Chapter 1

THE GLOBAL PROBLEM OF SALMONELLOSIS AND THE EMERGENCE OF MULTIPLE-DRUG RESISTANCE

1.1 THE THREAT OF INFECTIOUS DISEASES

Infectious diseases are, after cardiovascular diseases, the second leading cause of death worldwide (WHO, World Health Report, 2004). Among infectious diseases, acute lower respiratory tract infections, human immunodeficiency virus (HIV)/acquired immune deficiency syndrome (AIDS), diarrhoeal diseases, tuberculosis, and malaria predominate. Diarrhoeal diseases are the third most common cause of infectious disease, resulting in approximately 2, 5 million deaths per year (Lederberg, 1997). Estimates suggest that more than 80% of all diarrhoeal disease occurs in children younger than 5 years of age in developing countries and is attributable to unsafe water, sanitation and hygiene (WHO, World Health Report, 2003; Kosek *et al*., 2003).

1.2 SALMONELLAE AS PATHOGENS

Salmonellae can cause serious disease in both humans and animals, and infections with non-typhoidal salmonellae are a significant cause of illness and death worldwide (Maiorini *et al*., 1993). Salmonellae are widely distributed in the environment, and are found in livestock, wild animals, birds and reptiles, while their increasing prevalence in the global food chain results in diarrhoeal disease and the spread of antimicrobial resistance from animals to humans. Their pathogenic potential and abilities to harbour and spread resistance pose tremendous medical, public health and economic problems affecting animals and humans (Pegues *et al*., 2005). In order to understand the role of salmonellae as pathogens, it is important to study the organism and the pathogenic mechanisms by which it causes disease, as well as the clinical entities associated with the organisms and their treatment.

1.2.1 Structure and classification of salmonellae

Named after American veterinary pathologist, Daniel Salmon, but actually discovered by his assistant Theobald Smith, a subspecies of *Salmonella* isolated from pigs was described in 1885 (Smith, 1894). The antigenically complex *Salmonella* genus belongs to the family of *Enterobacteriaceae.* Salmonellae are gram-negative, flagellated facultatively anaerobic bacilli that measure 2µm to 3µm by 0.4µm to 0.6µm in size (Figure 1.1). Most strains are motile with peritrichous flagella but non-motile variants occur occasionally. Most strains grow on nutrient agar as smooth colonies, 2-4 mm in diameter.

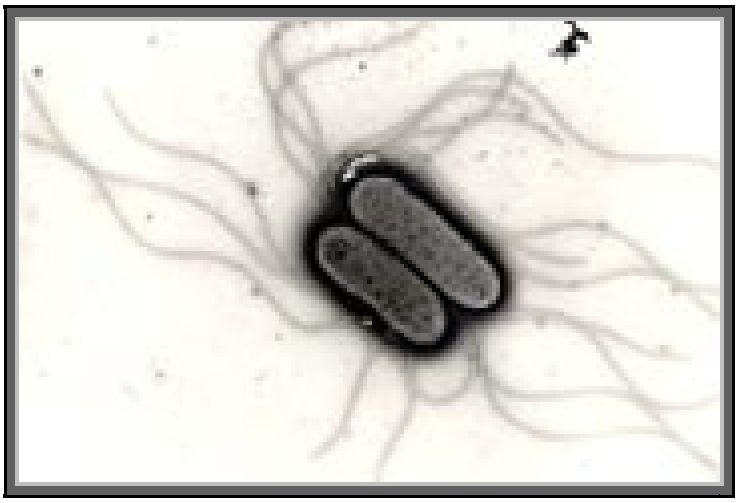


Figure 1.1 Transmission electron micrograph of two cells of *Salmonella* showing the flagella (http://www.schoolscience.co.uk/content/4/biology/sgm/sgmbugs2.html)

According to strict taxonomic criteria based on genome relatedness and biochemical reactions, the genus *Salmonella* has been assigned only two species, *Salmonella enterica* and *Salmonella bongori*. *S. enterica* is subdivided into six subspecies and many serotypes while *S. bongori* (formerly subspecies V) enjoys species recognition in its own right without subspecies, but accommodates some serotypes expressed according to their antigenic formulae (Tindall *et al*., 2005). *S. enterica* contains the following subspecies:

S. enterica subsp. *enterica*

S. enterica subsp. *arizonae*

S. enterica subsp. *diarizonae*

S. enterica subsp. *houtenae*

S. enterica subsp. *indica*

S. enterica subsp. *salamae*

Subspecies *enterica* is commonly isolated from man and warm-blooded animals (Popoff and Le Minor, 2001) whereas the other subspecies and *S. bongori* are associated with cold-blooded animals and the environment (Farmer *et al.,* 1984). The vast majority of human isolates (>99.5%) belong to *S. enterica* subsp. *enterica*.

Serotypes of all the subspecies, except *S. enterica* subsp. *arizonae* and *S. enterica* subsp. *diarizonae* (formerly subspecies IIIa and IIIb respectively), were given names before 1966. In 1966 the WHO Collaborating Centre for Reference and Research on *Salmonella* at the Pasteur Institute (Paris, France), began naming new serotypes only in the case of *S. enterica* subsp. *enterica* (formerly *S. enterica* subspecies) and designated antigenic formulae in the case of serotypes belonging to the other subspecies of *S. enterica* and *S. bongori (*Brenner *et al*., 2000).

1.2.2 Antigenic composition and nomenclature

Strains of the two recognized *Salmonella* spp., *Salmonella enterica* and *Salmonella bongori* (Tindall *et al*., 2005) are serotyped (using the slide and tube agglutination methods) and classified into serotypes (serovars) according to their polysaccharide O (somatic) surface, and when present, capsular Vi (possessed only by serovars *S.* Typhi*, S*. Paratyphi C and *S*. Dublin), and flagellar (H) antigens according to the Kauffmann-White scheme (Kauffmann, 1934). H antigens are further divided into phase 1 and phase 2, indicating that a serovar can switch from one antigen phase to another. Serovars of *Salmonella* may possess one (monophasic) or two (diphasic) H antigen phases. There are approximately 2500 serovars designated within both species (Popoff and Le Minor, 2001). The specific O antigens define serovars into *Salmonella* serogroups, designated as groups A, B, C1, C2, D and E.

The full description/antigenic formula of a salmonella isolate is given in the following sequence: O antigens, Vi: H antigen phase 1: H antigen phase 2 e.g., *Salmonella* Typhimurium would be I 1,4,[5],12 : i: 1,2 (Le Minor and Popoff, 1987; Reeves *et al*., 1989; Tindall *et al*., 2005). The name (e.g., *Salmonella* Dublin) usually refers to the location where the *Salmonella* serotype was first isolated although the early descriptions often refer to the type of disease and the animal from which it was originally isolated e.g. *Salmonella* Typhimurium and *Salmonella* Cholerasuis).

Salmonella nomenclature is controversial and complex since the original taxonomy of the genus was not based on DNA relatedness, but rather names were given according to clinical manifestations e.g. *Salmonella* Typhi, *Salmonella* Enteritidis etc. Antigenic analysis was adopted into the Kauffmann-White scheme in 1966 and each *Salmonella* serovar was considered as a species (Kauffmann, 1966). The vernacular terminology is still preferred by some in medical practice, e.g., *Salmonella typhi* for the taxonomically correct *Salmonella enterica* subspecies *enterica* serotype Typhi (not italicized), *Salmonella typhimurium* for *Salmonella enterica* subsp. *enterica* serotype Typhimurium (Brenner *et al*., 2000). The Judicial Commission of the International Committee on the Systematic Prokaryotes (2005) has however recently advised that the taxonomically validated proposals that the *Salmonella* genus should comprise two species only [(*Salmonella enterica* and *Salmonella bongori* of Le Minor and Popoff (1987) and Reeves *et al*., (1989)] with their subspecies and serotypes as summarized above, be used rather than vernacular epithets (Tindall *et al*., 2005).

1.2.3 Pathogenesis

The mechanisms of *Salmonella* invasion are complex and include initial binding to epithelial cell receptors and the stimulation of non-phagocytic cells to internalize bacteria. Virulence-associated genes are acquired during the evolution of pathogenic bacteria via horizontal gene transfer and are often clustered on plasmids or on chromosomal ‘pathogenicity islands’ (PIs) (Wallis and Galyov, 2000).

After being ingested, the organisms must pass the gastric acid barrier before they reach the intestine where they can cause disease. The infecting inoculum required to cause symptomatic disease in healthy adults must be relatively high (more than 10^5 colony-forming units), although it can be lower when protected by food (Blazer and Newman, 1982). After ingestion, the organisms colonize the ileum and colon, invade the intestinal epithelium, and proliferate within the epithelium and lymphoid follicles (Figure 1.2). Invasion of the epithelium involves an initial binding to specific receptors on the epithelial cell surface and occurs by the organism inducing the enterocyte membrane to undergo 'ruffling', thereby stimulating pinocytosis of the organisms. Two SPI-1 translocated proteins, SipC and SipA, promote membrane ruffling. Attachment and invasion, also known as bacteria-mediated endocytosis, are under distinct genetic control and involve multiple genes in both chromosomes and plasmids (Francis *et al*., 1992).

Salmonellae encode a type III secretion system (T3SS) within *Salmonella* pathogenicity island 1 (the SPI-1 T3SS) that is required for endocytosis and intestinal epithelial invasion (Kubori *et al*., 1998; Chakravortty *et al*., 2005). SPI-1 translocated proteins also contribute to intestinal inflammation and fluid secretion. In the mouse enteric fever model, salmonellae adhere to and enter the M (microfold)-cells within the Peyer's patches, unlike the bovine and rabbit model where they prefer to adhere and invade intestinal enterocytes (Watson *et al*., 1995).

After crossing the epithelial barrier, salmonellae invade macrophages, which shield the bacterium from effectors of humoral immunity and also expose it to the microbicidal and nutrient-poor environment of the phagosome. Encoded in *Salmonella* pathogenicity island 2 (SPI-2), a second T3SS system is expressed which translocates proteins across the membrane of the salmonella-containing vacuole (SCV) into the macrophage cytosol which appears to create an intracellular compartment favorable for bacterial replication (Shea *et al*., 1996).

Figure 1.2 Colour-enhanced scanning electron micrograph showing *Salmonella* Typhimurium (red) invading cultured human cells (http://www2.niaid.nih.gov/Biodefense/Public/Images.htm).

The innate immune system is able to suppress initial *Salmonella* replication, but final clearance of infection and adequate immunity requires a Th-1 type CD4 T-cell response, as well as production of specific antibodies by B-cells (Hess *et al*., 1996). Cell-mediated immune control of *Salmonella* infection in humans is important as individuals with HIV infection or other forms of immune deficiency, including immune suppression following organ transplantation are extremely susceptible to generalized or localized salmonellosis following bacteraemia (Angulo and Swerdlow, 1995).

1.2.4 Clinical manifestations

Salmonella species cause infections ranging in severity from mild self-limiting gastroenteritis to life-threatening typhoid fever and bacteraemic salmonellosis. A study by Lee *et al*. (1994) has shown the median duration of hospitalization to be 4 days for infections with susceptible strains and 5 days for infections with resistant strains in severe cases of diarrhoea.

The incubation time is 6 to 48 hours, although longer and shorter periods have been reported. The disease is characterized by loose or watery stools, lasting 2 to 5 days but

diarrhoea may be prolonged for several weeks. Laboratory diagnosis is by isolation of the bacterium from food or patients' stools (Pegues *et al*., 2005).

Clinical symptoms include abdominal cramps, diarrhoea, vomiting, fever, chills, and malaise. Four clinical types of *Salmonella* infection may be distinguished (Bopp *et al*., 1999):

i) Gastroenteritis, the most frequent manifestation, ranging from mild to fulminant diarrhoea, accompanied by low-grade fever and varying degrees of nausea and vomiting;

ii) Bacteraemia or septicaemia resulting in generalized salmonellosis and in some patients localization to target sites including bones and joints without major gastrointestinal symptoms characterized by high, spiking fever and positive blood cultures;

iii) Enteric fever, a severe systemic illness classically caused by *S*. Typhi, can also be caused by *S*. Paratyphi A, *S*. Paratyphi B and *S*. Paratyphi C. A less severe syndrome can however be rarely caused by any *Salmonella* serotype, usually manifesting as mild fever and diarrhoea.

iv) A carrier state, in which persons with previous infection may continue to excrete the organism in their faeces for up to 1 year following remission of symptoms.

1.2.5 Treatment

1.2.5.1 Appropriate antimicrobial agents

The β-lactam antibiotics amoxicillin and cefotaxime or ceftriaxone for antimicrobial susceptible, and imipenem for ESBL strains are, together with fluoroquinolones, the mainstay for the treatment of serious salmonella infections. Cotrimoxazole and chloramphenicol are also agents with proven efficacy but their use is limited due to resistance problems and potentially severe side effects while aminoglycosides, although they have excellent *in vitro* activity, are not very effective against intracellular salmonellae.

1.2.5.2 Indications and treatment options

Salmonella gastroenteritis is usually a self-limiting disease with fever, lasting 3 to 7 days. Antibiotic treatment is not required for *Salmonella* gastroenteritis but is essential for enteric fever, invasive salmonellosis, and for patients at risk of extra-intestinal disease. Therapy should primarily be directed at the replacement of fluid and electrolyte losses. Fluoroquinolones, such as ciprofloxacin and ofloxacin, and extended-spectrum cephalosporins (ESCs), such as ceftriaxone and cefotaxime, have been used for the indications mentioned above but resistance to these agents has been reported (Parry, 2003). Based on the current trends, imipenem/meropenem appears to be the best option for therapy of serious infections caused by ESBL-producing Enterobacteriaceae.

Salmonella infections may warrant quinolone or other antimicrobial therapy when systemic spread is considered a risk or suspected and for children less than 6 months of age; however, like other antimicrobial agents, quinolones may prolong shedding of NTS (DuPont, 1997). Antimicrobial prophylaxis has been required to control institutional outbreaks, especially in long-term care facilities or paediatric wards, where compliance with infection control measures may be difficult (Lightfoot *et al*., 1990).

The β-lactam antibiotics amoxicillin and cefotaxime or ceftriaxone for 'susceptible' isolates and imipenem/meropenem for ESBL infections, together with fluoroquinolones are the major candidates for the treatment of invasive salmonellosis. The interaction between fluoroquinolone and salmonellae will be dealt with briefly in this dissertation while that between β-lactam agents and salmonellae will be presented in more detail, also at the molecular level.

1.3 GLOBAL SIGNIFICANCE OF SALMONELLAE

1.3.1 Increasing trend of salmonellosis

The incidence of NTS infections has increased globally, but with marked differences among countries (http://www.who.int/salmsurv/en/). In the United States it is estimated that there are one to three million cases of *Salmonella* infections, resulting in 400-600 deaths each year (Parry, 2003). In 2002, the incidence rate of salmonellosis (17.7 per 1000,000 population) was highest among 10 potentially foodborne diseases under active surveillance and this high ranking varied little by geographic region in the USA (CDC, 2000).

Studies in the United States indicate that non-typhoidal *Salmonella* (NTS) serotypes are the second most common agents among the bacterial diarrhoeal pathogens (Slutsker *et al*., 1997). NTS gastroenteritis and septicaemia have increasingly been noted in persons with AIDS and related immunocompromised individuals from diverse high-risk groups (Mayer and Hanson, 1986).

