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

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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 Accessed 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

β-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 β-lactams have been described. Penicillin, penicillin derivatives, cephalosporins, cephemycins, carbapenems, monobactams and monocarbarms are classified as β-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).

β-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 β-lactams:

- **production of β-lactamases**
  β-lactamases hydrolyse the active β-lactam ring. The destruction of β-lactams by β-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 β-lactamase Mediated Resistance

The first plasmid-encoded β-lactamase that was able to destroy extended-spectrum β-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 β-lactam substituents, thus increasing resistance to these β-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 cephemycins 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 β-lactamases, the OXA-type class D β-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 β-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 β-lactamases of Klebsiella oxytoca. They efficiently hydrolyse many newer broad-spectrum oxyimino-β-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 β-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 delimit the β-lactam binding site, namely the terminal part of the B3 β-strand and the omega loop. The Asp240Gly substitution in the terminal part of the B3 β-strand is responsible for increased flexibility of the β-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 β-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 *Eserichia 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
CMY-2 confers resistance to various extended spectrum cephalosporins, including ceftiraxone, which is the antibiotic of choice for invasive Salmonella infections in children. The movement of the \textit{ampC} gene on to plasmids and transmission to other organisms is of major concern (Hanson \textit{et al.}, 2002). The expression of a plasmid-mediated CMY-2 \(\beta\)-lactamase has been responsible for most ceftiraxone resistance in \textit{Salmonella} spp. (Li \textit{et al.}, 2005). In Africa the first report of the CMY-2 gene in \textit{Salmonella} was from an Algerian clinical isolate of \textit{S. enterica} serotype Senftenberg in 1997 (Koeck \textit{et al.}, 1997), and in 2004 Kruger \textit{et al.}, reported the CMY-2 gene in \textit{Salmonella} Typhimurium and \textit{S. enterica} serotype Schwarzengrund from South Africa.

Many organisms producing class C \(\beta\)-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 \(\beta\)-lactamases have been reported when these patients were treated with cephalosporins. It is imperative that \textit{Salmonella} spp. producing plasmid-mediated class C \(\beta\)-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 \(\beta\)-lactamases (functional group 2f) with potent carbapenemase activities (Miriagou \textit{et al.}, 2003). KPCs are capable of hydrolysing carbapenems, cephalosporins, and aztreonam, and they are inhibited by clavulanic acid and tazobactam (Cai \textit{et al.}, 2008). The only carbapenemase reported in \textit{Salmonella} spp. is of the KPC-type. KPC-producing \textit{K. pneumoniae} strains have been found in hospitals in the USA and subsequently KPC-2 was found in a \textit{Salmonella} serotype \textit{Cubana} isolate also in a hospital in the USA. The emergence of \textit{Salmonella} and \textit{K. pneumoniae} strains producing plasmid-mediated KPC-type \(\beta\)-lactamases in the USA further underlines the potential for exchange of resistance determinants between salmonellae and nosocomial enterobacteria (Miriagou \textit{et al.}, 2004). The KPC-type reportedly confers
resistance to all β-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* (Aret 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 β-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 β-lactamases, thus, refers to their preferred penicillin substrate. Most OXA-type β-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 β-lactamase in *Salmonella* spp. and the first, OXA-30, β-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 β-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 β-lactamases are inhibited less efficiently by clavulanate than the majority of the other group 2 β-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 β-lactamase mediated resistance on Salmonella spp. in South Africa motivated this study.
1.7. Aim

To characterise resistance to β-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


2.2 Submitted Paper

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

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Abstract

β-Lactamase-mediated resistance was investigated in 59 putative extended-spectrum β-lactamase (ESBL)-positive Salmonella spp. from the pediatric 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 π 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.

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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
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 (bioMérieux sa, Marcy l'Étoile, 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, cefazidime, ceftiofur, ceftriaxone, cefepime, ceftazidime (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 1x 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 blatem, blasHV and blctxM

times

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 blatem and blasHV genes was carried out as described by Essack et al. [13]. The amplification mixture for the detection of blctxM 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 MICs 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, cefto-

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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, cefotaxim 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 cephalosporin-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 Ω238S 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 (>128 µg/mL). In contrast to TEM- and SHV-type cephalosporin-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].

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].

