn JP and the Colombian nosocomial resistance study group (2004). CTX-M-12 β-lactamase in a *Klebsiella pneumoniae* clinical isolate in Colombia. Antimicrob. Agents Chemother. 48: 629-631.
Wu TL, Chia JH, Su LH, Kuo AJ, Chu C, Chiu CH (2003). Dissemination of extended-spectrum β-lactamase-producing Enterobacteriaceae in paediatric intensive care units J. Clin. Microbiol. 41: 4836-4838
Yan JJ, Hsueh PR, Lu JJ, Chang FY, Shyr JM, Wan JH, Liu YC, Chuang YC, Yang YC, Tsao SM, Wu HH, Wang LS, Lin TP, Wu HM, Chen HM, Wu JJ (2006). Extended-spectrum β-lactamases among clinical isolates of *Escherichia coli* and *Klebsiella pneumoniae* from seven medical centres in Taiwan. Antimicrob. Agents Chemother. 50: 1861-1864.
Yong D, Lim YS, Yum JH, Lee H, Lee K, Kim EC, Lee BK, Chong Y (2005). Nosocomial outbreak of pediatric gastroenteritis cause by CTX-M-14 type extended-spectrum β-lactamase-producing strains of *Salmonella enterica* serovar London. J. Clin. Microbiol. 43: 3519-3521.

Available online at www.sciencedirect.com
ScienceDirect
Diagnostic Microbiology and Infectious Disease 62 (2008) 86–91
DIAGNOSTIC MICROBIOLOGY AND INFECTIOUS DISEASE
www.elsevier.com/locate/diagmicrobio

Characterization of extended-spectrum β-lactamases in *Salmonella* spp. at a tertiary hospital in Durban, South Africa

Govinden Usha[a,*], Mocktar Chunderika[a], Moodley Prashini[b], Sturm Adriaan Willem[b], Essack Sabiha Yusuf[a]

[a]*School of Pharmacy and Pharmacology, University of Kwazulu Natal, Durban, Kwazulu Natal 4000, South Africa*
[b]*Medical Microbiology, University of Kwazulu Natal, Durban, Kwazulu Natal 4000, South Africa*

Received 24 January 2008; accepted 29 April 2008

Abstract

Extended-spectrum β-lactamases (ESBLs) were characterized in 41 *Salmonella* spp. isolates from patients admitted to a pediatric ward of a tertiary hospital in Durban, South Africa. The most common (17/ 41) serotype was *Salmonella enterica* serotype Typhimurium, followed by *S. enterica* serotype Isangi (16/41), *S. enterica* serotype Saint-paul (2/41), *S. enterica* serotype Kissi (2/41), *S. enterica* serotype Kivu (2/41), and *S. enterica* serotype Reading (1/41). All isolates were resistant to ampicillin, amoxicillin–clavulanate, piperacillin, ceftazidime, and aztreonam but susceptible to meropenem. SHV-12 found in 39% of the isolates was the most common ESBL. TEM-63 was produced in 29% and TEM-116 in 10% of the isolates, and TEM-131 was found in 1 isolate. Other ESBLs that were identified included SHV-2 ($n = 2$), CTX-M-3 ($n = 1$), CTX-M-15 ($n = 2$), and CTX-M-37 ($n = 5$). In addition, CMY-2 ($n = 3$) and the OXA-1($n = 1$) β-lactamase were also detected. The diversity of ESBLs suggests that its incidence in *Salmonellae* needs to be monitored.

Keywords: *Salmonella* spp.; Extended-spectrum β-lactamase; Multidrug resistance

1. Introduction

Salmonellae are major pathogens in humans as well as in animals and comprise more than 2000 serotypes. The enteric fever salmonellae including *Salmonella enterica* serotype Typhi are strict human pathogens, whereas all other serotypes are primarily pathogens of other mammals but may also cause disease in man. Nonenteric fever salmonellae are implicated in foodborne gastroenteritis worldwide. Although antibiotics are not usually recommended in cases of salmonella enterocolitis, they become an essential part of management if the infection spreads beyond the gut. Invasive complications including meningitis, arthritis, and deep-seated abscesses are more common in infants, the elderly, and immunocompromised people (Yates and Amyes, 2005). Because there is widespread resistance against conventional antibiotics such as ampicillin, chloramphenicol, and cotrimoxazole, these can no longer be used for empiric treatment (Su et al., 2004). Invasive disease is, therefore, treated with fluoroquinolones or extended-spectrum cephalosporins (Yates and Amyes, 2005). Ceftriaxone is commonly used to treat children with invasive infections or severe diarrhea caused by salmonellae; however, ceftriaxone-resistant salmonellae in humans as well as animals have frequently been reported from all inhabited continents including Africa (Kariuki et al., 2005; Kruger et al., 2004; Li et al., 2005). A recent study of multidrug-resistant *Salmonella* spp. in Kuwait and the United Arab Emirates reported a 5-fold rise in the resistance rate to the 3rd-generation cephalosporin ceftriaxone and cefotaxime (Rotimi et al., 2008).

Extended-spectrum β-lactamases (ESBLs) are predominantly associated with Enterobacteriaceae. ESBLs in salmonellae in Africa were 1st described in 1988 (Hammami et al., 1991) and are increasing in prevalence worldwide (Morris et al., 2006). ESBLs reported in *Salmonella* spp. include TEM, SHV, and CTX-M (Su et al., 2004). In this study, we

* Corresponding author. Tel.: +27-31-2607413; fax: +27-31-2607792.
E-mail address: govindenu@ukzn.ac.za (G. Usha).

0732-8893/$ – see front matter © 2008 Elsevier Inc. All rights reserved.
doi:10.1016/j.diagmicrobio.2008.04.014

characterized the ESBLs in a collection of putative ESBL-positive *Salmonella* spp. from a tertiary hospital in Durban, Kwazulu Natal (KZN), South Africa.

2. Materials and methods

2.1. Bacterial strains

Fifty-nine putative ESBL-positive isolates of *Salmonella* spp. were cultured from stool samples of neonates presenting with acute diarrhea in 2001 at the King Edward VIII hospital in Durban. All isolates were serotyped by the hospital laboratory using commercially available antisera (Bioweb, Johannesburg, South Africa) according to the Kauffman–White scheme for salmonella serotyping (Kauffman, 1972; Popoff, 2001). *Escherichia coli* 25922 was used as the control for susceptibility testing. *Klebsiella pneumoniae* ATCC 700603 was used as the control for ESBL detection. For polymerase chain reaction (PCR) studies, *E. coli* MEN (Barthélémy et al., 1992), *E. coli* CF 204 (Sirot et al., 1987), and *E. coli* CF 1004 (Chanal et al., 1996) provided positive controls for CTX-M-, TEM-, and SHV-type β-lactamases, respectively, with distilled water being the negative control.

2.2. Susceptibility testing and ESBL detection

Disc diffusion susceptibility tests for ampicillin, amoxicillin–clavulanate, ampicillin–sulbactam, piperacillin, ceftazidime, cephalothin, ceftriaxone, cefepime, cefuroxime, cefotaxime, cefoxitin, meropenem, aztreonam, and piperacillin–tazobactam were performed according to the National Committee for Clinical Laboratory Standards (2003) guidelines. Results were read with the Biomic automated reading system (Giles Scientific, New York, NY). Putative ESBL-positive isolates were determined at the hospital laboratory by the double disc synergy/disc approximation method using ceftazidime, amoxicillin–clavulanate, and cefotaxime on Mueller–Hinton agar. ESBL production was further determined by the E-test method according to the manufacturer's guidelines (AB BIODISK, Solna, Sweden). The presence of an ESBL was verified by the appearance of a phantom zone or deformation of the cefotaxime or ceftazidime ellipse. The final sample size of 41 was determined by a positive ESBL test in combination with elevated MICs to 1 or more 3rd-generation cephalosporins.

2.3. Isoelectric focusing of β-lactamases

Bacterial cells were broken by the freeze–thaw method (Livermore and Williams, 1996), and isoelectric focusing of crude extracts was performed in polyacrylamide gels containing ampholines with a p*I* range of 3.5 to 9.5 (Amersham Biosciences, Uppsala, Sweden). An isoelectric point marker p*I* (4.7–10.6) calibration kit served as the standard (BDH, England). β-lactamase bands were visualized with nitrocefin (Oxoid, Basingstoke, UK).