1.3.2 Impact of HIV/AIDS on salmonellae infection

Several factors may explain the severity of *Salmonella* infections in immunocompromised patients, including that *Salmonella* is an organism that can survive intracellularly for long periods of time, despite the action of humoral antibodies and antibiotics (Gelzer and Suter, 1959; Hopps and Smadel, 1961). It has been suggested that patients with AIDS, like those with Hodgkin's disease or other syndromes with T-cell defects, are at risk for severe infections with *Salmonella* (Jacobs *et al*., 1985).

The epidemic of HIV/AIDS in developing countries, particularly in sub-Saharan Africa, has reached a crisis point (Smego, 1999). South Africa has the highest number of HIV-infected people in the world, with an estimated 5 million infected by the virus (Fassin and Schneider, 2003) and the statistics are astounding: 1,700 new cases of HIV

infection each day, seroprevalence of more than 30% among antenatal clinic attendees in the most severely affected regions, 6-8 million people infected, and a growing number of HIV/AIDS orphans (Walker *et al*., 2003). A global total of 4.9 million people became newly infected with HIV in 2005 (including 700,000 children), of which 3.2 million new infections occurred in sub-Saharan Africa (Global Health Council, 2006). HIV-infected persons have an estimated 20- to 100-fold increased risk of salmonellosis compared with the general population (Celum *et al*., 1987). Among HIV-infected persons in Africa, *Salmonella* species are one of the most common causes of bacteraemia, are often multi-drug resistant and associated with high mortality (24% - 80%) and recurrence rates (43%) (Gordon *et al*., 2002). Recurrent NTS bacteraemia is regarded as an early marker for AIDS in HIV infected individuals (Gruenewald *et al.*, 1994).

In Kenya, NTS bacteraemia was detected in 11% of HIV-seropositive individuals on admission to hospital (Gilks *et al*., 1990). The clinical course of *Salmonella* infections in patients with AIDS is different from that seen in non-AIDS persons (Wolday and Erge, 1998). One striking difference is the incidence of bacteraemia (Jacobs *et al*., 1985). The frequency of *Salmonella* bacteraemia has been reported in several studies of paediatric bacteraemia from tropical Africa (Graham *et al*., 2000), including South Africa (Berkowitz, 1984), Nigeria (Alausa *et al*., 1977), Kenya (Nesbitt and Mirza, 1989), Gambia (Mabaey *et al*., 1987), Malawi (Gordon *et al*., 2001) and Rwanda (Lepage *et al*., 1987). NTS ranked as the most frequent cause of septicaemia in children in Rwanda (Lepage *et al*., 1987) and Zaire (Green and Cheesbrough, 1993). It was reported that the infections in patients tended to be persistent and relapses of bacteraemia were common.

Diarrhoea is characteristic of HIV/AIDS (Prasad *et al*., 2000) and approximately 90% of HIV/AIDS patients in Africa suffer from chronic diarrhoea (Janoff and Smith, 1998). The degree of morbidity and mortality due to diarrhoeal diseases in developing countries, particularly in Africa, is compounded by the epidemic of HIV/AIDS (Obi and Bessong, 2002).

Many HIV/AIDS patients in South Africa are residents of rural areas, which lack adequate sanitation, proper hygienic practices, and efficient supply of potable water (Grabow, 1996; Von Schirnding, 1993). Many rural households obtain water directly from streams, ponds, or rivers. These sources of water are contaminated, highly polluted, usually not treated, and thus represent sources of transmission of water-borne diseases exemplified by diarrhoea (Grabow, 1996).

1.3.3 Invasive salmonellosis in Africa

The emergence of multidrug-resistant *Salmonella* serotypes is widespread in Africa (Molyneux *et al*., 2000; Kariuki *et al*., 2005) and ESBLs have been found in many other enterobacterial species in South Africa (RSA) (Bell *et al*., 2002; Essack *et al*., 2001; Hanson *et al*., 2002; Pitout *et al*., 1998). In sub-Saharan Africa, where invasive infections caused by NTS serotypes are particularly common in children under the age of 5 years and adults with HIV infection, the routes of transmission have not been characterized (Parry, 2003). Bottone *et al*., (1984) showed that patients diagnosed with AIDS developed *Salmonella* bacteraemia as an early manifestation of their illness. Invasive salmonellosis is also common among children in tropical Africa, typically presenting as a non-specific febrile illness that is difficult to distinguish clinically from malaria (WHO, 1986). Several studies from sub-Saharan Africa have indicated *Salmonella* species as the main organism of blood-borne infection (Berkowitz, 1984; Nesbitt and Mirza, 1989). The progressive increase in invasive *Salmonella* species infection was associated with an increase in HIV-seropositivity (Crewe-Brown *et al*., 1998).

1.3.4 Outbreaks involving drug-resistant salmonellae

Outbreaks and epidemic situations due to MDR bacilli may occur simultaneously in different hospitals as a consequence of the sequential hospitalization of colonized patients in multiple hospitals.

Holmberg *et al.* (1987) reviewed data from the CDC for community-based and nosocomial outbreaks of non-typhoidal salmonellosis occurring in the United States between 1971 and 1980. In community-based outbreaks caused by drug-susceptible strains, the death rate was three (0.2%) of 1321, whereas for multidrug-resistant strains, it was 7 (3.4%) of 205. In nosocomial outbreaks, the comparable figures were two (1.0%) of 202 for susceptible strains and 30 (11.7%) of 256 for multidrug-resistant strains.

Many hospitals have experienced outbreaks of ESBL-producing organisms which are often fueled by the large number of patient transfers between units and between hospitals (Lucet *et al.*, 1999). Multidrug-resistant bacteria in both the hospital and community environment are an important concern to the clinician and the pharmaceutical industry, as it is the major cause of failure in the treatment of infectious diseases (Jones and Phaller, 1998). Once MDR strains are introduced into a hospital environment, they are difficult to eradicate as they are subjected to constant selection by different antibiotics. Routine surveillance of antimicrobial resistance in hospitals revealed trends in resistance patterns and provided evidence of nosocomial spread of drug-resistant bacteria worldwide.

1.3.5 The role of nosocomial infections

Nosocomial infections are associated with a longer hospital stay, account for substantial health care cost and resource utilization and are responsible for considerable mortality affecting all ages. Nosocomial infections are especially associated with patients at the extremes of life and there is an almost nine-fold greater risk of death in the neonatal period (Parry, 2003), while pneumonia in elderly patients in an institutional setting, (including hospitals) is associated with an excessively higher mortality rate (Bonten and Bergmans, 1999).

Three MDR types of bacteria causing acute infections have emerged as major problems responsible for nosocomial infections: methicillin-resistant *Staphylococcus aureus*

(MRSA), vancomycin-resistant *Enterococcus* (VRE) species (spp) and extended-spectrum beta-lactamase (ESBL)-producing gram-negative bacilli (Harbath *et al*., 2001). ESBL-producing gram-negative bacilli are of special clinical concern, as they may transfer resistance genes to other more pathogenic bacterial genera.

Several outbreaks caused by ESBL-producing salmonellae have been reported in the last several years following the overuse of extended-spectrum cephalosporins (Sanders and Sanders, 1992). In a surveillance study from the Centers for Disease Control [(CDC, Atlanta, United States of America (USA)], among 758 subjects with culture-confirmed NTS diagnosed between 1989-1990 in the USA, 232 (31%) were infected by *Salmonella* spp. resistant to at least one antimicrobial agent and 189 (25%) resistant to two or more antimicrobials (Lee *et al*., 1994).

Outbreaks involving resistant gram-negative bacteria in various hospitals have been reported and the globalization of this problem has now been recognized (Cantón *et al*., 2003). This situation may be due not only to the concurrent emergence of resistant bacteria simultaneously in different institutions, but also to the clonal spread of these bacteria among different institutions (Cantón *et al*., 2003).

1.4 EMERGENCE OF DRUG RESISTANCE IN SALMONELLAE

1.4.1 Selection pressure of antimicrobial agent usage

The use of antimicrobial agents exerts selective pressure for the emergence of drug resistance. The treatment of salmonellosis, exposing a high organism load to an antimicrobial drug, could select for resistant mutants especially when a favourable environment for the pathogen is created e.g. under inappropriate therapeutic conditions when inappropriate antimicrobials are prescribed or when dosages or dose intervals are incorrect.

Patients with severe infections require hospitalization, creating an ideal setting for the emergence of drug resistance. Bacterial diseases, including salmonellosis are also

increasingly moving from epidemic to endemic situations involving not only multiple-drug resistant (MDR) bacterial strains, but also plasmids and other mobile gene cassettes capable of disseminating resistance genes (Livermore, 2003; Norrby, 2005). These genetic elements spread initially within body sites colonized by indigenous bacterial species and then to other human hosts and in the process complicate and compromise the effective control of nosocomial infections (Cantón *et al*., 2003).

1.4.2 Extent of drug resistance in salmonellae

High rates of antimicrobial resistance (>50% to 100%) to chloramphenicol, trimethoprim/sulphamethoxazole and ampicillin have been reported from Africa, Asia, and South America (Pegues *et al*., 2005). Of particular concern is the worldwide emergence of multidrug-resistant *S*. Typhimurium DT104 that is resistant to at least five antimicrobials (ampicillin, chloramphenicol, streptomycin, sulphonamides, and tetracyclines) (Humphrey, 2001). Antibiotic resistance genes can be propagated by mobile genomic cassettes, including integrons and transposons that can reside in the chromosome and on plasmids (Summers, 2002).

Since 1988 onwards, NTS isolates with resistance to ESBLs have been reported from countries in North and West Africa, South America, the Middle East, Eastern Europe and East Asia, Russia, India, Turkey, Greece and the United States (Parry, 2003). Differences in geographical distribution of ESBLs may be based on different treatment and prophylactic protocols (Szabó *et al*., 1997). The growing incidence of ESBLs in *Salmonella* serotypes is alarming and is also well illustrated by the 1997-1999 SENTRY survey, in which 3.4% of the *Salmonella* strains studied were found to express ESBLs (Winokur *et al*., 2001). Surveillance data demonstrated an alarming increase in overall antimicrobial resistance among salmonellae from 20%-30% in the early 1990s to as high as 70% in some countries at the turn of the century (Su *et al*., 2004).

Salmonellae have been found to express a wide variety of ESBL types, including TEM, SHV, PER, GES and CTX-M enzymes (AitMhand *et al*., 2002; Baraniak *et al*., 2002;

Bradford *et al*., 1998; Casin *et al*., 2003; Hanson *et al*., 2002; Kruger *et al*., 2004; Revathi *et al*., 1998; Villa *et al*., 2000; Weldhagen and Prinsloo, 2004; Chouchani *et al*., 2006; Govinden *et al*., 2006). In a study in South India, 60% of *Salmonella* spp. expressed the TEM-1 β-lactamase (Nandiva and Amyes, 1990). Additionally, strains have also been detected which produce acquired AmpC-type β-lactamases (Hanson *et al*., 2002; Pitout *et al*., 2003). The emergence of the cefotaxime-hydrolyzing β-lactamase (CTX-M) among *Salmonella* species is of concern as it displays higher levels of resistance to CTX and ceftriaxone than CAZ and may be related to the increased usage of extended-spectrum cephalosporins in recent years (Bonnet, 2004).

In the early 1990s, *S.* Typhimurium DT104 was isolated and found to be resistant to five antimicrobial agents (ampicillin, chloramphenicol, streptomycin, sulfonamide, and tetracycline) and its prevalence in the United Kingdom increased from <1% in 1997-1980 to 34% in 1996 (Davis *et al*., 1999).

A concerning trend is the appearance of isolates with additional trimethoprim and low-level ciprofloxacin resistance. As a result of their wide spectrum of activity, quinolones have been extensively used. Ciprofloxacin was regarded as the most consumed antibacterial agent worldwide and has been blamed for the rapid development of bacterial resistance to these agents. There are a few reports of *Salmonella* strains fully resistant to fluoroquinolones (Nakaya *et al*., 2003; Tibbetts *et al*., 2003). In a European surveillance study of 27 000 isolates in 2000, low-level ciprofloxacin resistance was found in 13% of *S*. Typhimurium, 8% of *S*. Enteritidis, 53% of *S*. Virchow and 57% of *S*. Hadar isolates (Threlfall *et al*., 2003). A national survey, conducted in the United States in 1994-1995 demonstrated that, of 4008 *Salmonella* isolates tested, 21 (0.5%) were nalidixic acid resistant and one isolate was resistant to ciprofloxacin. This rate of nalidixic acid resistance increased 5-fold, to 2.5% (Mølbak *et al*., 1999).

Another alarming fact is the recent isolation of a *Salmonella* strain that was resistant to imipenem, producing the recently described KPC-2 β-lactamase, although susceptible to nalidixic acid and ciprofloxacin (Miriagou *et al*., 2003). The growing incidence of

resistance in *Salmonella* spp., especially that of ESBLs, is concerning as this species has a great potential to enter the microflora of farm animals and the food produced of them.

1.5 MOLECULAR INTERACTION BETWEEN FLUOROQUINOLONES AND SALMONELLAE

1.5.1 Target alterations

Resistance to fluoroquinolones in salmonellae is mostly due to mutations in the quinolone resistance-determining region (QRDR) of the DNA gyrase genes (Cloeckaert and Chaslus-Dancla, 2001). Fluoroquinolones inhibit DNA synthesis when the antibiotic interacts with the complex formed by the union of the DNA with the quinolone target, the DNA gyrase (*gyrA* and *gyrB*) and topoisomerase IV (*parC* and *parE*). The quinolones induce a conformational change in the enzyme and breaks the DNA as well as prevent re-ligation of the broken DNA strands, thus blocking progression of polymerase and DNA replication (Wentzell and Maxwell, 2000). It is thought that cell death occurs when DNA is released from the quinolone-gyrase-DNA complex.

In salmonellae, where DNA gyrase is the primary target of quinolone action, a single point mutation in the QRDR of *gyrA* and *gyrB* can mediate resistance to the non-fluorinated quinolone nalidixic acid and reduced susceptibility to fluoroquinolones such as ciprofloxacin, e.g., an MIC of 0.25µg/ml (Piddock, 1999). Resistance to nalidixic acid has been shown to be an indicator of low-level fluoroquinolone resistance (Crump *et al*., 2003; Hakanen *et al*., 1999a, 1999b; Ruiz *et al*., 2003).

1.5.2 The efflux system

Another mechanism that may cause fluoroquinolone resistance in salmonellae is the recently reported AcrAB-TolC efflux system and its regulatory genes, *marRAB* and *soxRS* (Cloeckaert and Chaslus-Dancla, 2001). Active efflux systems that act synergistically with the outer membrane could have a high level of participation in the

intrinsic and the acquired antibiotic resistance of gram-negative bacteria (Nikaido, 1998). When the *acrB* gene is inactivated, the resistance level to fluoroquinolones is significantly reduced. A recently identified efflux pump inhibitor, Phe-Arg-naphthylamide dihydrochoride (MC-207,110) has been suggested as a candidate for use in combination with fluoroquinolones to treat infections with *Salmonella* strains with high-level resistance to fluoroquinolones (Baucheron *et al*., 2002).

1.5.3 Outer membrane permeability

A few studies have reported on alterations of outer membrane protein expression of lipopolysaccharide in quinolone-resistant *Salmonella* (Giraud *et al*., 2000; Piddock *et al*., 1998). It has been hypothesized that increased amounts of lipopolysaccharide form a permeability barrier which acts preferentially against hydrophilic quinolones (Michéa-Hamzehpour *et al*., 1991). Lack of expression of the OmpF porin has also been reported in some quinolone-resistant *Salmonella* strains (Piddock *et al*., 1993). It has been shown that the upregulation of SoxS may be responsible for the lack of expression of OmpF. The interaction both of changes in cell envelope preventing the entry of antimicrobials and the active efflux system is complex.