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### Table 1

<table>
<thead>
<tr>
<th>Primer</th>
<th>Function</th>
<th>Sequence (5'-3')</th>
<th>Nucleotide positions</th>
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</tr>
<tr>
<td>TEM 1 (R)</td>
<td>Seq</td>
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<td>1-22</td>
</tr>
<tr>
<td>TEM 3 (R)</td>
<td>Seq</td>
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<td>1-22</td>
</tr>
<tr>
<td>TEM 4 (R)</td>
<td>Seq</td>
<td>GTGTGTTTACACATTTTGTTTG</td>
<td>1-22</td>
</tr>
<tr>
<td>SHV 1 (F)</td>
<td>Amp &amp; seq</td>
<td>CAGTGTGTTTACACATTTTGTTTG</td>
<td>1-22</td>
</tr>
<tr>
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<td>Amp &amp; seq</td>
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<td>1-22</td>
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<td>SHV 3 (R)</td>
<td>Seq</td>
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<tr>
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<tr>
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<tr>
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</tr>
<tr>
<td>CTX-MR</td>
<td>Amp &amp; seq</td>
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<td>1-22</td>
</tr>
</tbody>
</table>

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

### Table 2

<table>
<thead>
<tr>
<th>β-Lactam</th>
<th>MIC (µg/mL)</th>
<th>509</th>
<th>541</th>
<th>640</th>
</tr>
</thead>
<tbody>
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<td>Carbenicillin</td>
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<td>&gt;512</td>
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<td>&gt;512</td>
<td>&gt;512</td>
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</tr>
<tr>
<td>Ticarcillin</td>
<td>&gt;256</td>
<td>&gt;256</td>
<td>&gt;256</td>
<td></td>
</tr>
<tr>
<td>Cefotaxime</td>
<td>&gt;128</td>
<td>&gt;128</td>
<td>&gt;128</td>
<td></td>
</tr>
<tr>
<td>Cefazolin</td>
<td>&gt;128</td>
<td>&gt;128</td>
<td>&gt;128</td>
<td></td>
</tr>
<tr>
<td>Cefadolin</td>
<td>&gt;128</td>
<td>&gt;128</td>
<td>&gt;128</td>
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</tr>
<tr>
<td>Cefoxitam</td>
<td>&gt;128</td>
<td>&gt;128</td>
<td>&gt;128</td>
<td></td>
</tr>
<tr>
<td>Ceftriaxin</td>
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<td>&gt;96</td>
<td>&gt;96</td>
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<td>Cefepime</td>
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<td>&gt;64</td>
<td></td>
</tr>
<tr>
<td>Aztreonam</td>
<td>&gt;64</td>
<td>&gt;64</td>
<td>&gt;64</td>
<td></td>
</tr>
<tr>
<td>Amoxicillin/clavulanic acid</td>
<td>&gt;64</td>
<td>&gt;64</td>
<td>&gt;64</td>
<td></td>
</tr>
<tr>
<td>Ampicillin/sulbactam</td>
<td>&gt;48</td>
<td>&gt;48</td>
<td>&gt;48</td>
<td></td>
</tr>
<tr>
<td>Ampicillin</td>
<td>&gt;48</td>
<td>&gt;48</td>
<td>&gt;48</td>
<td></td>
</tr>
<tr>
<td>Piperacillin/tazobactam</td>
<td>8</td>
<td>8</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>Cefoxitin</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>Meropenem</td>
<td>1</td>
<td>0.5</td>
<td>1</td>
<td></td>
</tr>
</tbody>
</table>
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 Mongolia 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

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References

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

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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 (Sturenburg and Mack, 2003). Hence, ESBL are an increasingly important cause of resistance to multiple β-lactam drugs in gram-negative bacteria (Livermore and Hawkey, 2005). 'Classical' ESBL such as TEM (Temoniera) and SHV (sulthiyl variable) have evolved from the widespread plasmid encoded enzyme families (Sturenburg 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...
subclassified by amino acid sequence similarities. A phylogenetic 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 β-lactamases are closest in amino acid identity to the chromosomal cephalosporins 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 β-lactamase KLU-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 Klyuyvera strains and the CTX-M β-lactamase suggest that the natural β-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. cryorescens. This β-lactamase, designated KLCU-1, shares only 85 to 86% identity with the most closely related β-lactamases, which belong to the β-lactamases of the CTX-M-1 group, although an enzyme identical 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. ISECP1-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,-8 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 β-Lactamases**