2.4. PCR detection of bla_{TEM}, bla_{SHV}, bla_{CTX-M}, bla_{OXA}, bla_{CMY}, bla_{DHA}, and bla_{ACC} genes

All 41 isolates were screened for the presence of bla_{TEM}, bla_{SHV}, $bla_{\text{CTX-M}}$, bla_{OXA}, bla_{CMY}, bla_{DHA}, and bla_{ACC} genes. Bacteria were grown on Mueller–Hinton agar (Biolab, Johannesburg, South Africa) overnight and suspended in distilled water. DNA was extracted by heating the suspensions at 95 °C for 5 min. PCR amplification was then performed in a Gene Amp 9700 PCR System (Applied Biosystems, Foster City, CA). The primers (Inqaba Biotechnology, Pretoria, South Africa) are described in Table 1. PCR conditions for amplification of bla_{TEM} and bla_{SHV} genes were carried out as described by Essack et al. (2001). The amplification mixture for the detection of $bla_{\text{CTX-M}}$, bla_{CMY}, bla_{OXA}, bla_{DHA}, and bla_{ACC} genes was prepared in a final volume of 50 μL, containing purified water, 2 μL of the template DNA, 10 pmol of each primer, and 25 μL of master mix (Applied Biosystems). The PCR program for $bla_{\text{CTX-M}}$ consisted of an initial denaturation step at 94 °C for 3 min, followed by 25 cycles of DNA denaturation at 94 °C for 30 s, primer annealing at 54 °C for 1 min, and primer extension at 72 °C for 2 min, with a final extension step at 72 °C for 7 min. The PCR conditions used for the detection of bla_{CMY}, bla_{OXA}, bla_{DHA}, and bla_{ACC} genes were as previously described (Kaye et al., 2004; Perez-Perez and Hanson, 2002; Zhao et al., 2001). Five-microliter aliquots of PCR product were analyzed by gel electrophoresis. Gels were stained with ethidium bromide at 10 μg/mL and photographed under ultraviolet illumination.

2.5. Sequencing

The primers used for DNA sequencing are shown in Table 1. Sequencing of the amplified products was performed with the Spectrumedix model SCE 2410 automated sequencer (Spectrumedix, State College, PA), incorporating the ABI Big Dye Terminator Cycle Sequencing kit version 3.1 (Applied Biosystems). Sequences were analyzed using the BLAST 2.0 (Basic Local Alignment Search Tool) software available on the website of the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/blast/BLAST.cgi, accessed 16 July 2007).

3. Results

Table 2 shows the serotypes, isoelectric point(s), the extrapolated MIC values of selected β-lactams, and the various β-lactamases identified in the study. The most common serotype among the 41 isolates was *S. enterica* serotype Typhimurium (17/41), followed by *S. enterica* serotype Isangi (16/41). All 41 isolates were resistant to ampicillin (>48 μg/mL), amoxicillin–clavulanate (48 to >64 μg/mL), piperacillin (>512 μg/mL), and ceftazidime (64 to >128 μg/mL) but susceptible to meropenem (<0.5 to 1 μg/mL). Tazobactam was the most effective inhibitor

Table 1
Primers used in this study

Primer	Sequence (5′–3′)	Purpose of primer	Reference
TEM 1 (F)	ATGAGTATTCAACATTTCCGTG	Amp	Essack et al. (2001)
TEM 2 (R)	TTACCAATGCTTAATCAGTGAG	Amp/seq	Essack et al. (2001)
TEM 3 (R)	TTCTGTGACTGGTGAGTACT	Seq	Essack et al. (2001)
TEM 4 (R)	GAGTAAGTAGTTCGCCAGTT	Seq	Essack et al. (2001)
TEM 5 (F)	CTGCAGCAATGGCAACAAC	Amp/seq	Mocktar et al. (2008)
SHV 1 (F)	ATGCGTTATATTCGCCTGTG	Amp/seq	Essack et al. (2001)
SHV 2 (R)	GTTAGCGTTGCCAGTGCTCG	Amp/seq	Essack et al. (2001)
SHV 3 (R)	CCGTTTCCCAGCGGTCAAGG	Seq	Essack et al. (2001)
CTX-M-3A	GGTTAAAAAATCACTGCG	Amp/seq	Govinden et al. (2006)
CTX-M-1B	CCGTTTCCGCTATTACAA	Amp/seq	Govinden et al. (2006)
CTX-M (F)	TTTGCGATGTGCAGTACCAGTAA	Amp/seq	Edelstein et al. (2003)
CTX-M (R)	CGATATCGTTGGTGGTGCCATA	Amp/seq	Edelstein et al. (2003)
CMY (F)	GACAGCCTCTTTCTCCACA	Amp/seq	Zhao et al. (2001)
CMY (R)	TGGAACGAAGGCTACGTA	Amp/seq	Zhao et al. (2001)
CMY (F1)	GAAAAAATCGTTATGCTGC	Amp/seq	Mocktar et al. (2008)
CMY (R1)	CCGTATAGGTGGCTAAGTGC	Amp/seq	Mocktar et al. (2008)
CMY (F2)	GTGAAATCCAGCGTTATTGA	Amp/seq	Mocktar et al. (2008)
CMY (R2)	CTTTTCAAGAATGCGCCAGG	Amp/seq	Mocktar et al. (2008)
OXA-1F	ACACAATACATATCAACTTCGC	Amp/seq	Kaye et al. (2004)
OXA-1R	AGTGTGTTTAGAATGGTGATC	Amp/seq	Kaye et al. (2004)
DHA (F)	AACTTTCACAGGTGTGCTGGGT	Amp/seq	Perez-Perez and Hanson (2002)
DHA (R)	CCGTACGCATACTGGCTTTGC	Amp/seq	Perez-Perez and Hanson (2002)
ACC (F)	AACAGCCTCAGCAGCCGGTTA	Amp/seq	Perez-Perez and Hanson (2002)
ACC (R)	TTCGCCGCAATCATCCCTAGC	Amp/seq	Perez-Perez and Hanson (2002)

F = forward primer; R = reverse primer; amp = amplification; seq = sequencing.

because resistance to piperacillin–tazobactam was found in only 1 isolate. TEM-1 (51%) was the most predominant β-lactamase produced, and it was found most frequently in combination with SHV-12 with pI values ranging from 5.4 to 6.1 and 7.6 to 8.4, respectively. Isolates 509, 541, and 640 produced TEM-1 and CTX-M-37 (Govinden et al., 2006). CTX-M-15 was only found in 2 isolates, 464 and 580, whereas CTX-M-3 was found in a single isolate. OXA-1 with CTX-M-37 and TEM-1 was produced only in isolate 509b. A unique combination of TEM-63, CTX-M-37, and the AmpC-type β-lactamase, CMY-2 was found in isolate 376. CMY-2 was also found in isolate 262 with TEM-1 and SHV-2 and in isolate 370 with TEM-63. All isolates with CMY-2 had elevated cefoxitin MICs of >96 μg/mL. The other AmpC-type β-lactamases, DHA and ACC, were not found. To our knowledge, this is the 1st report of such β-lactamase gene combinations in *Salmonella* spp. from South Africa.

4. Discussion

In 2007, The Enteric Diseases Reference Unit of the National Institute for Communicable Diseases in South Africa reported 17.7% of nontyphoidal salmonella isolates (n = 1502) to be ESBL producers, and resistance to 5 or more antimicrobial agents was observed in 33.8% of the isolates (Keddy, 2008). The 1st salmonella strains with ESBLs in Africa were identified in 1988 in Tunisia (Hammami et al., 1991). An ESBL study of 160 *Salmonella* spp. from 13 hospitals in South Africa was reported in 2004 to produce 15.6% of TEM or SHV ESBLs (Kruger et al., 2004). A nosocomial outbreak of salmonella infection in pediatric patients caused by *Salmonella* Isangi producing ESBLs was 1st reported from the Chris Hani Baragwanath Hospital, Johannesburg, South Africa, in 2006 (Wadula et al., 2006). Salmonellae have, thus, acquired a variety of ESBLs (TEM, SHV, CTX-M) and may no longer be described as a rare producer of ESBLs.