1.6 MOLECULAR INTERACTION BETWEEN β-LACTAM ANTIBIOTICS AND SALMONELLAE

1.6.1 Mechanism of action of β-lactam agents

β-Lactam antibiotics interfere with the final stage of cell wall (peptidoglycan) synthesis (Danziger and Pendland, 1995) by inhibiting bacterial enzymes called penicillin-binding proteins (PBPs). PBPs are bifunctional enzymes present in the cytoplasmic membrane of bacteria. They catalyze the synthesis of peptidoglycan in bacterial cell walls by linking N-acetyl glucosamine to muramic acid (transglycosidase function) and through transpeptidase activity mediate cross-linking of peptidoglycan polymers by means of interlinking pentapeptide side chains. Peptidoglycan has a net-like structure (Figure 1.3

composed of saccharide chains cross-linked by peptides (Livermore and Williams, 1996) and protects the organism from osmotic rupture (Salton, 1964).

β-Lactamases are largely retained in the periplasmic space between the cytoplasmic and outer membranes (Figure 1.3). The efficacy of β-lactam antibiotics, specifically against gram-negative bacteria, is dependent on accessibility to its targets, the degree of resistance to enzymatic inactivation by β-lactamases present in the periplasm, and their ability to inhibit the target PBPs. Any alteration of these parameters may result in resistance (Bellido and Pechére, 1991).

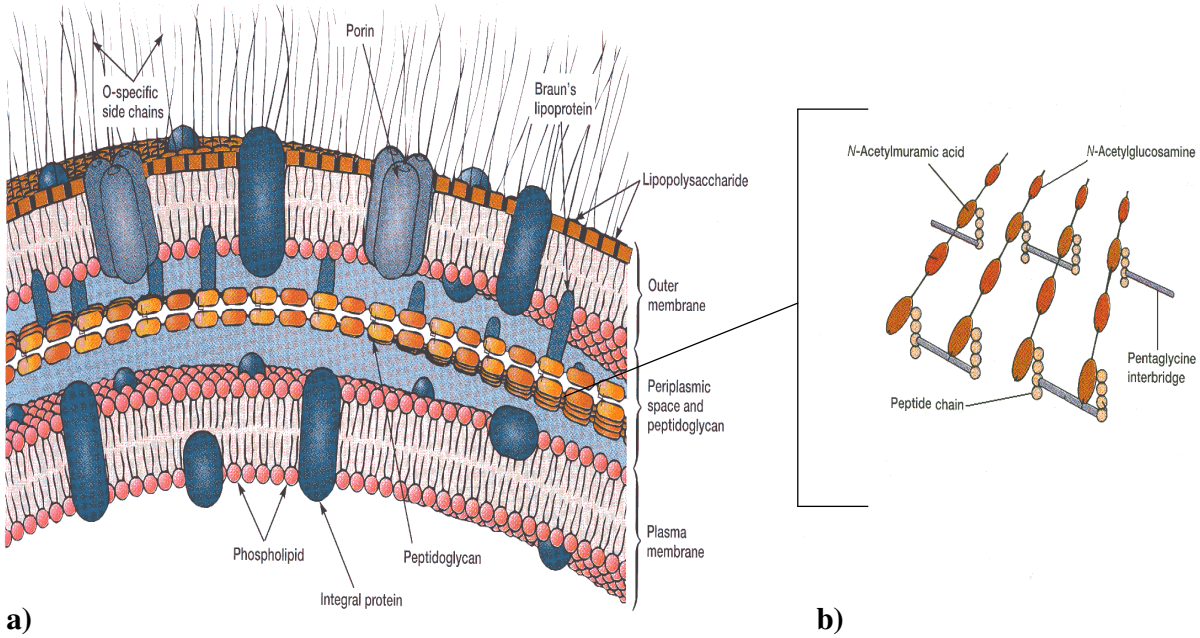


Figure 1.3 a) The gram-negative bacterial envelope. **b)** Peptidoglycan structure showing the polysaccharide chains, tetrapeptide side chains, and peptide interbridges. Adopted from (www.arches.uga.edu/~kristenc/cellwall.html)

1.6.2 Genetic basis of resistance to β-lactam agents

The molecular basis of resistance to β-lactam agents relates to either a) mutations affecting the affinity of PBPs for β-lactam agents e.g. penicillin resistance in pneumococci (Tomasz and Munoz, 1995) and gonococci (Dougherty, 1986) or b) the

acquisition of gene cassettes encoding β-lactamases which may be located in bacterial chromosomes or extrachromosomally-located on plasmids, transposons or integrons (Medeiros, 1997; Collis and Hall, 1995).

Prominent examples of genetically acquired resistance are the *mecA* gene of *Staphylococcus aureus* encoding PBP2A which takes over cell wall assembly, resulting in methicillin resistance (MRSA) (Chambers *et al*., 1985; de Jonge and Tomasz, 1993) and β-lactamases of gram-negative bacteria which hydrolyze β-lactam antibiotics (Medeiros, 1997; Chambers *et al*., 1985). The original β-lactamases are thought to have evolved from PBPs as a result of selective pressure exerted by β-lactam-producing soil organisms (Ghuysen, 1991). The first plasmid-mediated β-lactamase (TEM-1) was described in the early 1960s (Datta and Kontomichalou, 1965) and spread rapidly from *Escherichia coli* to other species of bacteria. The spectrum of β-lactam antibiotic substrates for evolving β-lactamases also progressed as a result of the selective pressures of newly introduced β-lactam agents. With the advent of the oxyimino-cephalosporins in the 1980s it was inevitable that extended-spectrum β-lactamases would develop (Medeiros, 1997; Kliebe *et al*., 1985).

1.7 EXTENDED-SPECTRUM BETA (β)-LACTAMASES (ESBLs)

1.7.1 Definition of extended-spectrum β-lactamases

Extended-spectrum β-lactamases are by definition, molecular class A or D β-lactamases which (i) are able to hydrolyze oxyimino-cephalosporins (cefotaxime, ceftazidime) at a rate equal to or higher than 10% of that for benzyl penicillin, (ii) have an active-site serine, and (iii) generally are inhibited by β-lactamase inhibitors such as clavulanic acid, sulbactam, or tazobactam (Bush *et al*., 1995; Livermore, 2005; Burwen *et al*., 1994). Up to the present time, ESBLs have only been described in gram-negative bacilli (Livermore, 1998).

1.7.2 Nature and function of β-lactamases

β-Lactamases split the amide bond of the β-lactam ring. They can be classified according to their phenotypic or functional properties based mainly on their preferred substrates and inhibition by clavulanate (Bush *et al*., 1995) while a molecular-based classification according to their amino acid sequences which are stable and not prone to distortion by mutations has been proposed by Ambler (Ambler *et al*., 1991). The latter classification divides β-lactamases into classes A to D. Classes A (generally TEM, SHV and CTX-M), C (AmpC-type) and D (OXA-type) constitute groups of serine-based enzymes and class B β-lactamases are metallo-enzymes requiring zinc for their activity (Bush *et al*., 1995; Ambler *et al*., 1991).

1.7.3 Evolution of ESBLs

1.7.3.1 Point mutations in the *bla* gene

Apart from conferring resistance to cefotaxime or ceftazidime, ESBLs also hydrolyse other broad-spectrum cephalosporins and monobactams such as aztreonam (Phillipon *et al*., 1989a; 1989b). They arise from *bla* genes encoding common plasmid-mediated SHV-1, TEM-1, and TEM-2 β-lactamases and evolve by mutations that alter the amino acid configuration around the active site of these enzymes so as to expand their spectrum of activity (Nukaga *et al*., 2003; Orencia *et al*., 2001). A website tracks the number and properties delineating these β-lactamase/ESBL enzymes (Jacoby and Bush, 1998)

Each ESBL has from one to four amino acid substitutions (point mutations) compared with the parent (SHV-1 and TEM-1) enzyme, constituting less than two percent of the protein sequence, and which sufficiently remodel the β-lactamase's active site to increase catalytic activity to encompass the broad-spectrum β-lactam antibiotics (Livermore, 1995). The most important substitutions are spectrum-extending mutations (Ambler position 164 in TEMs, 179 in SHVs and 238 in both). Phenotypic changes mediated by point mutations were initially clustered in five areas of the enzyme, which

are in close proximity to the active site cavity of the β-lactamases, creating novel enzyme-substrate interactions particularly with the oxyimino substituents of the newer cephalosporins (Du Bois *et al*., 1995) (Table 2.2).

The CTX-M-type β-lactamases have unique features and probably originated as a result of horizontal gene transfer and subsequent mutation from the chromosomal AmpC β-lactamases of *Klyuvera ascorbata* (99% homology with CTX-M-2). Their hydrolyzing capabilities are not due to a few amino acid substitutions but are 'intrinsic' which enables an ancestral enzyme to expand its substrate spectrum towards oxyimino-β-lactams.

1.7.3.2 Spread of ESBLs by plasmids and integrons

Acquisition of resistance determinants (ESBLs) is through mobile genetic elements including plasmids, transposons, and gene cassettes in integrons (Davies, 1994). Plasmids responsible for ESBL production tend to be large (80 kb or more in size), autonomous, extra-chromosomal, self-duplicating genetic elements that can carry resistance, virulence and other metabolic capacities. β-Lactamases encoded by transmissible plasmids were first observed in *Enterobacteriaceae* by Datta and Kontomichalou (1965) and have subsequently increased in frequency and spread to other groups of gram-negative aerobes (Dionisio, 2002).

Resistance genes of plasmids are often located within transposons and are often further clustered within elements called integrons. Transposons cannot replicate independently and must be maintained within a plasmid or a chromosome (Gentry, 1991). Integrons are frequently found within transposons and plasmids but are also found in bacterial chromosomes (Medeiros, 1997). Each resistance gene in an integron is encoded in a mobile gene cassette that can be excised and then incorporated into another integron on another genome (Negri, 2000). The conjugative transposons may be excised to form an intermediate that may transfer and regenerate a double-stranded circle in another bacterial cell and integrate into its chromosome (Tolmasky and Crosa, 1987).

1.7.4 Types of ESBLs

ESBLs may be divided into four groups: oxyimino TEM- or SHV-derived enzymes and non-TEM or non-SHV enzymes (OXA-derived ESBLs) (Sirot, 1995). More than 150 different natural ESBL variants are known at present. SHV and TEM ESBLs belong to class A enzymes and have serine as the main catalytic residue in their active site and are penicillinases and cephalosporinases usually found on plasmids or transposons (Ambler *et al*., 1991; Livermore, 1995; Gniadkowski, 2001).

TEM-1 β-lactamase activity is the most commonly encountered mechanism of resistance to the β-lactam group of drugs in gram-negative bacilli and is responsible for up to 50-60% of the plasmid-mediated ampicillin resistance in *E. coli* (Livermore, 1995). The TEM-type ESBLs are derivatives of TEM-1 and TEM-2. TEM-1 was first reported in 1965 from an *E. coli* isolate from a patient in Athens (Greece) named Temoneira (Datta and Kontomichalou, 1965).

The SHV family of β-lactamases is universally found in *K. pneumoniae* and evolved as a chromosomal gene in *Klebsiella* and was later incorporated into a plasmid, which has spread to other enterobacterial species. SHV refers to *s*ulf*h*ydryl *v*ariable (Sykes and Bush, 1982) and confers resistance to broad-spectrum penicillins such as ampicillin, ticarcillin and piperacillin but not to the oxyimino substituted cephalosporins (Livermore, 1995).

β-Lactamases that are not TEM and SHV derivatives include PER-1 (Danel *et al*., 1995), its close relative CTI-1 (Bauernfiend *et al*., 1994), MEN-1 (Bernard *et al*., 1992), as well as CTX-M which has mainly been found in strains of *Salmonella* Typhimurium and *E. coli* (Tzouvelekis *et al*., 2000). Paterson *et al*. (2003) reported the geographical spread of CTX-M-type ESBLs to Australia, Belgium, Turkey, and South Africa (Bradford *et al*., 1998; Chanawong *et al*., 2002; Coque *et al*., 2002; Tassios *et al*., 1999).

CTX-M type ESBLs are the most widespread enzymes and represent a new and rapidly growing family of molecular class A ESBLs that have been classified into five groups (group 1, 2, 8, 9, and 25/26) according to their amino acid similarities and have less than 40% homology with TEM- and SHV-types (Bonnet, 2004). In contrast to TEM- and SHV-type ESBLs, CTX-M hydrolyses and confers resistance to cefotaxime rather than ceftazidime. CTX-Ms were initially reported at the end of 1980s and have increased dramatically since 1995 and disseminated in most parts of the world. Bauernfeind *et al*., (1990), at the beginning of 1989, reported on a cefotaxime-resistant *E. coli* strain which produced the designated CTX-M-1. Simultaneously an explosive dissemination of cefotaxime-resistant *Salmonella* strains began in South America (Bauernfeind *et al*., 1992).

1.7.5 Global distribution of ESBLs

1.7.5.1 Geographical variation and type distribution

The first report of plasmid-encoded β-lactamases capable of hydrolyzing the extended-spectrum cephalosporins (ESCs) was published in 1983 (Knothe *et al*., 1983). Most ESBLs have been identified and reported on the mainland of Europe (Philippon *et al*., 1989a and 1989b; Jacoby and Sutton, 1991; Payne and Amyes, 1991). ESBL-producing clinical strains have been isolated from many parts of the world (Winokur *et al*., 2001). However, their frequency of occurrence varies widely. Specific ESBLs appear to be unique to a certain country or region and differences in geographical distribution of ESBLs may be based on different treatment and prophylactic protocols (Szabó *et al*., 1997), e.g. TEM-3 which is common in France, but has not been detected in the United States (Nordmann, 1998) and the prevalence of TEM-52 in Korea is unique to that country (Pai *et al*., 1999).

In contrast, the SHV-5 β-lactamase is commonly encountered worldwide and has been reported in Croatia, France, Greece, Hungary, Poland, South Africa, the United Kingdom, and the United States (Bedenic *et al*., 2001; D'Agata *et al*., 1998; Gaillot *et*

al., 1998; Gniadkowski *et al*., 1998a; Pitout *et al*., 1998; Shannon *et al*., 1998; Szabó *et al*., 1999; Vatopoulos *et al*., 1990: Liebana *et al*., 2004a).

In Europe, the prevalence of ESBL production among isolates varies greatly from country to country. In France, 40% of *K. pneumoniae* isolates were found to be ceftazidime resistant (Branger *et al*., 1998) and in Japan the incidence of β-lactam resistance due to ESBL production remains very low (Yagi *et al*., 2000). In recent surveys, a significant increase in the ESBL rate was reported from all parts of the world, including North America, South/Latin America, the Far East-Western Pacific area (Winokur *et al*., 2001; Bell *et al*., 2002) and Europe.

1.7.5.2 ESBLs identified in Africa and South Africa

- TEM-12, found in South Africa (Paterson *et al*., 2003), is only slightly more resistant to ceftazidime and aztreonam than TEM-1 and TEM-2 and not resistant to cefotaxime (Medeiros, 1997).

- TEM-63 seems to be unique to the African continent and has been reported from South Africa (Essack *et al.*, 2001; Paterson *et al.*, 2003; Kruger *et al*., 2004), Malawi (Gray *et al*., 2006) and Tanzania (Blomberg *et al*., 2005). It is only slightly more resistant to ceftazidime and aztreonam than TEM-1 and TEM-2.

- SHV-2 β-lactamase is prevalent in South Africa (Paterson *et al*., 2003), Tunisia, Senegal and Egypt (Pitout *et al*., 1998) and was associated with a substantial increase in the cefotaxime MICs of isolates but only a moderate increase in ceftazidime MICs (Du Bois *et al*., 1995).