**America**

In Argentina, a nationwide replacement of cefotaxime with ceftazidime in 1990 co-incided with severe infections, diagnosed with meningitis, septicemia, and enteritis. The infections emerged in August 1990 and were caused by multiresistant strains of Salmonella typhimurium which were resistant to ceftaxime but susceptible to ceftazidime, owing to the production of CTX-M-2 (Rasmussen and Holby, 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 Enterobacteriaceae strains collected in 1996 and 1997 from hospitals in Brazil identified CTX-M-8 in Citrobacter amalonaticus and CTX-M-2 in Proteus mirabilis (Bonne et al., 2000). CTX-M-8 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 (Bonne et al., 2001). During the period 2000 to 2002 CTX-M-14 β-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 β-lactam antibiotics (Bonne et al., 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 1996, Me et al. also reported a cefotaxime-resistant E. coli isolate containing Toho-2 (CTX-M-45). Surveys of ESBL-producing Enterobacteriaceae strains in Japan showed that the CTX-M-2 and CTX-M-3 enzymes predominated. At least three outbreaks involving CTX-M enzymes have occurred in Japan, implicating clonal E. coli spread (Bonne et al., 2004). The molecular types of CTX-M-β-lactamases in Japan were investigated in 1397 gram-negative bacilli collected between 2001 and 2003. 317 isolates were positive for CTX-M-type β-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>
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</table>

Table 1. CTX-M-4 (Type 1)
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 β-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 β-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 β-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 cefazidime-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-

Table 1. Contd.

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* Amino acid sequences of CTX-M-14 and CTX-M-18 are identical.

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 β-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 Holby, 2004).

The CTX-M enzyme was first characterized in Western Europe in two E. coli strains isolated in 1969 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 66-year-old man in Germany during treatment of postoperative peritonitis with cefuroxime (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 (Leffond-Guibout et al., 2004).

Paterson et al. (2003) identified 1 CTX-M-2 type β-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 (Naierri 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. morganii 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 (Mugnaioli et al., 2005). The first description of CTX-M-15 producing K. pneumoniae in Portugal was reported in 2005 (Conceição 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, 39 E. coli isolates and 11 Klebsiella sp. 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 (Chmelinsky et al., 2005). ESBL production was demonstrated in five independent, multidrug-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 blaCTX-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 β-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 newborn 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 (Ali-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. CTX-M-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 2005 of ESBL positive Salmonella sp. revealed the presence of a CTX-M-38 enzyme (Genbank accession number DG864700). 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 by 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 33 strand and possibly other regions. This increased flexibility is correlated with higher ceftazidime 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 β-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 Asp240Gly 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 cephalosporin activity found in the TEM and SHV ESBLs but did not result in increased hydrolysis of ceftazidime 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 (Potrel 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 (Sturenburg 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 under-developed continents where technology for the analysis of ESBL enzymes is scarce. The loss of the oxyiminocephalosporins 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 into the quantitative global distribution is lacking. A coordinated study to obtain this information is urgently needed.

REFERENCES


Al-Agamy MH, Ashour ME, Wiegand I (2006). First description of CTX-M genes from one country to another, which were highly conserved.


REFERENCES


REFERENCES


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Characterization of extended-spectrum $\beta$-lactamases in Salmonella spp. at a tertiary hospital in Durban, South Africa

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Abstract

Extended-spectrum $\beta$-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 ($n = 1$) $\beta$-lactamase were also detected. The diversity of ESBLs suggests that its incidence in Salmonellae needs to be monitored.

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Keywords: Salmonella spp.; Extended-spectrum $\beta$-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., 2005).

Extended-spectrum $\beta$-lactamases (ESBLs) are predominantly associated with Enterobacteriaceae. ESBLs in salmonellae in Africa were 1st described in 1988 (Hammany 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...
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 (Sirota et al., 1987), and E. coli CF 1064 (Chamal 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, cefazidime, cephalexin, cephradine, cefotaxime, cefoxitin, meropenem, aztreonam, and piperacillin–tazobactam were performed with the Spectrumedix model SCE 2410 automated reading system (Spectrumedix, State College, PA), incorporating the ABI Big Dye Terminator Cycle Sequencing kit (Applied Biosystems). 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 reading system (Spectrumedix, State College, PA). The primers (Inqaba Biotechnology, Pretoria, South Africa) are described in Table 1.