TEM-63 in *K. pneumoniae* was reported for the 1st time from Durban, South Africa, in 2000 by Essack et al., and there were further reports in South Africa of TEM-63 in strains of *Proteus* spp., *Enterobacter* spp., *E. coli*, *Enterobacter cloacae* (Gray et al., 2006), and *Salmonella* spp. (Kruger et al., 2004). An ESBL study in sub-Saharan Africa in Tanzania detected TEM-63 in *E. coli* and *K. pneumoniae* (Blomberg et al., 2005). The 1st report of TEM-63 outside the African continent in *Salmonella* Isangi, from a single patient, was also found in an ESBL study in Netherlands in 2005. It is unknown if the patient had traveled to South Africa (Hasman et al., 2005). In this study, 29% (12/41) of the isolates contained TEM-63 and was most commonly found in *Salmonella* Isangi. The amino acid changes of TEM-63 compared with TEM-1 were L21F, which lies in the signal peptide, E104K, which occurs in many TEM ESBLs, and R164S, which widens the binding cavity to accommodate the bulky side chains of oxyimino-aminothiazolyl cephalosporins. The R164S substitution also leads to high resistance to ceftazidime but low levels of resistance to cefotaxime (Orencia et al., 2001), as can be seen with the 9 isolates (216, 256, 259, 296, 420, 482, 793, 895, and 1048)

Table 2
Characterization of ESBL-positive *Salmonella* strains

S. enterica serotype	Isolate no.[a]	Isoelectric point (s)	β-lactamase (s)	MIC (μg/mL)								
				TZP	CEF	CXM	FOX	CRO	CTX	CAZ	FEP	ATM
ND	356	5.6; 8.2	TEM-1; SHV-12	6	>128	24	8	20	32	64	10	12
Typhimurium	15	6.1; 8.2	TEM-1; SHV-12	10	>128	64	4	16	48	>128	10	32
1,4,5,12: i 1,2[b]	175	5.6; 7.7; 8.2	TEM-1; SHV-12	4	>128	32	4	8	12	>128	<1	>64
	403	5.5; 8.1	TEM-1; SHV-12	32	>128	16	4	64	64	>128	12	>64
	565	5.5; 8.2	TEM-1; SHV-12	10	>128	24	4	16	48	>128	6	20
	611	5.8; 7.5	TEM-1; SHV-12	20	>128	>96	6	>128	>256	>128	>96	>64
	620	5.4; 8.2	TEM-1; SHV-12	6	>128	>96	4	>128	96	>128	24	>64
	646	5.5; 8.2	TEM-1; SHV-12	6	>128	32	8	16	24	>128	2	>64
	669	5.7; 8.2	TEM-1; SHV-12	4	>128	24	6	8	16	>128	<1	>64
	709	5.9; 8.4	TEM-1; SHV-12	10	>128	>96	10	48	96	>128	8	>64
	873	5.4; 7.6	TEM-1; SHV-12	10	>128	>96	6	96	64	>128	1	>64
	914	5.4; 8.1	TEM-1; SHV-12	4	>128	24	4	16	10	>128	4	>64
	919	5.7; 8.4	TEM-1; SHV-12	20	>128	>96	4	64	>256	>128	64	>64
	951	5.6; 8.4	TEM-1; SHV-12	128	>128	64	4	>128	>256	>128	64	20
	1048	6.0	TEM-63	6	32	10	4	6	4	>128	8	>64
	376	5.4; 7.2; 8.3	TEM-63; CTX-M-37; CMY-2	8	>128	48	>96	32	96	>128	<1	12
	948	5.3	TEM-116	4	>128	12	4	10	16	>128	6	>64
	288	5.4	TEM-116	4	>128	48	4	16	24	>128	4	>64
Isangi 6,7 14:d 1,5[b]	262	5.4; 8.3	TEM-1; SHV-2; CMY-2	20	>128	>96	>96	64	>256	>128	10	>64
	905	5.6; 8.0	TEM-1; SHV-12	10	>128	48	8	32	16	>128	6	>64
	464	5.5; 7.9	TEM-1; CTX-M-15	4	>128	48	4	128	64	>128	8	>64
	509[c]	5.8; 6.8; 7.2	TEM-1; CTX-M-37	8	>128	>96	6	>128	>256	>128	>96	>64
	541[c]	5.8; 7.2	TEM-1; CTX-M-37	6	>128	>96	4	>128	>256	>128	64	>64
	640[c]	5.8; 6.8; 7.2	TEM-1; CTX-M-37	10	>128	>96	6	>128	>256	>128	>96	>64
	216	6.0	TEM-63	8	48	12	4	6	6	>128	16	>64
	256	6.1	TEM-63	8	64	<2	6	8	6	>128	16	>64
	259	6.1	TEM-63	4	12	12	6	4	4	>128	12	32
	296	5.4	TEM-63	4	>128	10	2	8	6	>128	24	>64
	420	5.6	TEM-63	8	48	10	4	6	4	>128	48	>64
	482	5.5	TEM-63	4	64	12	8	16	8	>128	32	>64
	793	5.6	TEM-63	4	64	20	6	4	4	64	12	>64
	509b	5.5; 7.4	TEM-63; CTX-M-37; OXA-1	20	>128	12	2	>128	>256	>128	>96	>64
	317	5.4	TEM-116	6	48	10	6	8	8	>128	48	>64
	954	5.3	TEM-116	4	>128	32	4	48	16	>128	4	>64
Saint-paul 1,4,5,12: eh 1,2[b]	895	6.0	TEM-63	4	16	48	4	4	4	>128	8	>64
	402	5.5; 8.1	TEM-1; SHV-2	6	>128	32	6	64	>256	>128	<1	>64
Kivu 6,7: d 1,6[b]	580	5.9	TEM-63; CTX-M-15	6	>128	>96	6	64	>256	>128	>96	>64
	572	5.5; 8.0	TEM-1; CTX-M-3	6	>128	>96	8	>128	96	>128	64	48
Kissi 6.7: d 1,2[b]	370	5.6; 8.2	TEM-63; CMY-2;	32	>128	>96	>96	96	>96	>128	16	>64
	467	5.4	TEM-131	32	>128	48	4	48	48	>128	32	>64
Reading 1,4,5,12: eh 1,5[b]	493	5.4; 8.2	TEM-1; SHV-12	4	>128	>96	4	>128	>96	>128	12	>64

TZP = piperacillin–tazobactam; CEF = cephalothin; CXM = cefuroxime; FOX = cefoxitin; CRO = ceftriaxone; CTX = cefotaxime; CAZ = ceftazidime; FEP = cefepime; ATM = aztreonam; ND = serotype could not be determined.

[a] Hospital isolate number.

[b] Antigenic formula (somatic [O] antigen, flagellar [H] antigen phase 1, flagellar [H] antigen phase 2).

[c] Govinden et al., 2006.

expressing only TEM-63 with ceftazidime MICs of 64 to >128 μg/mL and cefotaxime MICs of 4 to 8 μg/mL. The M182T mutation is found only in combination with other amino acid substitutions, suggesting that it may correct defects introduced by other mutations that alter the specificity, thus, conferring a selective advantage during the evolution of drug resistance (Sideraki et al., 2001). TEM-131 from *Salmonella* spp. in South Africa was 1st reported in an ESBL study by Kruger et al. (2004). Only isolate 467 produced TEM-131, which differs from TEM-63 by 1 amino acid substitution, A237T. As reported by Knox (1995), a clear indication of the utility of threonine at position 237 comes from a crystallographic mapping of the binding of cefotaxime to the structurally homologous DD-peptidase. Cefotaxime with its branched oximino substituent was found tilted out of the binding site and unable to form the expected hydrogen bond to the backbone CO group at position 237. Instead, cefotaxime's acylamino NH group donates a hydrogen bond to the side-chain OH group of threonine, which exists at this position in the DD-peptidase. Thus, the replacement of A237 with a hydrogen bond acceptor such as threonine enhances the binding of

cefotaxime and ceftazidime (Knox, 1995). TEM-131 also has the R164S substitution with the ceftazidime MIC of >128 μg/mL and the cefotaxime MIC of 48 μg/mL. Four of the isolates (948, 954, 317, and 288) produced TEM-116, which differs from TEM-1 by 2 amino acid changes: V84I and A184V. This is the 1st report of TEM-116 from *Salmonella* spp. in South Africa. TEM-116, which was also identified in Korea, was reported by Jeong et al. (2004) to have possibly evolved directly from TEM-1.