- SHV-5 is among the most predominant ESBLs worldwide (Tzouvelekis *et al*., 1998) including South Africa (Pitout *et al*., 1998; Paterson *et al*, 2003) and increases the hydrolysis of ceftazidime and aztreonam (Amyes, 1997), while retaining resistance to cefotaxime. There have been reports of SHV-5

hyperproduction resulting in increased resistance to cefepime (Tzouvelekis *et al*., 1998).

- SHV-12 has been isolated in *S.* Enteritidis and *S.* Babelsberg from Mali (Weill *et al*., 2004a). The first report of SHV-12 in *S.* Newport concerned an isolate from Tanzania and constituted the first account of SHV-12 in sub-Saharan Africa (Blomberg *et al*., 2005).

- CTX-M-3 was found in sub-Saharan Africa in Bangui (Frank *et al*., 2006).

- CTX-M-12 was reported in *Klebsiella pneumoniae* isolates from Kenya (Kariuki *et al*., 2001).

- CTX-M-15 was detected in Malawi and has been reported world-wide and is prevalent in Cameroon, Tanzania, Southwest Nigeria and Bangui (Central African Republic) in Africa (Gangoue-Pieboji *et al*., 2005; Blomberg *et al*., 2005; Soge, *et al*., 2006; Frank *et al*., 2006).

- CTX-M-37 has recently been identified in *S*. Isangi from a paediatric ward in Durban, South Africa (Govinden *et al*., 2006). This is the first report of CTX-M-type enzymes in *Salmonella* spp. in South Africa.

1.8 *SALMONELLA ENTERICA* SUBSPECIES *ENTERICA* SEROTYPE ISANGI

1.8.1 History and global distribution

Salmonella enterica subspecies *enterica* serotype Isangi (*S*. Isangi) was first isolated in 1946 in Stanleyville (now known as Kisangani), in what is now the Democratic Republic of Congo (DRC) (Kelterborn, 1967). It was then infrequently isolated during the next twenty-four years. Initially in 1947, Kauffmann combined *Salmonella* serotype Mission with *S.* Isangi, and in the latest Kauffmann-White Scheme, *S.* Isangi is considered as an independent serotype belonging to group C, which includes other serotypes such as *S*. Infantis, *S*. Bovismorbificans and *S*. Muenchen.

There are only a few reports in the literature on *S.* Isangi and it has only been isolated from eight countries (Kinshasa [Democratic Republic of Congo], Pasteur Institute [Paris, France], Bulgaria, India, Nigeria, Western Georgia, the Netherlands and Luthuania). South Africa can now be added to the list.

The following map depicts regions from where *S.* Isangi has been reported worldwide between 1946 and 2003. The prevalence in these parts of the world is briefly described (see map and text for numbers **i** to **vii** below).

Figure 1.4 Occurrence of *Salmonella* Isangi worldwide, 1946 to 2006.

i) Kinshasa, Democratic Republic of Congo (DRC)

S. Isangi was isolated in 1968 at the University of Kinshasa from an adult European female. Four more *S.* Isangi cultures were isolated in the DRC in 1968, three in 1969 and 28 in 1970 (Gatti *et al.*, 1972). In December 1970, *S.* Isangi caused an extensive outbreak in the paediatric wards of the University Hospital in Kinshasa leading to a marked upsurge in the incidence of this serotype (Krubwa *et al.*, 1976). By 1973 it became the predominant serotype in Kinshasa and two thirds of the *Salmonella* strains isolated in that city were *S.* Isangi.

Since 1970, *S.* Isangi, rarely isolated since its discovery in Zaire in 1946 (Kauffman, *et al*., 1947; Kelterborn, 1967), suddenly appeared first (accounting for 60% of salmonellosis diagnosed) among all isolates in Kinshasa, then in other regions of Zaire and in a short while advanced to first place in the inventory of serotypes in that country (Gatti *et al*., 1972; Kashemwa *et al*., 1973). The *S*. Isangi epidemic entered into a fairly stable endemic pattern, without the tendency to supersede and was also found to be multi-drug resistant, although no antimicrobials were specified in the study.

ii) Pasteur Institute, Paris

The National Salmonellae Centre at the Pasteur Institute, Paris, reported the isolation of *S.* Isangi from *Salmonella* received at the centre during the years 1973-1976, (Le Minor and Le Minor, 1978) probably imported from West Africa. In France, Jurukov and Simeonov (1967) reported a case of salmonellosis caused by *S.* Mission var. Isangi in 1967.

iii) Bulgaria

S. Isangi was first isolated in 1956 in this country (Asseva *et al*., 2006). *S.* Isangi was isolated from HUMANA milk (a commercial brand name for a mother's milk substitute), in Bulgarian hospitals (Avramova *et al*., 1979). The children were from a particular day-care centre. The strain harboured two stable plasmids that were associated with an unusually high level of resistance to ampicillin (MIC 65 mg/ml) and cephalothin, streptomycin and sulphonamides (sulphamethoxazole). It was reported that the salmonella strains produced high levels of β-lactamase and carried two stable plasmids. In a recent study by Asseva *et al*., (2006) 9 *S*. Isangi isolates, producing CTX-M-3 β-lactamases, were detected between 1999 and 2004 in Bulgaria.

iv) India

Yadava *et al.,* (1986) reported the first two cases of *S.* Isangi in India at Ranchi (Bihar) in 1986, isolated from a slaughterhouse for pigs. These isolates were unlikely to have been exposed to selective pressures of antimicrobial agents used in humans, either as an animal commensal or the environment.

v) South-West Nigeria

S. Isangi was isolated from stool samples from children under five years of age with diarrhoea from Abeokuta (Ogun State, Nigeria) in June 1986 (Mascher *et al*., 1988).

S. Isangi was also identified from household wall lizards, which are an important reservoir for *Salmonella* in West Africa. No antimicrobial susceptibility was reported.

vi) Western Georgia

S. Isangi caused an outbreak at a wedding party in Abasha, Western Georgia on 8 September 2002. 118 out of 164 attendees were affected, of which 116 had to be hospitalised. The outbreak was associated with the consumption of infected chicken. *S.* Isangi was isolated from 16 clinical samples out of 76 tested as well as from the chicken consumed at the wedding party. *S.* Isangi has never been isolated in Georgia prior to this incidence and the origin of the strain could not be established (Imhoff, 2003). No antimicrobial susceptibility was reported.

vii) The Netherlands

In a study to determine the genetic determinants responsible for ESBL resistance of *Salmonella* isolated from poultry meat and hospitalized humans (Hasman *et al*., 2005), ESBL-producing *S*. Isangi was isolated from three patients in 2001 in Holland and from two patients in 2002. The isolates were fully resistant to amoxicillin, cephalothin, cefuroxime, ceftazidime and cefotaxime as well as gentamicin, streptomycin, trimethoprim, sulphamethoxazole and tetracycline. TEM-63 was isolated in 2002 and CTX-M-28 in 2 patients from 2001 and a patient from 2002.

viii) Lithuania

Salmonella Isangi was isolated from chicken and humans in Lithuania over the period from 2000 to 2004. One *S*. Isangi was isolated from a chicken sample in 2002 and one isolate from humans in 2002 and 2003 (Pieskus *et al*., 2006). No antimicrobial susceptibilities were reported. Chicken products, especially eggs, were considered to be the main source of human infections.

1.8.2 Emergence of *Salmonella* Isangi in South Africa

Evidence of extensive nosocomial transmission of multidrug-resisistant *S.* Isangi in South African hospitals, supported by molecular typing, is presented in this dissertation.

The Enteric Diseases Reference Unit (EDRU) serves the public sector of South Africa and is responsible, in collaboration with the CDC, for 'enhanced' surveillance of enteric organisms such as *Salmonella* species (including *Salmonella* Typhi), *Shigella* species, *Vibrio cholerae* and diarhoeagenic *Escherichia coli*. The unit collects data on patients presenting throughout South Africa with both invasive and non-invasive disease caused by the abovementioned organisms. Serogrouping, serotyping and antimicrobial susceptibility testing are routinely performed on all organisms submitted to the unit (http://www.nicd.ac.za).

Prior to 2002, *S.* Isangi was an uncommon isolate in South Africa, not appearing in the top five most common *Salmonella* serotypes submitted from most provinces in South Africa to the EDRU (Kruger, unpublished observation). In 2002, *S.* Isangi advanced to second place; accounting for approximately 30% of all isolates received at the EDRU of the National Institute of Communicable Diseases (NICD) in that year, and has been the second most frequently (after *S.* Typhimurium) isolated *Salmonella* serotype at the EDRU in Johannesburg, South Africa, since 2002. More than 99% of all *S.* Isangi serotyped at EDRU exhibits MDR patterns. Surveillance data from the last 4 years shows the prevalence of *S.* Isangi in all the provinces of Southern Africa.

When *S.* Isangi was first isolated in large numbers at EDRU in November 1999, it was thought to be the cause of several nosocomial outbreaks unique to the larger tertiary academic hospitals in Gauteng. By the end of 2001, *S.* Isangi was isolated throughout South Africa. Studies on *S.* Isangi isolates received during the period 2000-2002 by the EDRU will be recorded in the subsequent chapters of this dissertation.

1.9 MOLECULAR CHARACTERIZATION OF SALMONELLAE

1.9.1 Molecular typing of *Salmonella* species

Primary methods based on phenotypic properties, biochemical and serological, have been used for differentiation of strains, but modern typing methods are based on characterization of the genotype of the organism by analysis of chromosomal or plasmid DNA. Many epidemiological typing studies have used pulsed-field gel electrophoresis (PFGE) as a basis of identification of clones in *Salmonella* (Bender *et al*., 2001; Valdezate *et al*., 2000; Maslow *et al*, 1993) and have proven to be highly discriminatory when applied to most serotypes.

PFGE is helpful for the investigation of clonal relationships between and within serotypes. While PFGE and ribotyping methods (Liebana *et al*., 2001a) are powerful, they are also considerably more time-consuming than plasmid profiling and require more advanced techniques and equipment. Several studies have shown the stability of plasmid profile analysis of *Salmonella* species as a useful method for grouping strains with the same serotype obtained from a single outbreak (Schmidt *et al*., 1982; Threllfall *et al*., 1986).

Recently, a method called variable number tandem repeat (VNTR) analysis has been adopted for typing purposes (Lindstedt *et al*., 2003). The VNTR method was originally described in eukaryotes (Jeffreys *et al*., 1988) but is being increasingly used in prokaryotes when complete bacterial genomes were sequenced and several bacterial strains have VNTR in their genome. The VNTR method lends itself to a high level of polymorphism, which has a high discriminatory capacity. VNTR has been reported to be more discriminatory than PFGE (Lindstedt *et al*., 2003).

The most reliable and effective approach to fingerprinting of *Salmonella* for epidemiological investigations is a combination of methods. Such genetic information, used in conjunction with antibiotic resistance profiles, would help to detect the

emergence of potential new strains by genetic variation and spread of antimicrobial resistance among existing strains.

1.9.2 Molecular detection of ESBLs

PCR-based approach

New techniques have recently been described to identify ESBLs. Chanawong *et al*., (2001) demonstrated that the combination of polymerase chain reaction (PCR), restriction fragment length polymorphism and restriction site insertion PCR techniques can be readily applied to the epidemiological study of SHV β-lactamases. Randegger and Hachler (2001) used a real-time PCR for the rapid identification of SHV-ESBLs and the technique is based on the detection of PCR end products by fluorescently labeled hybridization probes followed by melting curve analysis. PCR has been applied successfully to characterize CTX-M β-lactamases, but detection of all members from the five groups requires multiple PCRs with group-specific primers (Cantón *et al*., 2002) or consensus primers which only amplifies a few CTX-M alleles. Recently a rapid and accurate multiplex PCR assay (CTX-Mplex PCR) for the amplification of all CTX-M genes and the differentiation of the five groups has been described (Xu *et al*., 2005).

The variety of molecular techniques used for diagnostic applications demonstrate that no universal technique exists which is optimal for detection of nucleic acids. The choice of a particular technique is dependent on the information required. The easiest and most common molecular method used to detect the presence of a β-lactamase belonging to a family of enzymes is the polymerase chain reaction (PCR) with oligonucleotide primers that are specific for a β-lactamase gene. However, PCR will not discriminate among different variants of ESBLs e.g. TEM, SHV or CTX-M. Nucleotide sequencing remains the standard approach to the determination of specific β-lactamase genes present in strains.

In Chapter 5, Chapter 6 and Chapter 7 of this dissertation, molecular techniques employed in this study will be discussed in more detail.

1.10 AIM AND OBJECTIVES OF THE STUDY

The aim of the research presented in this dissertation was to describe the mechanism of resistance and molecular characterization of multidrug resistant *Salmonella* serotype Isangi isolates causing nosocomial infections in South Africa. The objectives were as follows:

- To investigate strain relatedness and cluster formation by PFGE and plasmid profiling;
- To undertake and demonstrate the typing of β-lactamases in selected *S*. Isangi isolates on the basis of antibiograms and plasmid profiling;
- To investigate the performance of screening methods for ESBL detection in *S*. Isangi isolates;
- To study the phenotypic expression of ESBLs in *S*. Isangi by means of MIC profiles of extended-spectrum cephalosporins and disc-diffusion patterns in the presence of clavulanic acid, a beta-lactamase inhibitor;
- To identify and characterize the TEM and SHV genes by PCR and DNA sequencing.

Chapter 2

SUSCEPTIBILITY OF *Salmonella* Isangi BY MINIMAL INHIBITORY CONCENTRATION (MIC) DETERMINATION USING Etest® TECHNOLOGY

2.1 ANTIMICROBIAL SUSCEPTIBILITY TESTING: INTRODUCTION

Antibiotics are one of the most important medical discoveries of the twentieth century, yet their widespread use and often misuse gradually undermined their usefulness. Antimicrobial resistance monitoring enables us to review the current status of antimicrobial resistance locally, nationally and globally and may be helpful in minimizing the consequences of drug resistance and limit the emergence and spread of drug resistant pathogens.

There are two main types of antibiotic resistance in bacteria, i) intrinsic, whereby the bacteria are naturally resistant to an antibiotic due to inherent features, and ii) acquired, whereby resistant strains emerge in a previously sensitive population, usually after antibiotic exposure (Gentry, 1991). The presence of intrinsic resistance is well established e.g. resistance of most gram-negative bacteria to the glycopeptide agents vancomycin and teicoplanin, and does not require routine susceptibility testing. Laboratory monitoring therefore concentrates on the changing susceptibility patterns of bacteria which acquire resistance due to mutations in target-controlling genes and the acquisition of resistance – mediating plasmids, transposons or integrons.

Susceptibility to antimicrobial agents is measured quantitatively by the minimal inhibitory concentration (MIC) method performed in broth or agar dilutions. The MIC value is the lowest concentration of a specific antimicrobial agent that inhibits visible growth of the test organism (CLSI, 2006). MIC testing is important in clinical laboratories and serves to assist with the treatment and management of patients with problem infections, as well as to confirm and monitor resistance of microorganisms to

antimicrobial agents. In surveillance studies MIC technology is also widely used to determine the activity of new antimicrobial agents. Susceptibility testing is traditionally performed either by a disc-diffusion procedure where the size of the zone of inhibition is proportional to the antibiotic concentration, or by using dilution technology (broth or agar). The disc-diffusion method is used to provide a qualitative result (sensitive, intermediate, or resistant) whereas the MIC testing methods can be expressed either quantitatively (the MIC) or qualitatively (when the MIC is interpreted to guide treatment options). The disc-diffusion agar test is the most widely used laboratory technique for antimicrobial susceptibility testing. The inhibition zone around the disc is measured (mm) and is inversely proportional to the MIC value of the isolate.