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 pH range of 3.5 to 9.5 (Amersham Biosciences, Uppsala, Sweden). An isoelectric point marker pH (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 blaTEM, blaSHV, blaCTX-M, blaOXA, blaCMY, blaDHA, and blaACC genes

All 41 isolates were screened for the presence of blaTEM, blaSHV, blaCTX-M, blaOXA, blaCMY, blaDHA, and blaACC 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 blaTEM and blaSHV genes were carried out as described by Essack et al. (2001). The amplification mixture for the detection of blaCTX-M, blaCMY, blaOXA, blaDHA, and blaACC 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 blaCTX-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 blaCMY, blaOXA, blaDHA, and blaACC 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 Spectrumsedi 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 cefazidime (64 to >128 μg/mL) but susceptible to meropenem (<0.5 to 1 μg/mL). Tazobactam was the most effective inhibitor
because resistance to piperacillin-tazobactam was found in only 1 isolate. TEM-1 (51%) was the most predominant \( \beta \)-lactamase produced, and it was found most frequently in combination with SHV-12 with \( P_i \) values ranging from 5.4 to 6.1 and 7.6 to 8.4, respectively. Isolates 509, 541, and 640 were further reports in South Africa of TEM-63 in Africa (Hasman et al., 2005). In this study, 29% (12/41) of the isolates contained TEM-63 and was most commonly produced only in isolate 509b. A unique combination of TEM-63, CTX-M-37, and the AmpC-type \( \beta \)-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 \( >96 \) \( \mu \)g/mL. The other AmpC-type \( \beta \)-lactamases, DHA and ACC, were not found. To our knowledge, this is the 1st report of such \( \beta \)-lactamase gene combinations in \textit{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 \textit{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 \textit{Salmonella} strains with ESBLs in Africa were identified in 1988 in Tunisia (Hammani et al., 1991). An ESBL study of 160 \textit{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 \textit{Salmonella} Isangi producing ESBLs was 1st reported from the Chris Hani Baragwanath Hospital, Johannesburg, South Africa, in 2006 (Wadula et al., 2006). \textit{Salmonella}eae 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 \textit{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 \textit{Proteus} spp., \textit{Enterobacter} spp., \textit{E. coli}, \textit{Enterobacter cloacae} (Gray et al., 2006), and \textit{Salmonella} spp. (Kruger et al., 2004). An ESBL study in sub-Saharan Africa in Tanzania detected TEM-63 in \textit{E. coli} and \textit{K. pneumoniae} (Blomberg et al., 2005). The 1st report of TEM-63 outside the African continent in \textit{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 \textit{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 cefazidime but low levels of resistance to ceftaxime (Orenica et al., 2001), as can be seen with the 9 isolates (216, 256, 259, 296, 420, 482, 793, 895, and 1048).

Table 1

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5'-3')</th>
<th>Purpose of primer</th>
<th>Reference</th>
</tr>
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<td>TEM 1 (F)</td>
<td>ATGATTATTCAACATTTTCCGTG</td>
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<td>Essack et al. (2001)</td>
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<td>TEM 2 (R)</td>
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<td>CTX-M-3A</td>
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<td>CTX-M (R)</td>
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<td>ACC (F)</td>
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Purpose of primer: Amp = amplification; Seq = sequencing.

\( F = \) forward primer; \( R = \) reverse primer.
expressing only TEM-63 with ceftazidine MICs of >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 such mutations alter the specificity, thus, conferring a selective advantage during the evolution of drug resistance (Sidetakis et al., 2001). TEM-131 from Salmonella spp. in South Africa was first 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

Table 2
Characterization of ESBL-positive Salmonella strains

<table>
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<tr>
<th>S. enterica serotype</th>
<th>Isolate no.*</th>
<th>Isoelectric point (pI)</th>
<th>β-lactamase (s)</th>
<th>MIC (μg/mL)</th>
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<td></td>
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<td>TEM-1, SHV-12</td>
<td>TZP</td>
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<td>&gt;128</td>
</tr>
<tr>
<td></td>
<td>467</td>
<td>5.4</td>
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</tr>
<tr>
<td></td>
<td>493</td>
<td>5.4, 8.2</td>
<td></td>
<td>&gt;128</td>
</tr>
</tbody>
</table>

**TZP** = piperacillin-tazobactam; **CEF** = cefazolin; **CMX** = cefmetazole; **FOX** = cefoxitin; **CRO** = ceftriaxone; **CTX** = cefotaxime; **CAZ** = ceftazidime; **FEP** = cephepine; **ATM** = aztreonam; **ND** = not done.