SHV-12 in 39% (16/41) of isolates was produced in combination with a TEM-type β-lactamase. The 1st report of SHV-12 in the genus *Salmonella*, published in 2001, described 5 strains of *S. enterica* serotype Keurmassar, which were isolated in 2000 in Dakar, Senegal (Weill et al., 2004). SHV-12 has a combination of amino acid changes of SHV-2, SHV-2a, and SHV-5 (http://www.lahey.org/Studies/, accessed July 16, 2007). Except for SHV-2 in isolate 262 and 402, SHV-2a and SHV-5 were not detected, suggesting that SHV-12 may have evolved from SHV-2 or it could have been acquired from other strains such as *K. pneumoniae*. Kruger et al. (2004) reported SHV-12 and SHV-5 from *Salmonella* spp. in South Africa.

CTX-M-3, CTX-M-15, and CTX-M-37 were detected in this study. CTX-M-37 was 1st reported in 2005 from Mongolia and then in South Africa (Govinden et al., 2006). Several countries have reported the occurrence of CTX-M-15 either in an outbreak or from ESBL studies (Govinden et al., 2007). CTX-M-15 was only found in 2 isolates in this study. The expression of bla_{CMY-2} gene was reported as being responsible for most ceftriaxone resistance in *Salmonella* spp. This gene was 1st identified in a multiresistant *S. enterica* serotype Senftenberg strain isolated in 1994 (Li et al., 2005). Although ceftriaxone resistance was noted in this study, it was not solely attributable to CMY-2, which was detected in only 3 of the isolates that had MICs of 32 to 96 μg/mL for ceftriaxone. According to the South African Standard Treatment Guidelines (STG) and Essential Drugs List (EDL) (National Essential Drugs List Committee, 2006), ceftriaxone is prescribed for pediatric use in salmonella infections, and quinolones are used if there is a cephalosporin allergy. Ciprofloxacin and ceftriaxone are prescribed for adults. Ceftriaxone resistance was found in 41% (17/41) of the isolates, and the treatment with ceftriaxone for pediatric and possibly adult salmonella infections would soon be compromised. Ceftriaxone should not be used therapeutically for ESBL-producing *Salmonella* spp. According to the results of this study, the STG and the EDL needs to be revised urgently to maintain the efficacy of ceftriaxone. This study shows that ESBL-positive *Salmonella* spp. can be multidrug resistant, with the propensity to harbor TEM, SHV, CTX-M, CMY, and OXA β-lactamases in unique combinations, which is not frequently reported. Failure to identify drug-resistant salmonellae may affect the choice of appropriate alternative antibiotics in the treatment of patients with invasive salmonellosis (Su et al., 2004).

Acknowledgments

This study was supported by grants from the Medical Research Council, National Research Foundation, and the University of Kwazulu Natal, Durban, South Africa.

References

Barthélémy M, Péduzzi J, Bernard H, Tancréde C, Labia R (1992) Close amino acid sequence relationship between the plasmid-mediated extended-spectrum β-lactamase MEN-1 and chromosomally-encoded enzymes of *Klebsiella oxytoca*. *Biochim Biophys Acta* 1122:15–22.

Blomberg B, Jureen R, Manji KP, Tamin BS, Mwakagile DS, Urassa WK, Fataki M, Msangi V, Tellevik MG, Maselle SY, Langeland N (2005) High rate of fatal cases of pediatric septicemia caused by gram-negative bacteria with extended-spectrum β-lactamases in Dar es Salaam, Tanzania. *J Clin Microbiol* 43:745–749.

Chanal C, Sirot D, Romaszko JP, Bret L, Sirot J (1996) Survey of prevalence of extended spectrum β-lactamases among Enterobacteriaceae. *J Antimicrob Chemother* 38:127–132.

Edelstein M, Pimkin M, Palagin I, Edelstein I, Stratchounski L (2003) Prevalence and molecular epidemiology of CTX-M extended spectrum β-lactamase production in *Escherichia coli* and *Klebsiella pneumoniae* in Russian hospitals. *Antimicrob Agents Chemother* 47:3724–3732.

Essack SY, Hall LM, Pillay DG, McFadyen ML, Livermore DM (2001) Complexity and diversity of *Klebsiella pneumoniae* strains with extended-spectrum β-lactamases isolated in 1994 and 1996 at a teaching hospital in Durban, South Africa. *Antimicrob Agents Chemother* 45:88–95.

Govinden U, Mocktar C, Moodley P, Sturm AW, Essack SY (2006) CTX-M-37 in *Salmonella enterica* serotype Isangi from Durban, South Africa. *Int J Antimicrob Agents* 28:288–291.

Govinden U, Mocktar C, Moodley P, Sturm AW, Essack SY (2007) Geographical evolution of the CTX-M β-lactamase—an update. *African J Biotechnol* 6:831–839.

Gray KJ, Wilson KW, Phri A, Corkill JE, French N, Hart A (2006) Identification and characterization of ceftriaxone resistance and extended-spectrum β-lactamases in Malawian bacteraemic Enterobacteriaceae. *J Antimicrob Chemother* 57:661–665.

Hammami AG, Arlet G, Ben Redjeb S, Grimont F, Ben Hassen A, Rekik A, Philippon A (1991) Nosocomial outbreak of acute gastroenteritis in a neonatal intensive care unit in Tunisia caused by multiply drug resistant *Salmonella wien* producing SHV-2 β-lactamase. *Eur J Clin Microbiol Infect Dis* 10:614–616.

Hasman H, Mevius D, Veldman K, Olesen I, Aarestrup FM (2005) β-Lactamases among extended-spectrum β-lactamase (ESBL)-resistant salmonella from poultry, poultry products and human patients in the Netherlands. *J Antimicrob Chemother* 56:115–121.

Jeong SK, Bae IK, Lee JH, Sohn SG, Kang GH, Jeon GJ, Kim YH, Jeong BC, Lee SH (2004) Molecular characterization of extended-spectrum β-lactamases produced by clinical isolates of *Klebsiella pneumoniae* and *Escherichia coli* from a Korean nationwide survey. *J Clin Microbiol* 42:2902–2906.

Kariuki S, Revathi G, Kariuki N, Muyodi J, Mwituria J, Munyalo A, Kagendo D, Murungi L, Hart CA (2005) Increasing prevalence of multidrug-resistant non-typhoidal salmonella, Kenya 1994–2003. *Int J Antimicrob Agents* 25:38–43.

Kauffman F (1972) Serologic Diagnosis of *Salmonella* species. Copenhagen, Denmark: Munksgaard.

Kaye KS, Gold HS, Schwaber MJ, Venkataraman L, Oi Y, De Girolami PC, Samore MH, Anderson G, Rasheed JK, Tenover FC (2004) Variety of β-lactamases produced by amoxicillin–clavulanate-resistant *Escherichia coli* isolated in the Northeastern United States. *Antimicrob Agents Chemother* 48:1520–1525.

Keddy K (2008) Non typhoidal *Salmonella Enterica*. *Commun Dis Surveill Bull* 6:21–28.

Knox JR (1995) Extended-spectrum and inhibitor-resistant TEM-type β-lactamases: mutations, specificity, and three-dimensional structure. *Antimicrob Agents Chemother* 39:2593–2601.

Kruger T, Szabo D, Keddy KH, Deeley K, Marsh JW, Hujer AM, Bonomo RA, Patterson DL (2004) Infections with nontyphoidal *Salmonella* species producing TEM-63 or a novel TEM enzyme, TEM 131 in South Africa. *Antimicrob Agents Chemother* 48:4263–4270.

Li WC, Huang FY, Liu CP, Weng LC, Wang NY (2005) Ceftriaxone resistance of nontyphoidal *Salmonella enterica* isolates in Northern Taiwan attributable to production of CTX-M-14 and CMY-2 β-lactamases. *J Clin Microbiol* 43:3237–3243.

Livermore DM, Williams JD (1996) Mode of action and mechanisms of bacterial resistance. In: *Antibiotics in Laboratory Medicine*. Lorian V, Ed. 4th ed. Baltimore, MD: The Williams & Wilkins Co., pp. 502–578.

Mocktar C, Govinden U, Sturm, Essack SY (2008) CMY-20 A novel AmpC-type β-lactamase from South African clinical *Escherichia coli* isolates. *Diagn Microbiol Infect Dis* 60:405–408.

Morris D, Whelan M, Corbett-Feeney G, Cormican M, Hawkey P, Li X, Doran G (2006) First report of extended-spectrum-β-lactamase-producing *Salmonella enterica* isolates in Ireland. *Antimicrob Agents Chemother* 50:1608–1609.