2.2 METHODS FOR MIC DETERMINATION

2.2.1 Conventional routinely used methods

Traditional methods for determining MIC are broth dilution (tube or microtitre plates), or agar dilution tests, where a standard inoculum of the test organism is exposed to two-fold serial dilutions of an antibiotic, distributed in tubes of broth or agar plates.

In the agar dilution method, different concentrations of antibiotics are incorporated into the agar and bacteria are inoculated onto the surface of the agar before it is incubated and subsequently read for growth inhibition. The broth dilution method involves diluting antimicrobials to obtain diminishing concentrations in broth to which bacteria have been added. Visible growth after incubation in tubes/wells containing the different antibiotic concentrations denotes lack of inhibition. The lowest dilution showing no inhibition is the MIC (Andrews, 2001).

The Etest® method is an indirect MIC method in which an impregnated strip with a concentration gradient of an antibiotic is placed onto the surface of an agar plate inoculated with the culture. The MIC depends on the size of inhibition zone and is read off a calibrated scale on the strip. It is universally accepted that the range of antibiotic concentrations used for determining MICs be made up in doubling dilution (two-fold)

steps (log_2 serial dilutions) and be expressed in mg/L. In the case of the Etest® where the concentration scale on the strip is continuous, MIC determination is not dependent on dilution steps, allowing intermediate MIC reading.

2.2.2 The Etest®

Although the agar or tube dilution methods, depending on the organism tested, are considered the 'Gold standard' for antimicrobial susceptibility testing (CLSI, 2006), they are laborious and time-consuming. The Etest®, also known as the epsilometer test, has been proposed (Bolmström *et al.,* 1995) as an alternative method to the broth and agar dilution methods and employs 'exponential gradient' testing methodology where 'E' in Etest® refers to the Greek symbol epsilon (ε).

The Etest® is a quantitative method for antimicrobial susceptibility testing in which a predefined stable antimicrobial gradient is present on a thin inert carrier strip. When this Etest® strip is applied onto an inoculated agar plate, there is an immediate release of the drug into the medium. Following incubation, a symmetrical inhibition ellipse is produced. The intersection of the inhibitory zone edge and the calibrated carrier strip indicates the MIC value which is read off a scale stretching over a wide concentration range (>10 log_2 dilutions). The test is easy to perform but requires experience and skill to read the MIC with precision and accuracy. A number of recent studies have demonstrated the Etest® as an excellent method for multi-centre assessment of antimicrobial activity of new compounds as well as a means of performing surveillance of antimicrobial resistance (Doern *et al*., 1998; Jones and Phaller, 1998).

2.2.3 Standardization of MIC methodology

The Clinical and Laboratory Standards Institute (CLSI) is an international, interdisciplinary, non-profit, non-governmental organization composed of medical professionals, government, industry, healthcare providers, educators etc. It promotes accurate antimicrobial susceptibility testing (AST) and appropriate reporting by

developing standard reference methods, interpretative criteria for the results of standard AST methods and establishing quality control parameters for standard test methods. It also provides testing and reporting strategies that are clinically relevant and cost-effective. Interpretative criteria of CLSI are developed based on international collaborative studies and evidence correlating MICs with clinical outcome. It is also updated regularly. The 'SIR' system is used to classify the susceptibility of the tested microorganisms: an isolate is defined as being susceptible (S), intermediately resistant (I) or resistant (R). Based on study results, CLSI interpretative criteria are revised frequently. CLSI is approved by FDA-USA and recommended by the WHO.

In this chapter the antimicrobial susceptibility status of *S*. Isangi isolates submitted to the EDRU will be described. Resistance to representatives of classes of antibiotic agents, potentially useful for the treatment of salmonellosis, was determined using Etest® technology.

2.3 MATERIALS AND METHODS

2.3.1 Bacterial strains for MIC testing

2.3.1.1 Control strains

The following strains were used as quality control test organisms for susceptibility testing:

- *Escherichia coli* ATCC® 25922: Used as a β-lactamase (ESBL)-negative control strain.
- *Staphylococcus aureus* ATCC® 25923: This is the recommended CLSI (2000) reference strain used for susceptibility testing and evaluation of Mueller-Hinton agar.
- *Escherichia coli* ATCC® 35218: Used for β-lactam/β-lactamase inhibitor combinations.

2.3.1.2 Test isolates

S. Isangi isolates (279 in total) were investigated between January 2000 and December 2002 and subjected to MIC testing using Etest® strips. Only one isolate per infection episode was included. Six *S*. Isangi isolates did not produce an ESBL and were also subjected to MIC testing. The identification of the isolates was performed at local laboratory level by conventional biochemical tests (Ewing, 1986) and submitted to the EDRU as *Salmonella* spp. for serotyping. Serotyping was performed on all isolates, using the method of slide agglutination on the basis of lipopolysaccharide (O) and flagellar (H) antigens, according to the Kauffman-White scheme for *Salmonella* serotyping (Kauffman, 1972; Popoff, 2001). Commercially available antisera (Bio-Rad, Marnes-la-Coquette, France) were used according to the manufacturer's instructions.

Of the 204 patients whose ages were known, 86.3% (176/204) were ≤5 years of age and 83.3% (170/204) were ≤2 years. Only 34/204 patients were adults. The gender status of 107 was available and 60/107 (56%) were male and 47/107 (43.9%) female. Most of the isolates were from stool cultures and is a reflection of the focus of surveillance at the time of this study. Table 2.1 shows the number of *S*. Isangi isolates that were recovered from clinical specimens.

Table 2.1 Number of *Salmonella* Isangi isolated from clinical specimens

Clinical source	**Number of isolates**	
	n	**(%)**
Stool culture	188	(67.4%)
Blood culture	34	(12.2%)
Body fluids*	16	(5.7%)
Rectal swab	15	(5.4%)
Urine	11	(4%)
Pus swab	4	(1.4%)
Sputum	3	(1%)
Pus	1	(0.4%)
Unknown	7	(2.5%)
Total	**279**	**100%**

*Normally sterile body site e.g. CSF, lung fluid and pleural fluid
n = Total number of isolates

2.3.2 Antimicrobial agents and CLSI breakpoints

The class of antimicrobial agents, concentration ranges, as well as their interpretive breakpoints to determine MICs is shown in Table 2.2. Cefoxitin (FOX) discs were included in the test battery to screen for, and exclude AmpC beta-lactamase producing strains (Livermore, 1995). Etest® strips were manufactured by AB Biodisk (Solna, Sweden) and used according to the manufacturer's instructions.

Table 2.2 List of Etest® strips used to determine minimal inhibitory concentrations of *Salmonella* Isangi isolates and interpretive standards for MIC breakpoints.

Class and representative antimicrobial (abbreviation)*		Concentration range (µg/ml)	Interpretive criteria† (µg/ml) S	R
Cephalosporins:				
3rd generation	Ceftazidime (TZ)	0.002-32	≤8	≥32
	Cefotaxime (CT)	0.016-256	≤8	≥64
4th generation	Cefepime (PM)	0.016-256	≤8	≥32
Carbapenems:	Imipenem (IP)	0.002-32	≤4	≥16
Quinolones:				
1st generation	Nalidixic acid (NA)	0.016-256	≤8	≥32
2nd generation	Ciprofloxacin (CI)	0.002-32	≤1	≥4
Aminoglycosides:	Gentamicin (GM)	0.064-1024	≤4	≥8
Tetracyclines:	Tetracycline (TC)	0.016-256	≤4	≥16
Combination:	Trimethoprim/ Sulphamethoxazole (1/19) (TS)	0.002-32	≤2	≥8

* Abbreviations are those of the manufacturer. † 'R', resistant; 'S', susceptible.
Isolates with MICs between R and S (intermediate resistant) are labeled resistant for the purpose of the present study.

2.3.3 Preparation of the inoculum for MIC testing

Isolates of *S.* Isangi were grown overnight on 5% blood agar plates (Oxoid, Uniparth Ltd., Basingstroke, Hampshire, England) at 37°C and checked for pure growth. A few

colonies were resuspended in 0.9% saline (Diagnostic Media Products (DMP), NHLS, Johannesburg) to give an inoculum density equivalent to 0.5 McFarland (10^7 and 10^8 cfu/ml) opacity standard [(0.048M $BaCl_2$ (1.17% w/v $BaCl_2.2H_2O$) to 99.5ml of 0.18M H_2SO_4 (1% v/v)] (BioMérieux, Basingstroke, UK). A sterile cotton swab was dipped into the suspension and streaked across the surface of Mueller-Hinton (MH) II agar (Oxoid) plates to ensure even inoculum distribution (CLSI, 2000). After application of the appropriate antimicrobial agents (Etest® strip), the plates were incubated aerobically at 37°C for 18 hours (overnight). The MICs of all 279 *S.* Isangi isolates were determined by reading from the scale on the strip where the ellipse of growth inhibition intercepted the strip.

2.4 RESULTS

2.4.1 Species and serotype confirmation of isolates

All 279 isolates were confirmed as *Salmonella* species, using standard biochemical tests, then serotyped as *Salmonella enterica* subspecies *enterica* serotype Isangi (characterised by the antigenic formula I; 6,7,14: d: 1,5).

2.4.2 MIC status of all isolates

All 279 isolates were susceptible to cefoxitin when screened with the disc-diffusion method indicating that no AmpC β-lactamases were present and only one resistance phenotype was observed among all *S.* Isangi isolates. Of the 273 isolates, 166 (60.8 %) were resistant (≥32µg/ml) to NA and 265 (97%) isolates were resistant (≥4µg/ml) to TS.

The MICs of six of the 279 isolates tested during the study period which were shown not to produce ESBL (see Chapter 4) are given in Table 2.3. All 273 ESBL-positive isolates were resistant to TZ (≥32µg/ml) and TET (≥16µg/ml) and all except two isolates were resistant to GM (≥16µg/ml). Of the 273 isolates 212 (78.2%) were susceptible to CT and 137 (50.5%) to PM. CT detected only 59 (21.7%) isolates when

the CLSI breakpoint of 8µg/ml was used to determine susceptibility to this agent. When the CLSI screening criteria (1µg/ml) for ESBL producers were used instead, CT detected all 273 isolates, except one (Table 2.4).

During the study period susceptibility to IP and CIP was sustained among all 279 (100%) isolates although one isolate showed intermediate resistance (2µg/ml) to IP. No decreased susceptibility to IP was detected and MICs around 0.25µg/ml remained constant within a relatively narrow range throughout 2000, 2001 and 2002 (See Table 2.4). There was a marked decrease in the susceptibility of *S.* Isangi to NA and CIP during the three year study from 2000 to 2002 especially from 2001 to 2002. There were increases in MIC_{50} levels of NA (4, 12 and >256µg/ml for 2000, 2001 and 2002 respectively) as well as CIP (0.012, 0.016 and 0.125µg/ml). The respective MIC_{90} levels for these two agents were 16, >256 and >256µg/ml for NA and 0.016, 0.125 and 0.19µg/ml for CIP. The modal values for NA and CIP clearly showed changing trends in susceptibility (See Table 2.5) and the MIC_{50} and MIC_{90} levels for NA and CIP are demonstrated in Figure 2.1. The MICs of the six ESBL-negative strains are depicted in Table 2.3 and a summary of the 273 ESBL-positive strains in Table 2.4.

Table 2.3 Minimal inhibitory concentrations (MICs) of the ESBL-negative *S*. Isangi isolates.

Hospital (Isolate number)	**MIC (µg/ml)**								
	TZ	**CT**	**FEP**	**CIP**	**TS**	**TET**	**GM**	**IP**	**NA**
Livingston (6832)	0.25	0.094	0.064	0.012	0.032	1	0.125	0.25	6
Pietersburg (7626)	0.5	0.064	0.064	0.008	**>32**	**32**	0.38	0.19	6
Tambo Memorial (9626)	0.5	0.125	0.125	0.008	**>32**	**192**	0.38	0.19	4
Greenpoint (13480)	0.25	0.25	0.125	0.016	0.125	2	0.25	0.25	6
Greenpoint (18909) [a]	0.25	0.19	0.125	0.19	**>32**	**64**	0.38	0.25	**>256**
Red Cross (14066)	0.38	0.25	0.19	0.16	**>32**	**256**	0.38	0.25	4

[a] MICs for NA and CIP have been confirmed
MICs in bold indicate resistance (including intermediately resistant)
TZ, ceftazidime; CT, cefotaxime; FEP, cefepime; CIP, ciprofloxacin; TS, cotrimoxazole; TET, tetracycline; GM, gentamicin; IP, imipenem; NA, nalidixic acid.

Table 2.4 Minimal inhibitory concentrations (MICs) of ESBL-positive *S*. Isangi isolates determined by the Etest® strips.

Antimicrobial [a]	Number of isolates inhibited at MIC in µg/ml [b] and cumulative percentages (in brackets)																						Total
	0.032	**0.094**	**0.38**	**0.5**	**1**	**1.5**	**2**	**3**	**4**	**6**	**8**	**12**	**16**	**24**	**32**	**48**	**64**	**96**	**128**	**192**	**256**	**>256**	
Cefotaxime																							
2000						1 (1.8)	11 (22)	11 (42)	15 (69)	4 (76)	1 (78)	5 (87)	4 (95)	2 (98)							1 (100)		55
2001							6 (19)	7 (41)	4 (53)	7 (75)	4 (88)		2 (94)	2 (100)									32
2002		1 (0.5)			2 (1.6)	8 (6)	40 (28)	41 (50)	30 (66)	14 (74)	5 (77)	7 (80)	9 (85)	3 (87)			1 (88)			1 (88)	4 (90)	18 (100)	184
Total		1			2	9	57	59	49	25	10	12	15	7			1			1	5	18	271 [c]
Cefepime																							
2000									1 (1.8)	5 (11)	15 (38)	6 (49)	9 (65)	5 (75)	2 (78)	2 (82)			1 (84)	1 (85)	6 (96)	2 (100)	55
2001									1 (3.1)	5 (19)	8 (44)	4 (56)	2 (63)	3 (72)	3 (81)						5 (97)	1 (100)	32
2002	1 (0.5)					1 (1.1)		2 (2.2)	19 (13)	50 (40)	30 (56)	14 (64)	15 (72)	10 (77)	7 (81)	7 (85)	2 (86)	2 (87)	1 (88)		17 (97)	6 (100)	184
Total	1					1		2	21	60	53	24	26	18	12	9	2	2	2	1	28	9	271 [d]
Tetracycline																							
2000													1 (1.8)	1 (3.6)	5 (13)	6 (24)	20 (60)	18 (93)	4 (100)				55
2001															1 (3.1)	2 (9.3)	10 (41)	12 (78)	4 (91)	1 (94)	2 (100)		32
2002															3 (0.5)	11 (7.5)	62 (41)	67 (77)	19 (87)	5 (90)	5 (92)	14 (100)	186
Total													1	1	9	19	92	97	27	6	7	14	273

Table 2.4 ***continued***

Antimicrobial [a]	**Number of isolates inhibited at MIC in µg/ml** [b] **and cumulative percentages (in brackets)**																					**Total**
	0.032	**0.094**	**0.38**	**0.5**	**1**	**1.5**	**2**	**3**	**4**	**6**	**8**	**12**	**16**	**24**	**32**	**48**	**64**	**96**	**128**	**192**	**256**	**>256**	
Gentamicin																							
2000															1 (1.8)	6 (13)	17 (44)	11 (64)	5 (73)	2 (76)		13 (100)	55
2001																	2 (9.3)	8 (31)	7 (53)	2 (59)	9 (88)	4 (100)	32
2002			1 (0.5)	1 (1.1)											2 (2.1)	4 (4.3)	29 (20)	37 (40)	25 (53)	12 (60)	29 (75)	46 (100)	186
Total			1	1											3	10	48	56	37	16	38	63	273
	0.004	**0.008**	**0.012**	**0.016**	**0.023**	**0.032**	**0.047**	**0.064**	**0.094**	**0.125**	**0.2**	**0.25**	**0.38**	**0.5**	**1**	**2**			**>32**				
Cotrimoxazole																							
2000									1 (1.8)		1 (3.6)	1 (5.5)							52 (100)				55
2001																			32 (100)				32
2002									1 (0.5)	4 (16)									181 (100)				186
Total									2	4	1	1							265				273

[a] All ceftazidime MICs were ≥32µg/ml on low concentration Etest strips.
[b] Total number of *S*. Isangi isolates excluding the six ESBL negative strains.
[c] Two strains were not tested for cefotaxime and cefepime.
[d] Eight strains were non-viable upon repeated subculture.
MICs in red indicate resistant strains (including intermediately resistant).