*Hospital isolate number.

†Antigenic formula (serotype [O] antigen, flagellar [H] antigen phase 1, flagellar [H] antigen phase 2).
pneumoniae. Kruger et al. (2004) reported SHV-12 and appropriate alternative antibiotics in the treatment of identify drug-resistant salmonellae may affect the choice of combinations, which is not frequently reported. Failure to be multidrug resistant, with the propensity to harbor TEM, spp. can the results of this study, the STG and the EDL needs to be compromised. Ceftriaxone should not be used therapeutically if resistance was found in 41% (17/41) of the isolates (948, 954, 317, and 288) produced TEM-116, which is not frequently reported. The expression of gene was reported as bla


catomyces 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

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Detection of mutations in the gyrA of clinical Salmonella spp

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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 β-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. Topoisomerase 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 (Kauffmann, 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) andQP1- 5’ GATAAAGTTTTTCAGCAAGGG 3’ andQP2 - 5’ ATCCAGATCGCAAAGGTTA 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.nih.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 gyrA (Cebrian et al., 2005).

Quinolone resistance in Enterobacteriaceae appears to be a staggered process, where an initial mutation in gyrA 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 nalidixic 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 β-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


Table: Mutations in gyrA of *Salmonella* isolates

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Serotype</th>
<th>MIC (µg/ml)</th>
<th>Mutations in gyrA</th>
<th>ESBL + (n = 21)</th>
<th>ESBL - (n = 8)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Nalidixic acid</td>
<td>Ciprofloxacin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>216</td>
<td>S. isangi</td>
<td>&gt;48</td>
<td>0.25</td>
<td>D87N</td>
<td>+</td>
</tr>
<tr>
<td>218</td>
<td>S. isangi</td>
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<td>0.25</td>
<td>D87N</td>
<td>-</td>
</tr>
<tr>
<td>259</td>
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<td>0.5</td>
<td>D87N</td>
<td>+</td>
</tr>
<tr>
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<td>S. isangi</td>
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<td>0.25</td>
<td>D87N</td>
<td>+</td>
</tr>
<tr>
<td>296</td>
<td>S. isangi</td>
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<td>0.25</td>
<td>D87N</td>
<td>+</td>
</tr>
<tr>
<td>370</td>
<td>S. kissi</td>
<td>&gt;48</td>
<td>0.25</td>
<td>D87N</td>
<td>+</td>
</tr>
<tr>
<td>493</td>
<td>S. reading</td>
<td>&gt;48</td>
<td>&lt;0.125</td>
<td>D87N</td>
<td>+</td>
</tr>
<tr>
<td>509</td>
<td>S. typhimurium</td>
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<td>D87N</td>
<td>+</td>
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<tr>
<td>606</td>
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<td>0.25</td>
<td>D87N</td>
<td>-</td>
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<tr>
<td>580</td>
<td>S. kivu</td>
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<td>0.5</td>
<td>D87N; S83L</td>
<td>+</td>
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<td>15</td>
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<tr>
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<td>D87G</td>
<td>+</td>
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<tr>
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<tr>
<td>518*</td>
<td>ND</td>
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<td>D87G</td>
<td>-</td>
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<td>&lt;0.125</td>
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<tr>
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<td>D87G; S83F</td>
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<td>D87G</td>
<td>+</td>
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<td>0.25</td>
<td>D87G</td>
<td>+</td>
</tr>
<tr>
<td>978</td>
<td>S. typhimurium</td>
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<td>&lt;0.125</td>
<td>D87G</td>
<td>-</td>
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<tr>
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<td>+</td>
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<tr>
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<td>-</td>
</tr>
<tr>
<td>2</td>
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<td>No change</td>
<td>-</td>
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<td>No change</td>
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<tr>
<td>467</td>
<td>S. kissi</td>
<td>10</td>
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<td>No change</td>
<td>+</td>
</tr>
<tr>
<td>611</td>
<td>S. typhimurium</td>
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<td>&lt;0.125</td>
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<td>+</td>
</tr>
<tr>
<td>669</td>
<td>S. typhimurium</td>
<td>4</td>
<td>&lt;0.125</td>
<td>No change</td>
<td>+</td>
</tr>
</tbody>
</table>

* 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 β-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.
Detection of different classes of β-lactamases in β-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, β-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.
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