National Committee for Clinical Laboratory Standards (2003) Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically. 6th ed. Wayne, PA: National Committee for Clinical Laboratory Standards.

National Essential Drugs List Committee (2006) Standard Treatment Guidelines and Essential Drugs List—Hospital Level-Adults. Pretoria, South Africa: National Department of Health.

Orencia MC, Yoon JS, Ness JE, Stemmer WPC, Stevens RC (2001) Predicting the emergence of antibiotic resistance by directed evolution and structural analysis. *Nat Struct Biol* 8:238–242.

Perez-Perez FJ, Hanson ND (2002) Detection of plasmid-mediated AmpC β-lactamase genes in clinical isolates by using multiplex PCR. *J Clin Microbiol* 40:2153–2162.

Popoff MY (2001) Antigenic Formula of the *Salmonella* Serovars. 8th ed. Paris, France: Institut Pasteur.

Rotimi VO, Jamal W, Pal T, Sonnevend A, Dimitrov TS, Albert MJ (2008) Emergence of multidrug-resistant *Salmonella* spp. and isolates with reduced susceptibility to ciprofloxacin in Kuwait and the United Arab Emirates. *Diagn Microbiol Infect Dis* 60:71–77.

Sideraki V, Huang W, Palzkil T, Gilbert HF (2001) A secondary drug resistance mutation of TEM-1 β-lactamase that suppresses misfolding and aggregation. *Proc Natl Acad Sci U S A* 98:283–288.

Sirot D, Sirot J, Labia R, Morand A, Courvalin P, Darfeuille-Michaud A, Perroux R, Cluzel R (1987) Transferable resistance to third generation cephalosporins in clinical isolates of *Klebsiella pneumoniae*: identification of CTX-1, a novel β-lactamase. *J Animicrob Chemother* 20: 323–334.

Su LH, Chiu CH, Chu C, Ou JT (2004) Antimicrobial resistance in nontyphoidal Salmonella serotypes: a global challenge. *Clin Infect Dis* 39:546–551.

Wadula J, Von Gottenberg A, Kilner D, de Jong G, Cohen C, Khoosal M, Keddy K, Crewe-Brown H (2006) Nosocomial outbreak of extended-spectrum β-lactamase producing *Salmonella* Isangi in paediatric wards. *Paediart Infect Dis J* 25:843–844.

Weill FX, Demartin M, Tande D, Espie E (2004) SHV-12–like extended-spectrum-β-lactamase–producing strains of *Salmonella enterica* serotypes Babelsberg and Enteritidis isolated in France among Infants adopted from Mali. *J Clin Microbiol* 42: 2432–2437.

Yates C, Amyes S (2005) Extended-spectrum β-lactamases in non-typhoidal *Salmonella* spp. isolated in the UK are now a reality: why the late arrival? *J Animicrob Chemother* 56:262–264.

Zhao S, White DG, McDermott PF, Friedman S, English L, Ayers S, Meng J, Maurer JJ, Holland R, Walker RD (2001) Identification and expression of cephamycinase bla_{CMY} genes in *Escherichia coli* and *Salmonella* isolates from food animals and ground meat. *Antimicrob Agents Chemother* 45:3647–3650.

Detection of mutations in the gyrA of clinical *Salmonella* spp

Govinden U[1*], Mocktar C[1], Moodley P[2, 3] Sturm AW[2] and Essack SY[1]
[1]School of Pharmacy and Pharmacology, University of Kwazulu-Natal, Private Bag X54001, Durban, 4000, South Africa
2. Department of Medical Microbiology, University of Kwazulu-Natal, Durban, 4000, South Africa
3. Department of Infection Control, University of Kwazulu-Natal, Durban, 4000, South Africa

Corresponding author Tel: + 2731-2608251; Fax + 2731-2607792;
E-mail:Govindenu@ukzn.ac.za

Abstract

The high prevalence of resistance to nalidixic acid and reduced susceptibility to ciprofloxacin of *Salmonella* spp. obtained from stool samples of neonates presenting with acute diarrhea in 2001 at the King Edward VIII hospital in Durban, South Africa, prompted this study to determine if there were any mutations in the QRDR of these isolates and to search for the *qnrA* gene. All isolates with nalidixic acid MICs > 48 µg/ml had the single mutation D87N, or D87G in the QRDR of the *gyrA* gene, and only 2 strains had an additional mutation; S83L and S83F respectively. The mutation S83T was present in only one isolate with the nalidixic acid MIC of 10 µg/ml whilst the 6 other strains with nalidixic acid MICs < 10 µg/ml had no changes in the QRDR of the *gyrA* gene. The *qnrA* gene was not found. These findings indicate that there are mutations in the gyrA of *Salmonella* isolates which could contribute to resistance to nalidixic acid with reduced susceptibility to ciprofloxacin and there is the co-expression of quinolone and extended-spectrum ß-lactam resistance among *Salmonella* spp.

Keywords: quinolone resistance, mutations in *gyrA*

Introduction

In gram-negative bacteria the principal target of quinolone including fluoroquinolone activity is the type II topoisomerase, DNA gyrase. DNA gyrase is a tetramer composed of two GyrA subunits (encoded by *gyrA* gene) and two GyrB subunits (encoded by *gyrB* gene) (Kilmartin et al., 2005). DNA gyrase catalyses the negative supercoiling of DNA and is therefore essential for maintenance of DNA topology. Topoisomearse 1V is also a tetrameric enzyme consisting of two ParC and two ParE subunits and is involved in the segregation of replicated daughter chromosomes during DNA replication. Topoisomerase 1V is a homologue of DNA gyrase and the parC and parE genes have strong sequence identity to gyrA and gyrB. Fluoroquinolones stabilize the breaks in the DNA made by the DNA gyrase or topoisomerase 1V, and the resulting drug/enzyme/DNA complex inhibits DNA synthesis (Hopkins et al., 2005).

The isolates were serotyped using commercially available antisera (Bioweb, South Africa) according to the Kauffman-White scheme for *Salmonella* serotyping (Kauffman, 1972, Popoff, 2001).

Susceptibility testing

Disc diffusion susceptibility tests for nalidixic acid and ciprofloxacin, were performed according to NCCLS guidelines (2003). Results were read with the Biomic automated reading system (Giles Scientific, New York).

PCR detection of *gyrA* and *qnrA* genes

Amplification of the *gyrA* and *QnrA* genes were done with primers gyrA- F 5' TGTCCGAGATGGCCTGAAGC 3', gyrA- R 5' CGTTGATGACTTCCGTCAG 3' (Giraud et al., 1999) and QP1- 5' GATAAAGTTTTTCAGCAAGAGG 3' and QP2 - 5' ATCCAGATCGGCAAAGGTTA 3' (Jacoby et al., 2003) respectively. Strains were grown overnight at 37°C in Mueller-Hinton broth. 1.5 ml of each culture was pelleted and cells were boiled in 200 ul of water. After centrifugation the supernatants were kept at -20°C. PCR was performed in a total volume of 50 ul, which contained 5 ul of supernatant, 25 ul of master mix (Applied Biosystems), 25 pmol of each primer and water. After an initial denaturation of 3 min at 94°C, amplification was performed over 30 cycles, each one consisting of 1 minute at 94°C, 1 minute at hybridization temperature 55°C, and 1 min at 72°C, with a final extension step of 10 min at 72°C. Five-microliter aliquots of PCR product were analysed by gel electrophoresis with 1 % agarose. Negative controls were PCR mixtures with the addition of water in place of template DNA. Gels were stained with ethidium bromide at 10 μg/ml and photographed with UV illumination. Sequencing of the amplified products was performed with the SpectruMedix model SCE 2410 automated sequencer (SpectruMedix, State College, PA), incorporating the ABI Big Dye Terminator Cycle Sequencing kit version 3.1 (Applied Biosystems, Foster City, CA). Sequences were analysed using the BLAST 2.0 (Basic Local Alignment Search Tool) software (http://www.ncbi.nlm.mih.gov/Blast/; accessed September 2007.