Table 2.5 Susceptibility of *S*. Isangi to nalidixic acid, ciprofloxacin and imipenem, 2000 to 2002.

Antimicrobial	Number of isolates inhibited at MIC (µg/ml) [a] and cumulative percentages (in brackets)																Total
	2	**3**	**4**	**6**	**8**	**12**	**16**	**24**	**32**	**48**	**64**	**96**	**128**	**192**	**256**	**>256**	
Nalidixic acid:																	
2000	5 (9)	8 (24)	**21** (62)	8 (76)	3 (82)	4 (89)	2 (93)									4 (100)	55
2001		2 (6)	**8** (31)	3 (41)	1 (44)	**7** (65)	1 (69)	1 (72)								**9** (100)	32
2002	1 (0.6)	3 (2.2)	8 (7)	5 (10)	3 (11)		5 (14)	1 (15)								**152** (100)	178
Total	6	13	37	16	7	11	8	2								165	**265** [b]
	0.004	**0.008**	**0.012**	**0.016**	**0.023**	**0.032**	**0.047**	**0.064**	**0.094**	**0.125**	**0.19**	**0.25**	**0.38**	**0.5**	**1**	**2**	
Ciprofloxacin:																	
2000	1 (1.8)	6 (13)	**34** (75)	10 (93)					2 (96)	2 (100)							55
2001			6 (18.8)	**16** (69)	1 (72)					6 (91)	3 (100)						32
2002		4 (2.2)	17 (11)	6 (15)		1 (15.1)	1 (15.6)	2 (17)	20 (27)	**107** (85)	20 (96)	5 (98)	2 (99.5)	1 (100)			186
Total	1	10	57	32	1	1	1	2	22	115	23	5	2	1			**273** [a]
Imipenem:																	
2000					1 (1.8)						18 (35)	**26** (82)	9 (98)	1 (100)			55
2001									1 (3)		9 (31)	**15** (78)	4 (91)	3 (100)			32
2002										5 (2.7)	**82** (47)	**79** (89)	15 (97)	3 (98.9)	1 (99)	1 (100)	186
Total					1				1	5	109	120	28	7	1	1	**273** [a]

[a] Total number of *S*. Isangi isolates, excluding six ESBL-negative strains; [b] Eight strains were non-viable upon repeated subculture.
Modal values are flagged in bold blue and resistant isolates are indicated in red (including intermediately resistant).

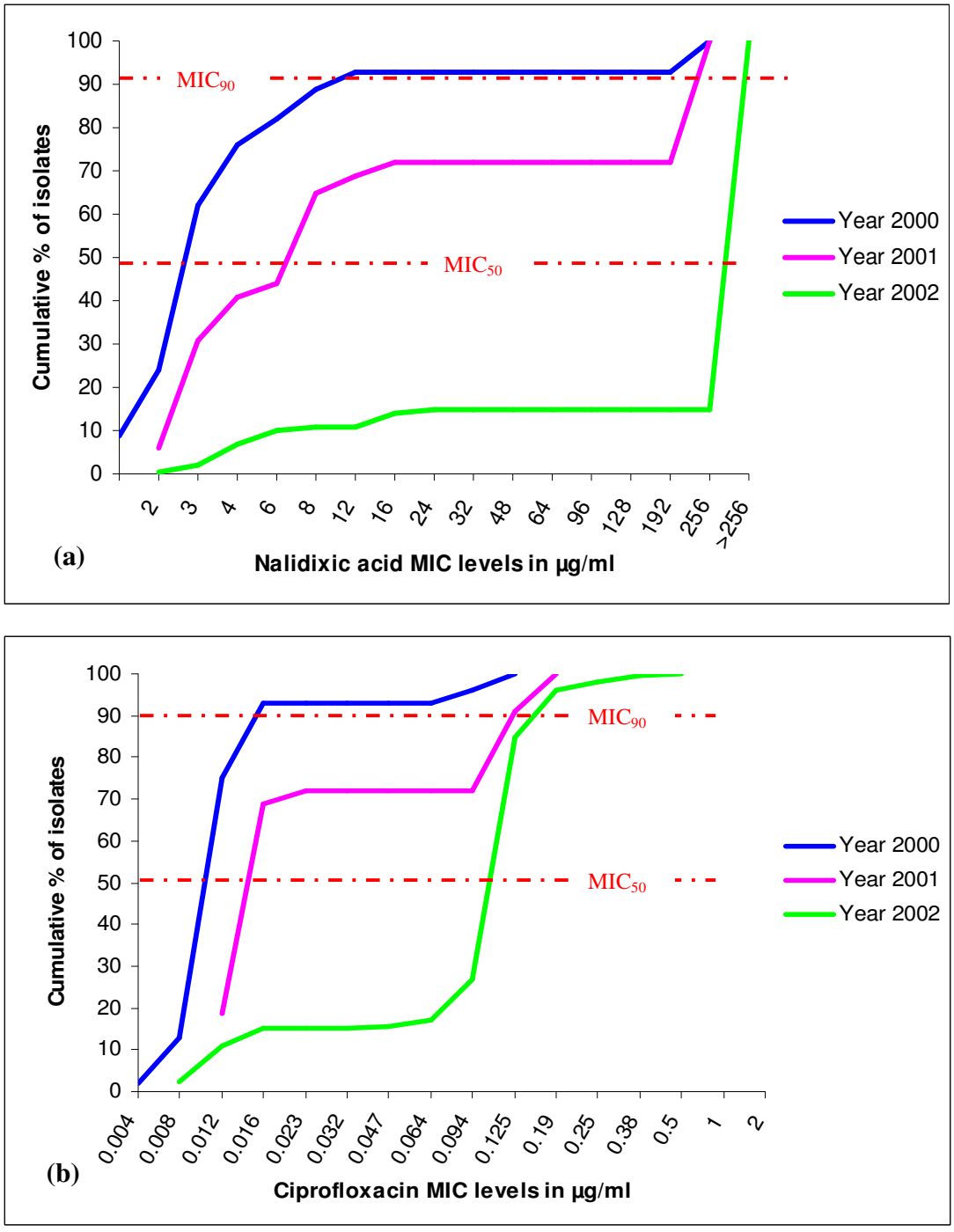


Figure 2.1 Increase in MIC levels of (a) nalidixic acid and (b) ciprofloxacin of *Salmonella* Isangi isolates during the period 2000 to 2002. MIC_{50} and MIC_{90} are the MICs at which 50% and 90% of the isolates were inhibited. The MIC_{50} and MIC_{90} reading should be taken at the concentration above the point where the percentile dotted line crosses the graph.

DISCUSSION

2.5.1 Species and serotype confirmation of isolates

Identification to the biochemical level is a pre-requisite for predicting resistance mechanisms from antibiogram data (Livermore, 1995). The Kauffmann and White scheme is based on the immunologically distinct variations in somatic O antigens of the cell wall and flagellar H antigens which provide each *Salmonella* serovar with its own unique antigenic combination (Kauffmann, 1954). A limitation of this system is that a small number of *S. enterica* serovars is responsible for a majority of infections. For organism tracing during an outbreak of salmonellosis, the need for a method to subdivide isolates within a serovar is essential. The classical method used has been phage typing but will not be covered in this dissertation (Callow, 1959; Hickman-Brenner *et al.,* 1991).

2.5.2 Age distribution of patients and rate of extra-intestinal infection

The predilection of *S*. Isangi for children of less than two years of age and its much less common occurrence in adults are clearly evident: In this study 83.3% (170/204) were ≤2 years and only 34/204 (16%) of the patients were adults. These data correlate with the study by Krubwa *et al*., (1976) where 73% of *S*. Isangi isolates were from children less than two years of age and only 6.3% were from adults.

Krubwa *et al*., (1976) also concluded that the *S*. Isangi epidemic was due to poor hygiene and was transmitted through the fecal-oral-route. It is likely that in the present study, overcrowding with person-to-person spread and poor infection control aggravated by staff shortages may have played a role in transmission in paediatric wards (Wadula *et al*., 2006). The presence of isolates from extra-intestinal sources (e.g. blood) constitutes the best indication of the invasive power of *S*. Isangi and in our study we found 25.4% of the isolates to be from extra-intestinal sources. Wadula *et al* (2006) reported that 26 out of 41 (75%) paediatric patients at CHB hospital with *S*. Isangi infections were HIV-positive.

2.5.3 MICs

The majority (78.3%) of ESBL-positive isolates (212/271) had MICs within the susceptible range to cefotaxime and cefepime (50.9%), although it is generally recommended that ESBL-positive isolates be considered resistant to all extended-spectrum β-lactam antimicrobials regardless of susceptibility test results (Emery and Weymouth, 1997). The findings of the present study reinforces the CLSI recommendation to screen for ESBL producers with both ceftazidime and cefotaxime as the ESBL status of all except one of the isolates would have been missed in this study if cefotaxime was used alone. The MICs of cefepime was higher than that for cefotaxime but lower than that for ceftazidime. Of a total of 271 isolates, 59 were resistant (>8µg/ml) to cefotaxime while 133 were resistant (>8µg/ml) to cefepime. This is in contrast to the findings by Govinden *et al*., (2006) where the three *S*. Isangi isolates tested by them had higher MICs for cefotaxime than cefepime due to the production of CTX-M-37. Using current CLSI breakpoints for cefepime, 47 of ESBL-producing isolates would have been regarded as susceptible to cefepime and the suggestion by Szabó *et al*., (2005) that cefepime breakpoints should be reconsidered is supported by our findings.

All 273 (100%) ESBL-positive isolates were resistant to ceftazidime, gentamicin and tetracycline and the MICs for ceftazidime were >32µg/ml. *S.* Isangi exhibited high levels of resistance to nalidixic acid (60.8%) and cotrimoxazole (97%). Susceptibility for imipenem was retained in all 279 isolates with MICs ranging 0.094-2µg/ml, although a gradual increase in imipenem MICs was observed between 2000 and 2002 (Table 2.5).

2.5.4 Quinolone resistance

Resistance to fluoroquinolone antibiotics is increasing. In 2000, 1.4% of *Salmonella* isolates in the United States were resistant to nalidixic acid and 0.4% were ciprofloxacin resistant (CDC, 2000).

A large percentage of isolates (38%) in 2000 in this study was susceptible to nalidixic acid at 4µg/ml. This susceptibility decreased in 2001 with 25% of isolates having MIC values of 4µg/ml, while 28% had MICs of >256µg/ml. In 2002, 85% of isolates showed were fully resistant to nalidixic acid with MICs >256µg/ml (Table 2.4). The sharp decline in susceptibility to nalidixic acid is alarming as it is correctly referred to as a marker for ciprofloxacin resistance in salmonellae (Hakanen *et al*., 1999a, 1999b). Single mutations in the *gyrA* or *gyrB* genes may confer full resistance to nalidixic acid while encoding low level resistance to ciprofloxacin. At least one additional mutation in these genes (and/or *parC* or *parE*) is required for full resistance to the fluoroquinolones (see section 1.5.1 of this dissertation). Decreased susceptibility to ciprofloxacin from 2000 to 2002 was also detected and MICs increased from 0.012µg/ml in 2000 (61.8% of isolates) to 0.016µg/ml in 2001 (50%) and 0.125µg/ml in 2002 (57.5%) (Table 2.4 and Figure 2.1). When an MIC of 0.125µg/ml to 1µg/ml was considered as decreased susceptibility to ciprofloxacin, resistance to nalidixic acid was observed with MICs >256µg/ml and correlates with findings from the studies performed by Threlfall and Ward (2001), Hakanen *et al*., (2001) and Ercis *et al*., (2006).

Although the ciprofloxacin MICs were still in the susceptible range it is likely that isolates with raised MICs have already acquired mutations in the *gyr*A and or *par*C genes. It is likely that only one further stepwise mutation may confer full resistance to ciprofloxacin. A similar trend of decreasing nalidixic acid susceptibility was recorded in a recent study performed in South Africa involving *E. coli* and klebsiellae isolates (Sein *et al.,* 2005).

Previous studies have shown that all *Salmonella* strains recovered from patients who failed fluoroquinolone therapy have been resistant to nalidixic acid, suggesting an alteration in DNA gyrase as a consequence of mutation (Launay *et al*., 1997). The recognition of strains with decreased susceptibility to nalidixic acid is of concern due to the increasing number of treatment failures in invasive salmonellosis reported in association with reduced fluoroquinolone susceptibility. Screening for reduced

susceptibility to ciprofloxacin (MIC ≥0.125µg/ml), using nalidixic acid disks, has been evaluated and demonstrates high sensitivity and specificity (Hakanen *et al*., 1999).

Antibiotic utilization patterns, including widespread cephalosporin use, have been associated with the emergence of ESBLs. In particular, the widespread use of ceftazidime has been implicated, and decrease in ceftazidime use has been associated with control of ESBL emergence in several instances (Meyer, *et al*. 1993; Patterson, *et al*. 2000; Rice, 1996; Rahal, *et al*., 2002). Reduction in the use of these agents and the use of extended-spectrum penicillins and combination therapy with aminoglycosides has been shown to restore bacterial susceptibility (Jones, 1992; Ballow and Schentag, 1992). It is a good recommendation that all isolates of the *Salmonella* species be tested for nalidixic acid resistance in order to avoid reporting false susceptibility to fluoroquinolones (Launay *et al*., 1997; Vasallo *et al*, 1998).

Chapter 3

PERFORMANCE OF METHODS FOR SCREENING AND CONFIRMATION OF ESBL EXPRESSION IN *Salmonella* Isangi

3.1 REQUISITES FOR ESBL DETECTION

Subsequent to the occurrence of ESBLs and AmpC-type β-lactamases, resistance to the extended-spectrum cephalosporins among members of the family *Enterobacteriaceae* has become a growing problem worldwide (Bradford, 2001). This creates a need for laboratory testing methods that will accurately identify these enzymes in clinical isolates.

The requisites for ESBL detection in routine diagnostic laboratories would ideally be reliable and easy-to-perform phenotypic tests that are affordable and readily available for the management of patients. Laboratory detection of ESBLs is however not straightforward and can be problematic because some ESBLs do not confer obvious resistance to all their substrates *in vitro* and up to 35% of ESBL producers have been reported as susceptible to cefotaxime (CTX) and ceftriaxone (CRO) in Europe (Babini and Livermore, 2000; Livermore and Yuan, 1996). The under-recognition of ESBL-mediated resistance has been due to the lack of a convenient and sensitive method for recognizing ESBL-producing strains (Cormican *et al.*, 1996) and the fact that the MIC values for CTX or CRO for ESBLs may be below the breakpoint for resistance defined by the CLSI.

Phenotypic screening for antimicrobial resistance in *S.* Isangi isolates by MIC determination was covered in Chapter 3 and screening and confirmatory testing by disc methodology for ESBL determination will feature in this chapter, while aspects of genetic characterization of ESBL-producing isolates will feature in Chapter 5, Chapter 6 and Chapter 7.

3.2 PHENOTYPIC TESTS FOR ESBL DETECTION

3.2.1 Recommended methods

ESBL detection tests that can conveniently be performed using the Kirby-Bauer disc-diffusion (KBDD) methodology have been proposed by CLSI (2006). The double-disc approximation test described by Jarlier *et al*., (1988) and the broth-dilution MIC reduction method (CLSI confirmatory test) are easy to perform and relatively inexpensive methods used by many clinical laboratories. None of the phenotypic tests are however sufficiently sensitive or specific to detect accurately all strains producing ESBLs. There is a need for improved detection of ESBLs in clinical isolates, especially gram-negative organisms (Paterson *et al*., 2003).

3.2.2 CLSI recommendations

The sensitivity of screening for ESBLs can vary depending on the type of antimicrobial agent tested. The CLSI (2006) at present does not have specific recommendations for salmonellae but recommends initial screening tests involving any one or more of CPD, CAZ, ASM, CTX or CRO for the detection of ESBL production in *Klebsiella pneumoniae*, *Klebsiella oxytoca*, *E. coli* and *Proteus mirabilis* using either disc-diffusion or broth dilution MICs. The screening test is followed by a phenotypic confirmation test by disc diffusion using CAZ or CTX with and without the presence of clavulanic acid (CV).

3.2.2.1 MIC-based method

For the MIC-based method, CPD, CAZ, CTX, CRO and ASM are incorporated into cation-adjusted Mueller-Hinton broth (CAMHB) at concentrations of 4µg/ml for CPD and 1µg/ml for the other β-lactam screening agents. When resistance based on presence of growth is evident in the case of the above-mentioned screening cephalosporins, CAZ and CTX alone and in combination with clavulanic acid (CV) are used at a standard

concentration of CV (4µg/ml) in all the serial dilutions. A ≥3 two-fold decrease in MIC within the combination concentrations constitutes ESBL confirmation.

3.2.2.2 Disc-diffusion-based methods

The current CLSI recommendations for screening and confirmation of ESBLs in *K. pneumoniae*, *K. oxytoca* and *E. coli* using the standard disc-diffusion on Mueller-Hinton agar (MH) method together with interpretative criteria are illustrated below:

Initial screening test	**Phenotypic confirmatory test***
Cefpodoxime (10µg) zone ≤17mm or	Ceftazidime (30µg) +
Ceftazidime (30µg) zone ≤22mm or	Ceftazidime/clavulanic acid (30/10µg)
Cefotaxime (30µg) zone ≤27mm or	***and***
Ceftriaxone (30µg) zone ≤25mm or	Cefotaxime (30µg) +
Aztreonam (30 µg) zone ≤27mm**	Cefotaxime/clavulanic acid (30/10µg)

(The use of more than one drug improves sensitivity of detection)

*A ≥5mm increase in zone diameter for antimicrobial agent tested in combination with clavulanic acid versus its zone when tested alone = ESBL positive.

** Not used in this study.

3.3 PERFORMANCE OF DISC-DIFFUSION-BASED METHODS

A number of different testing methods have been suggested since ESBLs were first described. These methods have generally been described for members of the Enterobacteriaceae and *Pseudomonas aeruginosa* (Bradford, 2001) and a brief overview of the methods and their advantages and disadvantages be presented below, followed by a comparison of their performance in *Salmonella* Isangi.

3.3.1 Overview of screening and confirmatory tests to determine ESBL production

The double-disc diffusion test and modifications thereof, the three-dimensional test and modifications thereof, the Vitek test (Sanders *et al*., 1996) and Etest® strips have all been introduced as rapid screening methods for ESBL production. The merits and shortcomings of each of the detection tests are outlined in Table 3.1.

Table 3.1 Comparison of clinical microbiology techniques for ESBL detection.

Test	Advantages	Disadvantages	Reference(s)
Standard CLSI interpretive criteria	Easy to use, used in every laboratory	ESBLs not always 'resistant'	(CLSI, 2006)
*__CLSI ESBL confirmatory test__	Easy to use and interpret	Sensitivity depends on choice of cephalosporin	(CLSI, 2006)
*__Double-disc approximation test (DDD)__	Easy to use and interpret	Distance of disc placement not standardized	(Jarlier *et al*., 1988; Thomson and Sanders, 1992; Florijn *et al*., 2002)
Three-dimensional test	Sensitive and easy to interpret	Labour intensive, not specific for all ESBLs	(Thomson and Sanders, 1992)
*__Etest® ESBL strip__	Easy to use	Less sensitive than DDD, sometimes difficult to interpret	(Vercauteren *et al.*,1997)
Vitek ESBL test	Easy to use and interpret	Reduced sensitivity	(Sanders *et al*., 1996; Tenover *et al*., 1997)
Oxoid combination disc method	Easy to use and interpret	Designed specifically for klebsiellae	(Carter *et al*., 2000)

This table has been adapted from Bradford, 2001.
*Methods evaluated in this chapter.

In the double-disc approximation methods, also known as double-disc diffusion (DDD) tests, first described by Jarlier *et al*., (1988), oxyimino-cephalosporin discs are placed peripherally on the sensitivity agar plate and CV usually in the form of co-amoxyclav (Augmentin) in the centre. The distance (centre- to centre) of 30mm between the discs

was suggested by Jarlier *et al*., (1988) but shorter distances (15mm-25mm) have been found to improve sensitivity (Coudron *et al*., 1997; MacKenzie *et al*., 2002; Jiang *et al*., 2006). The sensitivity of screening for ESBLs using this method and other phenotypic screening methods can also vary depending on the type of antimicrobial agent tested (Ho *et al*., 2000; Bradford, 2001; MacKenzie *et al*., 2002).

The DDD test has revealed sensitivities ranging from 79% to 97% and specificities ranging from 94% to 100% in previous reports (MacKenzie *et al*., 2002). Final standardization of the distance between discs however, still needs to be established. Different studies have reported the greater sensitivity or reliability of detection of one method over the other as well as the success of particular antibiotic substrates (Thomson and Sanders, 1992; Sanders *et al*., 1996; Vercauteren *et al*., 1997; Florijn *et al*., 2002).

The use of more than one of the five indicator cephalosporins suggested for screening e.g. CAZ and CTX, will improve the sensitivity of detection of ESBLs (M'Zali *et al*., 2000; Bradford, 2001). If necessary to rely on only one screening substance, either CPD or CAZ would be the best choice because they show the highest sensitivity of detection in evaluation studies (MacKenzie *et al*., 2002). It is most practical to screen with CAZ since almost all ESBLs show resistance to this compound (Livermore, 1995; Sirot, 1995; Bonafede and Rice, 1997) and other oxyimino-aminothiazolyl cephalosporins are less reliable indicators. It should particularly be noted that ESBL-producers often appear to be susceptible to cefuroxime (CXM) (Livermore and Brown, 2001). First and second-generation cephalosporins as well as cephamycins, including CXM are active *in vitro* but are not effective *in vivo* (Livermore *et al*., 2001). Commercial manufacturers have developed ESBL detection tests that can be used along with MIC test methods already in place in the clinical laboratory.

The **Vitek method** (Biomerieux, Hazlewood, MO) is an automated antimicrobial susceptibility test system that utilizes either CAZ or CTX alone and in combination with CV (4µg/ml). In this test, a predetermined reduction in growth in wells containing

CV compared to those containing drug alone indicates the presence of an ESBL (Sanders *et al*., 1996). ESBL detection in automated systems has been reported to be complex and misleading (Tzouvelekis *et al*., 1999).

Another method, the **three-dimensional test**, was described by Thomson and Sanders (1992). Following inoculation of the test organism onto the surface of a Mueller-Hinton plate, a slit is cut into the agar, into which a broth suspension of the test organisms is introduced. Antimicrobial discs are placed 3mm from the slit and distortion in the expected circular zone of inhibition is reported as a positive test.

The **Oxoid combination disc method** compares the zones of CPD (10µg) and CPD (10µg) plus CV (1µg) discs. The zone size of the discs with CV should be ≥5mm than those without the inhibitor to be called an ESBL-producer. This method was validated by the British Society of Antimicrobial Chemotherapy (BSAC) and CLSI methodology and found to detect ESBL-producing klebsiellae with 100% sensitivity and specificity (Carter *et al*., 2000).

CPD (with and without CV) has been incorporated into a new method, for the presumptive identification of ESBLs, called the **MAST ID™ ESBL disc method**. The test comprises three paired discs that contain CAZ (30µg), CTX (30µg) and CPD (30µg), with and without CV (10µg). An increase in ratio of 1.5 or greater in zone diameter of inhibition by the disc containing CV compared to cephalosporin disc alone indicates ESBL production. This test also incorporates the CLSI confirmatory test method: A ≥5mm increase in zone diameter for either antimicrobial agent tested in combination with clavulanic acid versus its zone when tested alone indicates ESBL production. According to the manufacturers' guidelines, a positive result from any or all of the pairs of discs indicates ESBL production.

The **Etest® ESBL strip** (AB Biodisk, Sweden) is a thin inert, plastic drug-impregnated strip, one side that generates a stable concentration gradient for ceftazidime (TZ) (0.5 to 35µg/ml) and the remaining side that generates a gradient of ceftazidime (0.125 to

8µg/ml) and clavulanic acid (TZL) (4µg/ml) (Cormican *et al*., 1996). A continuous and exponential gradient of antibiotic concentration is created under the strip. The method is based on the recognition of a reduction in TZ MIC in the presence of 4mg of clavulanic acid per liter. The strips are useful for both screening and phenotypic confirmation of ESBL production and have been recommended by the manufacturers for use in gram-negative nosocomial infections.

In this chapter the performance of the double-disc diffusion method, MAST ID™ ESBL discs and Etest® ESBL strips for the detection of ESBL expression, was evaluated.

3.4 MATERIALS AND METHODS

3.4.2 Bacterial strains for ESBL testing

3.4.2.1 Control Strains

Double-disc diffusion test (DDD):

- *Staphylococcus aureus* ATCC® 29213
  This is the recommended CLSI, (2000) reference strain used for susceptibility testing, evaluation of Mueller-Hinton agar and antimicrobial disc testing.

MAST ID™ESBL disc test:

- *Escherichia coli* ATCC® 25922 (ESBL-negative control strain)
  Should indicate ≤2mm increase in zone diameter for antimicrobial agent tested alone versus its zone when tested in combination with clavulanic acid.

- *Klebsiella pneumoniae* ATCC® 700603. ESBL-positive control (Previously K6, SHV-18 β-lactamase producer)
  Should indicate ≥5mm increase in ceftazidime and ≥3mm increase in cefotaxime zone diameter when tested in combination with clavulanic acid compared to that of the antimicrobial alone.

Etest ® ESBL strips:

- *Escherichia coli* ATCC® 35218 (ESBL-negative control)
  TEM-1 [non-ESBL] β-lactamase-producing isolate.
- *Klebsiella pneumoniae* ATCC® 700603 (ESBL-positive control)

3.4.2.2 Test Isolates

The 279 *S.* Isangi isolates, subjected to demonstration of ESBL activity using the double-disc diffusion method and Etest® ESBL strips were the same as those used in Chapter 2 (see Section 2.3.1.2, p37). Due to financial and time constraints and the fact that the MAST ID™ ESBL disc method was not proposed in the original planning of the dissertation, only 115 of the 279 isolates were used to evaluate the MAST ID™ ESBL disc method. The isolates are representative of the total collection with regard to time, hospital isolated from and resistance type.

3.4.3 Antimicrobial agents

The antimicrobials used in the ESBL screening methods are shown in Table 3.2. Etest® and Etest® ESBL strips were manufactured by AB Biodisk (Solna, Sweden), the antibiotic discs for the DDD test by Oxoid (Uniparth Ltd., Basingstroke, Hampshire, England) and the MAST ID™ ESBL discs by Mast Diagnostics (Mast Group Limited, Merseyside, United Kingdom).

3.4.4 Methods applied for demonstration of ESBL expression

The opacity of the suspensions (0.5 McFarland) of *S.* Isangi isolates was the same as that were used for MIC determination (see Chapter 2, section 2.3.3). These suspensions were used within 30min of preparation. A sterile cotton swab was dipped into the suspension and streaked across the surface of Mueller-Hinton (MH) agar plates to ensure even inoculum distribution (CLSI, 2000). After application of the appropriate antimicrobial agent/s (discs or Etest® ESBL strip) (Table 3.2), the plates were incubated aerobically at 37°C for 18 hours (overnight).

Table 3.2 Antimicrobial agents used to evaluate the three ESBL screening methods.

Method used and representative antibiotic/s*	Concentration	Reading
Double-disc diffusion (DDD):**		
Ceftazidime (CAZ)	30µg	Zone enhancement
Cefotaxime (CTX)	30µg	
Cefepime (FEP)	30µg	
Cefuroxime (CXM)	30µg	
Amoxycillin + clavulanic acid (AUG)	20µg/10µg	
MAST ID™ ESBL discs:		
Ceftazidime (CAZ)	30µg	Ratio: $\frac{\text{Ceph + CV}}{\text{Ceph}} < 1.5$
Ceftazidime (CAZ) + clavulanic acid (CV)	30µg/10µg	
Cefpodoxime (CPD)	30µg	
Cefpodoxime (CPD) + clavulanic acid (CV)	30µg/10µg	
Cefotaxime (CTX)	30µg	
Cefotaxime (CTX) + clavulanic acid (CV)	30µg/10µg	
Etest ® ESBL strips:		
Ceftazidime (TZ) + 4µg/ml clavulanic acid (TZL)	0.5-32/0.064 + 4µg/ml	Ratio ≤ 8 of zone enhancement
Cefepime (PM) + 4µg/ml clavulanic acid (PML)	0.25-16/0.064 + 4µg/ml	
Cefotaxime (CT) + 4µg/ml clavulanic acid (CTL)	0.25-16/0.064-4µg/ml	

When an Etest ® strip contains clavulanic acid, the letter 'L' is added.
* Abbreviations are those of the manufacturer. Ceph: cephalosporin
** Cefoxitin (FOX) disc at 30µg was also used to assist with the detection of AmpC.

3.4.4.1 Double-disc diffusion test (DDD)

The DDD test was performed, with some modification, according to the method of Jarlier *et al.* (1988) to screen for ESBL expression in all 279 *S.* Isangi isolates. MH agar plates were prepared as described in section 2.3.3 of Chapter 2. A disc containing amoxycillin-clavulanic acid (AUG) was placed in the centre of the inoculated susceptibility plate (Mueller Hinton Agar, DMP, NHLS, SA) 20mm (±2mm) centre to centre of discs containing extended-spectrum cephalosporins (Table 3.2) (Moland and Thomson, 1994). The cephalosporin discs were placed on the agar plate using a MAST disc dispenser (Davies Diagnostics). The diameters of any zones of inhibition were measured and recorded in millimeters (mm) to determine the presence or absence of ESBL production in all 279 *S.* Isangi isolates.