Results and Discussion

Of the 29 isolates studied, 37% (22/59) were resistant to nalidixic acid with MICs > 48 μg/ml, whilst 63 % (37/59) fell in the susceptible range with MICs from 4 to 10 μg/ml. All the isolates were susceptible to ciprofloxacin with MIC ranging from 0.125 μg/ml to 0.5 μg/ml. Sixteen resistant and 5 susceptible isolates to nalidixic acid were ESBL positive. Sequencing identified mutations in the QRDR of gyrA as per table. Mutations in gyrA were noted for ESBL positive and negative strains with resistance to nalidixic acid; the exception was isolate 376 that was

ESBL positive, susceptible to nalidixic acid and had a S83T mutation. No positive amplification product was obtained for the *qnrA* gene.

All isolates with nalidixic acid MICs > 48 had the single mutation D87N, or D87G in the QRDR of the *gyrA* gene, and only 2 strains; 580 and 695 had an additional mutation; S83L and S83F respectively. The mutation S83T was present in only one strain that had the nalidixic acid MIC of 10 µg/ml whilst the 6 other strains with nalidixic acid MICs < 10 µg/ml had no changes in the QRDR of the *gyrA* gene. An increase in the MIC to ciprofloxacin was noted in most strains with ciprofloxacin MIC > 0.125 µg/ml. Although ciprofloxacin MICs of < 1 µg/ml and > 4 µg/ml are accepted breakpoints for susceptibility and resistance to salmonellae, it has been suggested that fluoroquinolone-susceptible strains that test resistant to nalidixic acid may be associated with clinical failure or delayed response in fluoroquinolone-treated patients with extraintestinal salmonellosis (CLSI, 2008). Susceptibility testing for nalidixic acid is therefore encouraged although this drug is not used for the treatment of extraintestinal *Salmonella* infections. (Rodriguez et al., 2005). A single mutation in *gyrA* of *Salmonella* may be sufficient to cause high-level resistance to nalidixic acid but additional mutations may be required to attain high level fluoroquinolone resistance. Levy et al., (2004) showed that the relative frequency of mutations depended on the particular fluoroquinolone used for selection. Selection with enrofloxacin was more likely to yield S83F mutations, while selection with ciprofloxacin or nalidixic acid favoured recovery of D87G mutations (Hopkins et al., 2005). The most frequent mutations noted in this study were D87G and D87N. There is also a speculation that the reduced quinolone susceptibility may be due to decreased permeability or the presence of efflux pump mechanisms as exposure to low level quinolones can lead to inactivation of the efflux pump system and a reduction in susceptibility, even when there is no mutation in *gyr*A (Cebrian et al ., 2005).

Quinolone resistance in Enterobacteriaceae appears to be a staggered process, where an initial mutation in *gyr*A produces nalidixic acid resistance and decreased susceptibility to fluoroquinolones and facilitates the occurrence of a second mutation in the same gene or in other quinolone target encoding genes that will lead to full resistance. Therefore resistance to naldixic acid could be a good predictor for the emergence of fluoroquinolone resistance (Aznar et al., 2007). These findings indicate that there are mutations in the gyrA of *Salmonella* isolates which could contribute to resistance to nalidixic acid with increased MICs to ciprofloxacin. In addition there is the co-expression of quinolone and extended-spectrum ß-lactam resistance among *Salmonella* spp. The continued use of nalidixic acid and ciprofloxacin could result in further mutations in the DNA gyrase and increasing resistance to these antibiotics.

Acknowledgements

This study was funded by research grants from the MRC, NRF and the University of Kwazulu-Natal.

References

Aznar E, Alarcon T, Buendia B, García Peñuela Lopez-Brea M (2007). Detection of decreased susceptibility to fluoroquinolones in *Salmonella* spp. by five different methods including real-time polymerase chain reaction (PCR). Int J Antimicrob Agents 30: 67-71.

Cattoir V, Poirel L, Rotimi V, Soussy CJ, Nordmann P (2007). Multiplex PCR for detection of plasmid-mediated quinolone resistance qnr genes in ESBL-producing enterobacterial isolates. J.Antimicrob Chemother. 60: 394-397.

Cebrian L, Rodriguez JC, Escribano I, Royo G (2005). Characterisation of *Salmonella* spp. mutants with reduced fluoroquinolone susceptibility: importance of efflux pump mechanisms Chemother. 51: 40-43.

CLSI (2008). Performance standards for antimicrobial susceptibility testing; Eighteenth informational supplement. CLSI document M100- S 18, Wayne, PA: Clinical and Laboratory Standards Institute.

Giraud E, Cloeckaert A, Kerboeuf D, Chaslus-Dancla E (2000). Evidence for active efflux as the primary mechanism of resistance to ciprofloxacin in *Salmonella enterica* Serovar Typhimurium. Antimicrob Agents Chemother. 44: 1223-1228.

Govinden U, Mocktar C, Sturm AW, Moodley P, Essack SY (2008) Characterization of extended-spectrum ß-lactamases in *Salmonella* sp-p. at a tertiary hospital in Durban South Africa. Diagn Microb. Infect Dis. 62: 86-91.

Hopkins KL, Davies RH, Threlfall EJ (2005). Mechanisms of quinolone resistance in *Escherichia coli* and *Salmonella*: recent developments Int J Antimicrob Agents 25: 358-373.

Jacoby GA, Chow N, Waites KB (2003). Prevalence of plasmid-mediated quinolone resistance Antimicrob Agents Chemother. 47: 559-562.

Kauffman F (1972). Serologic diagnosis of Salmonella species. Copenhagen, Denmark: Munksgaard.

Kilmartin D, Morris D, O' Hare C, Corbett-Feeney G, Cormican M (2005). Clonal Expression may account for high levels of Quinolone resistance in *Salmonella enterica* serovar Enteriditis Appl. Environ. Microbiology: 2587-2591.

Levy DD, Sharma B, Cebula TA (2004). Single-nucleotide polymorphism mutation spectra and resistance to quinolones in *Salmonella enterica* serovar Enteritidis with a mutator phenotype. *Antimicrob Agents Chemother.* 48: 2355-2363.

NCCLS (2003). Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically. 6th ed. Wayne, PA: National Committee for Clinical Laboratory Standards.

Popoff MY (2001). Antigenic formula of the Salmonella serovars, 8th ed. Institut Pasteur, Paris, France.

Rodriguez- Avial I, Rodriguez-Avial C, López O, Picazo J J (2005). Trends in nalidixic acid resistance in nontyphoidal *Salmonella* isolated from 1999 to 2002: decreased susceptibility to 6 fluoroquinolones *Diagn. Microbiol. and Infect. Dis.* 52: 261-264.

Table : Mutations in *gyrA* of *Salmonella* isolates

Isolate	Serotype	MIC (µg/ml)		Mutations In *gyrA*	ESBL + (n = 21) ESBL - (n = 8)
		Nalidixic acid	Ciprofloxacin		
216	*S. isangi*	>48	0.25	D87N	+
218	*S. isangi*	>48	0.25	D87N	-
259	*S. isangi*	>48	0.5	D87N	+
262	*S. isangi*	>48	0.25	D87N	+
296	*S. isangi*	>48	0.25	D87N	+
370	*S. kissi*	>48	0.25	D87N	+
493	*S. reading*	>48	<0.125	D87N	+
509	*S. typhimurium*	>48	0.25	D87N	+
606	*S. typhimurium*	>48	0.25	D87N	-
580	*S. kivu*	>48	0.5	D87N ; S83L	+
15	*S. typhimurium*	>48	<0.125	D87G	+
256	*S. isangi*	>48	0.25	D87G	+
464	*S. isangi*	>48	0.125	D87G	+
518*	*ND*	>48	0.25	D87G	-
565	*S. typhimurium*	>48	<0.125	D87G	+
586	*S. typhimurium*	>48	0.25	D87G	-
620	*S. typhimurium*	>48	<0.25	D87G	+
695	*S. typhimurium*	>48	0.25	D87G ; S83F	-
709	*S. typhimurium*	>48	<0.125	D87G	+
873	*S. typhimurium*	>48	<0.125	D87G	+
951	*S. typhimurium*	>48	0.25	D87G	+
978	*S. typhimurium*	>48	<0.125	D87G	-
376	*S. typhimurium*	10	<0.25	S83T	+
31	*S. typhimurium*	8	<0.125	No change	-
2	*S. typhimurium*	8	<0.125	No change	-
420	*S. isangi*	6	<0.125	No change	+
467	*S. kissi*	10	<0.125	No change	+
611	*S. typhimurium*	6	<0.125	No change	+
669	*S. typhimurium*	4	<0.125	No change	+

* Serotype could not be determined

CHAPTER THREE - LIMITATIONS, CONCLUSIONS, RECOMMENDATIONS

3.1 LIMITATIONS

The PER-1 β-lactamase was not considered in this study as it is endemic to Turkey and Argentina. The carbapenemase (KPC-type) was reported in a *Salmonella* serotype *Cubana* isolate in a hospital in the United States in 2003. It has not been reported since and was thus not included in this study. Other examples of non TEM, non SHV ESBLs that have been described but not reported in *Salmonella* such as GES, BES, SFO, TLA, IBC and VEB-1 were also not included in this study (http://www.lahey.org/Studies/; Accessed 17 August 2008; Paterson and Bonomo, 2005).

3.2 CONCLUSIONS

β-lactamases were characterized in 41 *Salmonella* spp. isolates from patients in a pediatric ward of a tertiary hospital in Durban South Africa.

- The most common serotype (17/ 41) was *Salmonella enterica* serotype Typhimurium.
- All isolates were multi-drug resistant with meropenem being the most effective β-lactam antibiotic. Ceftriaxone resistance was found in 17/41 (41 %) of the isolates. Ceftriaxone should thus not be used therapeutically for ESBL producing *Salmonella* spp. and the South African STG and the EDL needs to be reviewed in this regard.

- TEM-1 was the most predominant β-lactamase. The evolution of the TEM-type β-lactamase was evident in the identification of TEM-1, TEM-63, TEM-116 and TEM-131.
- The most common ESBL was SHV-12 whilst SHV-2 was found only in 2 isolates.
- CTX-M-3, -15, -37, CMY-2 and the OXA-1 β-lactamases were also detected.
- This is the first report of TEM-116 and CTX-M-37 in *Salmonella* spp.
- The unique co-expression of TEM-63, CTX-M-37 and the AmpC-type β-lactamase CMY-2, is to our knowledge, the first report of such β-lactamase gene combinations in *Salmonella* spp. globally.
- All isolates with nalidixic acid MICs > 48 µg/ml had the single mutation D87N, or D87G in the QRDR of the *gyrA* gene, and only 2 strains had an additional mutation; S83L and S83F respectively. The *qnrA* gene was not found.
- The co-expression of quinolone and extended-spectrum ß -lactam resistance among *Salmonella* spp. was noted.

This study shows that ESBL positive *Salmonella* spp. can be multi-drug resistant with the propensity to harbour TEM, SHV, CTX-M, CMY and OXA β-lactamases in unique combinations. Co-resistance to quinolones and ESBLs will limit therapeutic options for *Salmonella* infections. Failure to identify drug-resistant salmonellae may affect the choice of appropriate antibiotics in the treatment of patients, especially pediatric patients with invasive salmonellosis.

3.3 RECOMMENDATIONS FOR FURTHER STUDY

Detection of different classes of ß-lactamases in ß-lactam resistant *Salmonella* spp. is of critical clinical importance, since it can often provide valuable information to clinicians leading to more effective and appropriate use of antimicrobials. It also serves as a powerful epidemiologic tool for the infection control purpose (Doi and Paterson, 2007).

There is very little published information on fluoroquinolone, ß-lactamase or ESBL studies on *Salmonella* spp. in South Africa. Focused surveillance studies are needed in hospitals and the community to determine and monitor the extent and spread of resistance in *Salmonella*. Studies should be inclusive of ESBL identification on the molecular level and other types of resistance with emphasis on the mechanisms involved. The use of ceftriaxone, quinolones and fluoroquinolones needs to be closely monitored.

REFERENCES

1. Abassi MS, Torres C, Achour W, Vinue L, Saenz Y, Costa D, Bouchami O, Hassen AB (2008). Genetic characterization of CTX-M-15 -producing *Klebsiella pneumoniae* and *Escherichia coli* strains isolated from stem cell transplant patients in Tunisia. *International Journal of Antimicrobial Agents* 32: 308-14.
2. Aznar E, Alarcon T, Buendia B, García Peñuela Lopez-Brea M (2007). Detection of decreased susceptibility to fluoroquinolones in *Salmonella* spp. by five different methods including real-time polymerase chain reaction (PCR). *International Journal of Antimicrobial Agents* 30: 67-71.
3. Arlet G, Barrett TJ, Butaye P, Cloeckaert A, Mulvey MR, White DG (2006). *Salmonella* resistant to extended-spectrum cephalosporins: prevalence and epidemiology. *Microbes and Infection* 8:1945-1954.
4. Babic M, Hujer AM, Bonomo RA (2006). Whats new in antibiotic resistance? Focus on beta-lactamases. *Drug Resistance Updates* 9: 142-156.
5. Bonnet R, Sampio JLM, Labia R, De Champs C, Sirot D, Chanal C, Sirot R (2000). A novel CTX-M ß-lactamase (CTX-M-8) in cefotaxime resistant Enterobacteriaceae isolated in Brazil. *Antimicrobial Agents and Chemotherapy* 44: 1936-1942.
6. Cai JC, Zhou HW, Zhang R, Chen GX (2008). Emergence of *Serratia marcescens*, *Klebsiella pneumoniae*, and *Escherichia coli* isolates possessing the plasmid-mediated carbapenem-hydrolysing ß-lactamase KPC-2 in intensive care units of a Chinese hospital. *Antimicrobial Agents and Chemotherapy* 52: 2014-2018.
7. Canton R, Coque TM (2006). The CTX-M β-lactamase pandemic. *Current Opinion in Microbiology* 9: 466 – 475.
8. Cardinale E, Colbachini P, Perrier-Gross-Claude JD (2001). Dual emergence in food and humans of a novel multi-resistant serotype of *Salmonella enterica* subsp. Enterica serotype 35:c:1,2. *Journal of Clinical Microbiology* 39: 2373-2374.

9. Cattoir V, Poirel L, Rotimi V, Soussy CJ, Nordmann P (2007). Multiplex PCR for detection of plasmid-mediated quinolone resistance qnr genes in ESBL-producing enterobacterial isolates. *Journal of Antimicrobial Chemotherapy* 60: 394-397.
10. Clinical and Laboratory Standards Institute (2008). Performance standards for antimicrobial susceptibility testing; Eighteenth informational supplement M100-S 18; vol. 28 (1). CLSI, Wayne, PA, USA.
11. Doi Y and Paterson DL (2007). Detection of plasmid-mediated class C ß-lactamases. *International Journal of Infectious Disease* 11: 191-197.
12. Fabrega A, Sanchez-Cespedes J, Soto S, Vila J (2008). Quinolone resistance in the food chain. *International Journal of Antimicrobial Agents* 31: 307-305.
13. Fierer J and Guiney DG (2001). Diverse virulence traits underlying different clinical outcomes of *Salmonella* infection. *Journal of Clinical Investigation* 107: 775-780.
14. Geddes AM, Klugman KP, Rolinson GN (2007). Introduction: historical perspective and development of amoxicillin/clavulanate. *International Journal of Antimicrobial Agents* 30: 109-112.
15. Godet OB, Salem YB, Fabre L, Demartin M, Grimont PAD, Mzoughi R, Weill FX (2005). Nosocomial outbreak caused by *Salmonella enterica* serotype Livingstone producing CTX-M-27 in a neonatal unit in Sousse, Tunisia. *Journal of Clinical Microbiology* 43: 1037-1044.
16. Graham SM, Molyneux EM, Walsh AL, Chesebrough JS, Molyneux ME, Hart CA (2000). Nontyphoidal *Salmonella* infections of children in tropical Africa. *Pediatric Infectious Disease Journal* 19: 1189-1196.
17. Hakanen AJ, Kotilainen P, Pitkanen S, Huiko S, Siitonen A, Huovinen P (2006). Reduction in fluoroquinolone susceptibility among non-typhoidal strains of *Salmonella enterica* isolated from Finnish patients. *Journal of Antimicrobial Chemotherapy* 57: 569-572.
18. Hanson ND, Moland ES, Hossain A, Neville SA, Gosbell IB, Kenneth ST (2002). Unusual *Salmonella enterica* serotype *Typhimurium* isolate producing CMY-7,

SHV-9 and OXA-30 beta-lactamases. *Journal of Antimicrobial Chemotherapy* 49: 1011-1014.

19. Hopkins KL, Threlfall EJ, Karisik E, Wardle JK (2008). Identification of novel plasmid-mediated extended-spectrum β-lactamase CTX-M-57 in *Salmonella enterica* serovar Typhimurium. *International Journal of Antimicrobial Agents* 31: 85-86.
20. http://www.who.int/whr/2005/annexes-en.pdf Accessed 17 August 2008).
21. .http://www.lahey.org/Studies/; Accessed 17 August 2008).
22. Kariuki S, Revathi G, Kariuki N, Muyodi J, Mwituria J, Munyalo A, Kagendo D, Murungi L, Hart CA (2005). Increasing prevalence of multi-drug-resistant non-typhoidal *Salmonella*, Kenya 1994-2003. *International Journal of Antimicrobial Agents* 25: 38-43.
23. Keddy K (2008). Non typhoidal *Salmonella Enterica*. *Communicable Diseases Surveillance Bulletin* 6: 21-28.
24. Kimura S, Ishii Y, Tateda K, Yamaguchi K (2007). Predictive analysis of ceftazidime hydrolysis in CTX-M-type beta-lactamase family members with a mutational substitution at position 167. *International Journal of Antimicrobial Agents* 29: 326-331.
25. Knox JR (1995). Extended-spectrum and inhibitor-resistant TEM-type beta-lactamases: mutations, specificity, and three-dimensional structure. *Antimicrobial Agents and Chemotherapy* 39: 2593-2601.
26. Koeck JL, Arlet G, Philipon A, Basmaciogullari S, Thien HV, Buisson Y, Buisson Y, Cavallo JD (1997). A plasmid-mediated CMY-2 ß-lactamase from an Algerian clinical isolate of *Salmonella Senftenberg*. *FEMS Microbiology Letters* 152: 255-260.
27. Kruger T, Szabo D, Keddy KH, Deeley K, Marsh JW, Hujer AM, Bonomo RA, Patterson DL (2004). Infections with nontyphoidal *Salmonella* species producing TEM-63 or a novel TEM enzyme, TEM 131 in South Africa. *Antimicrobial Agents and Chemotherapy* 48: 4263-70.
28. Li WC, Huang FY, Liu CP, Weng LC, Wang NY (2005). Ceftriaxone resistance of nontyphoidal *Salmonella enterica* isolates in Northern Taiwan attributable to

production of CTX-M-14 and CMY-2 ß-lactamases. *Journal of Clinical Microbiology* 43: 3237-3243.

29. Livermore DM (1995). β-lactamases in laboratory and clinical resistance. *Clinical Microbiology Reviews* 8: 557-84.
30. Livermore DM and Williams JD (1996). Mode of action and mechanisms of bacterial resistance. In Antibiotics in Laboratory Medicine, 4th ed. (Lorain, V. Ed) pp. 502-577. Williams and Wilkins, Baltimore.
31. Matagne A, Brasseur L, Frere JM (1998). Catalytic properties of class A ß-lactamases, efficiency and diversity. *Biochemical Journal* 330: 581-595.
32. Mhand RA, Brahimi N, Moustaoui N, Mdaghri NE, Amarouch H, Grimont F, Bingen E, Benbachir M (1999). Characterisation of extended spectrum beta-lactamase producing *Salmonella Typhimurium* by phenotypic and genotypic Typing methods. *Journal of Clinical Microbiology* 37: 3769-3773.
33. Miriagou V, Filip R, Coman G, Tzouvelekis LS (2002). Expanded spectrum cephalosporin resistant *Salmonella* strains in Romania. *Journal of Clinical Microbiology* 40, (11), 4334-4336.
34. Miriagou V, Tzouvelekis LS, Rossiter S, Tzelepi E, Angulo FJ, Whichard JM (2003). Imipenem resistance in a *Salmonella* clinical strain due to plasmid-mediated class A carbapenemse KPC-2. *Antimicrobial Agents and Chemotherapy* 47: 1297-1300.
35. Miriagou V, Tassios PT, Legakis NJ, Tzouvelekis LS (2004). Expanded-spectrum cephalosporin resistance in non-typhoid *Salmonella. International Journal of Antimicrobial Agents* 23: 547-555.
36. Moland ES, Kim SY, Hong SG, Thomson KS (2008). Newer β-lactamases: Clinical and laboratory implications, Part 1. *Clinical Microbiology Newsletter* : 30 (10), 71-77.
37. Mulvey MR, Soule G, Boyd D, Demczuk W, Ahmed R, and the multi-provincial *Salmonella Typhimurium* case control study group (2003). Characterisation of the first extended spectrum beta-lactamse-producing *Salmonella* isolate identified in Canada. *Journal of Clinical Microbiology* 41: 460-462.

38. National Department of Health (2006). Standard Treatment Guidelines and Essential Drugs List-Hospital Level-Adults. National Department of Health, Pretoria South Africa.
39. Nikaido H, Vaara M (1985). Molecular basis of bacterial outer membrane permeability. *Microbiology Reviews* 45: 1-32.
40. Paterson DL, Bonomo RA (2005). Extended- spectrum β-lactamases: a clinical update. *Clinical Microbiology Reviews* 18: 657-686.
41. Okumura R, Hirata T, Onodera Y, Hoshino K, Otani T, Yamamoto T (2008). Dual-targeting properties of the 3-aminopyrrolidyl quinolones, DC-159A and sitafloxacin, against DNA gyrase and topoisomerase IV: contribution to reducing *in vitro* emergence of quinolone resistant *Streptococcus pneumoniae*. *Journal of Antimicrobial Chemotherapy* 62: 98-104.
42. Poole K (1994). Bacterial multi-drug resistance: emphasis on efflux mechanisms and *Pseudomonas aeruginosa*. *Journal of Antimicrobial Chemotherapy* 34: 435-456.
43. Rasmussen WJ, Hoiby N (2006). OXA- type carbapenemases. *Journal of Antimicrobial Chemotherapy* 57: 373 -383.
44. Rodriguez-Avial I, Rodriguez-Avial C, López O, Picazo JJ (2005). Trends in nalidixic acid resistance in nontyphoidal *Salmonella* isolated from 1999 to 2002: decreased susceptibility to 6 fluoroquinolones. *Diagnostic Microbiology and Infectious Disease* 52: 261-264.
45. Rossolini GM, D'Andrea MM, Mugnaioli C (2008). The spread of CTX-M-type extended-spectrum ß-lactamases. *Clinical Microbiology and Infection* 14: 33-41.
46. Rotimi VO, Jamal W, Pal T, Sonnevend A, Dimitrov TS, Albert M J (2008). Emergence of multidrug-resistant *Salmonella* spp. and isolates with reduced susceptibility to ciprofloxacin in Kuwait and the United Arab Emirates. *Diagnostic Microbiology and Infectious Disease* 60: 71-77.
47. Su LH, Chiu CH, Chu C, Ou JT (2004). Antimicrobial resistance in nontyphoidal *Salmonella* serotypes: a global challenge. *Clinical Infectious Disease* 39: 546-551.

48. Tzouvelekis LS, Tzelepi E, Tassios PT, Legakis NJ (2000). CTX-M-type β-lactamases: an emerging group of extended-spectrum enzymes. *International Journal of Antimicrobial Agents* 14: 137-142.
49. Vahaboglu H, Dodanli S, Eroglu C, Ozturk R, Soyletir G, Yildrim I, Avkan V (1996).Characterisation of multiple-antibiotic-resistant *Salmonella Typhimurium* strains: Molecular epidemiology of PER-1-producing isolates and evidence for nosocomial plasmid exchange by a clone. *Journal of Clinical Microbiology* 34: 2942 – 2946.
50. Wadula J, Von Gottenberg A, Kilner D, de Jong G, Cohen C, Khoosal M, Keddy K, Crewe-Brown H (2006). Nosocomial outbreak of extended-spectrum beta-lactamase producing *Salmonella Isangi* in paediatric wards. *Pediatric Infectious Disease Journal* 25: 843-844.
51. Waxman DJ, Strominger JL (1983). Penicillin-binding proteins and the mechanism of action of β-lactam antibiotics. *Annual Review of Biochemistry* 52: 825-869.
52. Williams JD (1999). β-lactamases and β-lactamase inhibitors. *International Journal of Antimicrobial Agents* 12 Suppl. 1: S3 - S7.
53. Wu JJ, Ko WC, Wu HM, Yan JJ (2008). Prevalence of Qnr determinants among bloodstream isolates of *Escherichia coli* and *Klebsiella pneumoniae* in a Taiwanese hospital, 1999-2005. *Journal of Antimicrobial Chemotherapy* 61: 1234-1239.