Experience in our laboratory with *Salmonella* Isangi isolates suggested that a distance of 20mm between discs was superior to the 30mm described by Jarlier *et al*., (1988). Enhancement of the zone of inhibition around the cephalosporin disc toward the clavulanate-containing disc was interpreted as synergy and indicated the presence of ESBL expression (Jarlier *et al*., 1998). Cefoxitin (FOX) resistance in Enterobacteriaceae is diagnostic of AmpC enzyme production and was included in the test battery to screen for, and exclude AmpC beta-lactamase producing strains (Livermore, 1995).

3.4.4.2 MAST ID™ ESBL discs

This is a phenotypic confirmatory test and compares the zone sizes given by discs containing a cephalosporin with and without clavulanic acid (CV). If an ESBL is produced, the zones are enlarged for the discs containing the inhibitor. See Table 3.2 for the list of antimicrobial agents used. The diameter of any zones of inhibition were measured and recorded in millimeters (mm). The following calculation was used to determine the presence of ESBL production in a sample of 115/279 *S*. Isangi isolates:

Zone diameter ratio (mm):

$$\frac{\textit{Cephalosporin + clavulanic acid}}{\textit{Cephalosporin}} \quad \geq \mathbf{1.5} = \text{ESBL positive}$$

$$\textit{Cephalosporin} \quad < \mathbf{1.5} = \text{ESBL negative}$$

3.4.4.3 Etest ® ESBL strips

The MIC on both ends of the strip was interpreted as the point of intersection of the inhibition ellipse with the Etest® strip edge. The presence of an ESBL was confirmed by the appearance of a phantom zone or deformation of the ellipse or when the MIC was reduced by $\geq$3 $\log_2$ dilutions in the presence of CV. See Table 3.2 for the list of antimicrobial agents used. The following calculation was used to determine the presence or absence of ESBL production:

MIC ratio (µg/ml):

Cephalosporin + clavulanic acid / *Cephalosporin*	**≥8**	=	ESBL positive
	<8	=	ESBL negative

3.5 RESULTS

3.5.1 Double-disc diffusion (DDD)

Synergism with AUG against CAZ (43.7%), CTX (68.9%) and FEP (99.2%) was detected. Although the synergy effect was observed in 273/279 isolates, details of individual tests were only recorded in 137 isolates is summarized in Table 3.3. All 279 isolates were susceptible to FOX and six of the 279 isolates were ESBL negative. In this test FEP was the most effective agent to screen for ESBL production as synergy was more pronounced with this antimicrobial than CAZ or CTX. Although synergy with CXM was only detected in 17.6% of isolates, all 273 isolates were fully resistant to this agent.

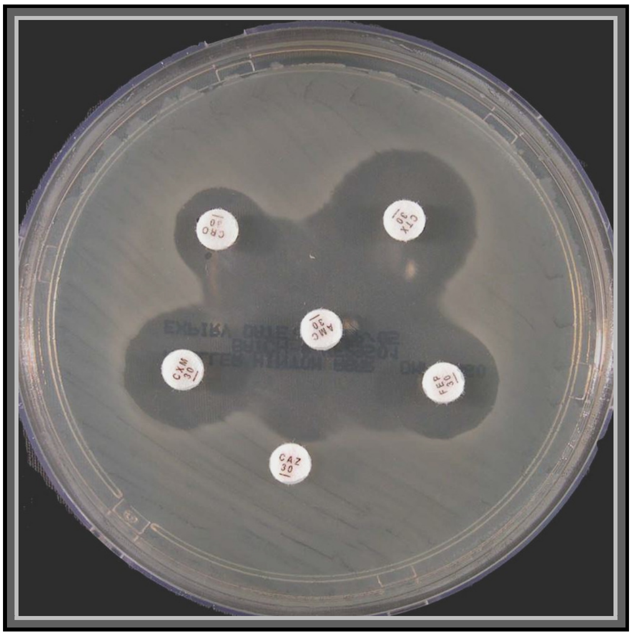


Figure 3.1 Demonstration of ESBL production with the double-disc diffusion (DDD) test. From the top of the plate clockwise: cefotaxime (CTX), cefepime (FEP), ceftazidime (CAZ), cefuroxime (CXM), ceftriaxone (CRO), and Augmentin (amoxycillin/clavulanic acid) (AMC) in the centre of the Mueller-Hinton agar plate.

Table 3.3 Summary of the DDD method with each cephalosporin tested for synergy against Augmentin.

Antimicrobial agent	Number (%) of *Salmonella* Isangi isolates			
	Tested	**Resistant**[a]	**Synergy +ve**	**Synergy not demonstrated**
Ceftazidime	137	136 (99.2)	60/137 (43.7)	77 (56.2)
Cefotaxime	87	82 (94.2)	60/87 (68.9)	27 (31.0)
Cefepime	135	73 (54.1)	134/135 (99.2)	1 (0.7)
Cefuroxime	136	136	24/136 (17.6)	0

[a] According to CLSI disc-diffusion testing criteria

3.5.2 MAST ID™ ESBL discs

Ninety-three percent (107) of isolates showed synergy with CAZ 77.4% with CPD, while only five (4.3%) isolates were positive for ESBL production with CTX (Figure 3.2). This means that in 82.6% (95) of the isolates, ESBL production would not have been detected using CTX as a screening agent alone. When disc zone sizes are below or above the test ranges, interpretation is non-determinable (ND) (Figure 3.3) and should be further investigated with the CLSI reference method and/or genotyping. Forty-eight isolates were non-determinable when CPD was used (21.7%), 13% with CTX and 6.9% with CAZ. The six isolates that were negative in the DDD test were also ESBL negative with this test.

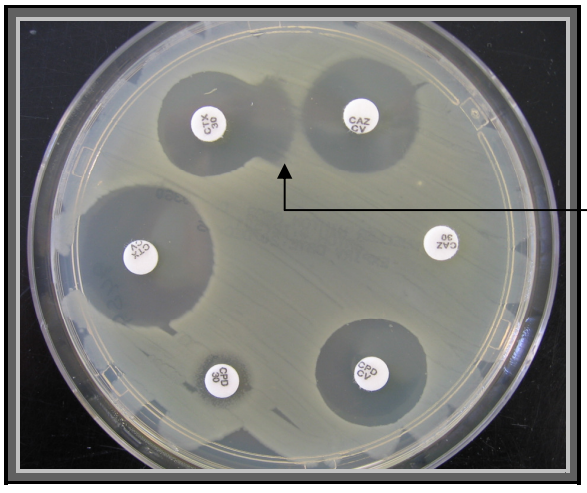


Figure 3.2 Demonstration of ESBL production with the MAST ID™ ESBL disc test. From the top of the plate clockwise: ceftazidime + clavulanic acid (CAZ/CV), ceftazidime (CAZ), cefpodoxime + clavulanic acid (CPD/CV), cefpodoxime (CPD), cefotaxime + clavulanic acid (CTX/CV), cefotaxime (CTX).

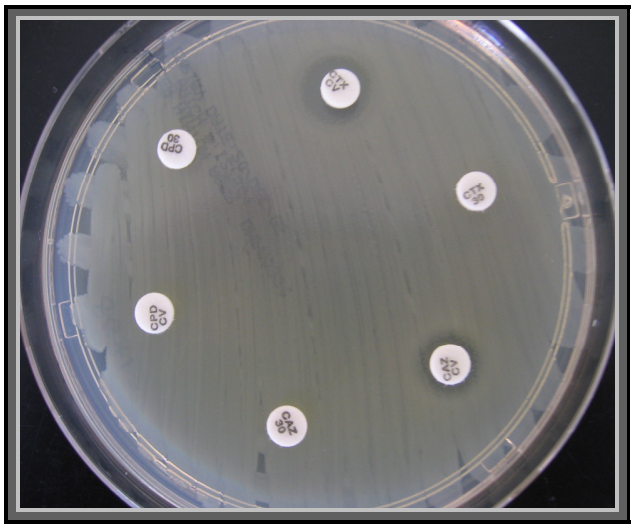


Figure 3.3 Demonstration of non-determinable (ND) results with the MAST ID™ ESBL disc test. From the top of the plate clockwise: cefotaxime + clavulanic acid (CTX/CV), cefotaxime (CTX), ceftazidime + clavulanic acid (CAZ/CV), ceftazidime (CAZ), cefpodoxime + clavulanic acid (CPD/CV), cefpodoxime (CPD).

Table 3.4 Summary of the results of the MAST ID™ ESBL disc method.

Antimicrobial Agent*	Number (%) of *Salmonella* Isangi isolates			
	Tested	**Resistant**	**Synergy**	**Non-determinable**
CAZ/CAZ + CV	115	115 (100)	107 (93%)	8 (6.9%)
CPD/CPD + CV	115	114 (99.1)	89 (77.4%)	25 (21.7%)
CTX/CTX + CV	115	20 (17.4)	5 (4.3%)	15 (13%)

CAZ, ceftazidime; CPD, cefpodoxime; CTX, cefotaxime; CV, clavulanic acid
* Resistant to single agent according to CLSI criteria.

3.5.3 Etest ® ESBL strips

Of the 273 isolates, 256 (93.7%) tested positive with CT/CTL (phantom zone, deformation of the ellipse and clear cut positive) (Figures 3.4c, 3.4b and 3.4a), while 17 (6.2%) were ND (Figure 3.4d). The MIC reading had to be extrapolated for 86 (31.5%) isolates when CT/CTL was used as the CTL diffused over onto the opposite side of the strip. PM/PML detected 252 (93.3%) isolates and 12 (4.4%) were ND. The MIC had to be extrapolated for only two isolates with PM/PML. TZ/TZL detected 205 (75.1%) isolates of which 68 (25%) were ND.

Phantom zones and ellipse deformations were more frequent with CT/CTL and PM/PML than with TZ/TZL strips. According to the Etest interpretation guidelines, deformation of the ellipse or the presence of a phantom zone is indicative of ESBL production even if the MIC ratio of <8 is not achieved. As with the MAST ID™ ESBL, when MIC values are below or above the test ranges, interpretation was ND (Figure 3.4d). This may suggest the presence of IRT (inhibitor resistant TEM) or AmpC enzymes or that the MIC values are outside the test device range.

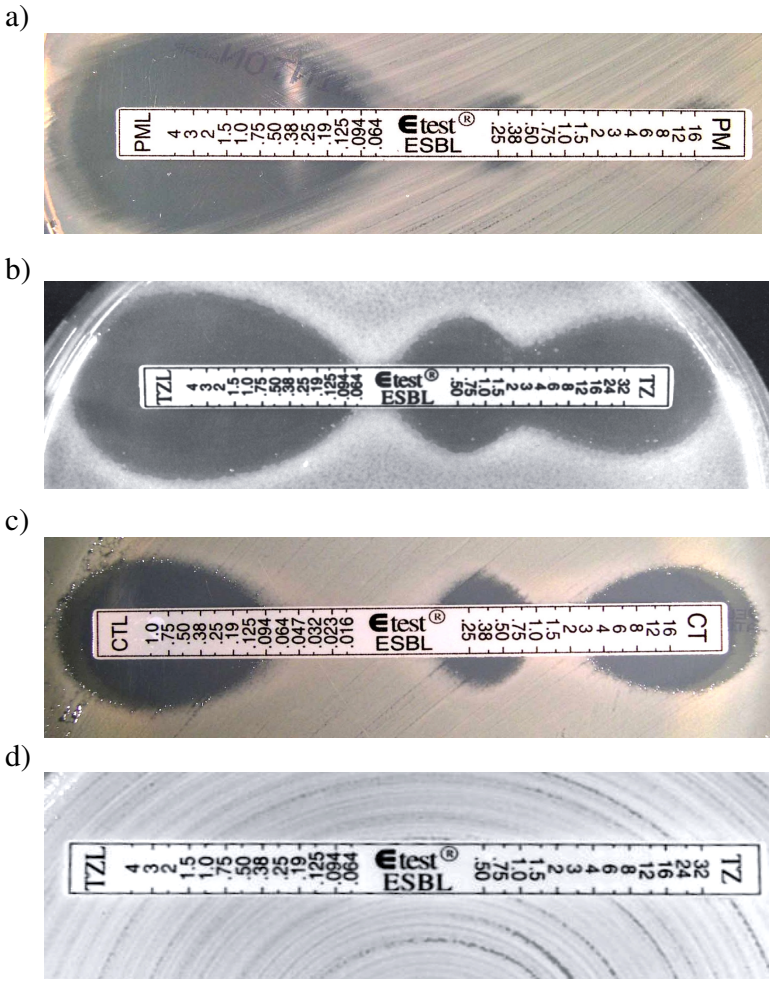


Figure 3.4 Growth-inhibition patterns of the Etest® ESBL strips of cefotaxime (CT), ceftazidime (TZ) and cefepime (PM). a) Clear-cut ESBL positive, b) deformation of inhibition ellipse indicating a positive ESBL, c) Phantom zone indicating an ESBL positive result, d) A non-determinable (ND) result.

Table 3.5 Summary of the results of the Etest ® ESBL strips method.

Antimicrobial agent	Number (%) of *Salmonella* Isangi isolates				
	Tested	Synergy finding			
		Typical	Phantom zone	Deformation	Non-determinable
TZ + TZL	273	205 (75.1%)	0	0	68 (24.9%)
PM + PML	264 [a]	44 (16.6%)	206 (78%)	2 (0.8%)	12 (4.5%)
CT + CTL	273	27 (9.9%)	143 (52.4%)	86 (31.5%)	17 (6.2%)

[a] Nine isolates were not tested.
TZ, ceftazidime; PM, cefepime; CT, cefotaxime; L, clavulanic acid.

Table 3.6 compares the performance of the three ESBL detection methods used with the interaction of CV or AMC with CAZ, CTX, FEP and CPD.

Table 3.6 Comparative performance of three methods for ESBL detection in *Salmonella* Isangi.

Method	Percentage of *Salmonella* Isangi isolates reacting with CV or AUG and				
	CAZ	CTX	FEP	CPD	Any
DDD (n=137):					
Synergy positive	43.7	68.9[a]	**99.2[b]**	N/T	100
Synergy not demonstrated	56.2	31.0[a]	0.7 [b]	N/T	0
Resistant	99.2	94.2[a]	54.1[b]	N/T	100
MAST ID (n=115):					
Synergy positive	**93.0**	4.3	N/T	77.4	100
Non-determinable	6.9	13.0	N/T	21.7	0
Susceptible	100	17.4	N/T	99.1	100
Etest ESBL (n=273):					
Synergy positive	75.1	**93.7**	**93.3[c]**	N/T	100
Non-determinable	24.9	6.2	4.5[c]	N/T	0
Resistant	100	100	100[c]	N/T	100

Percentages in bold indicates the successful antimicrobial agent/s per method.
n = number of isolates tested in the ESBL method;
[a] Only 87 isolates tested;
[b] 135 isolates tested;
[c] 270 isolates tested.
N/T = not tested

3.6 DISCUSSION

The performance of the three disc diffusion methods used in this study will be discussed separately.

3.6.1 DDD method

In order to detect TEM- and SHV-derived ESBL-producers, it has been recommended by the UK National Guidelines Laboratories and the British Society for Antimicrobial Chemotherapy (BSAC) that CAZ *and* CTX or CPD should be included in all first-line susceptibility testing in isolates of *Salmonella* species, and ESBL-production should be suspected in those that show resistance (Health Protection Agency, 2006; BSAC, 2006).

In the present study the DDD test confirmed 273/279 isolates as ESBL producers. Both CAZ (99.2%) and CXM (100%) were effective agents to determine candidate isolates for ESBL-based resistance. When looking at phenotypic confirmatory testing, FEP detected synergy in 99.2% of the isolates, while CXM and CAZ only detected 17.6% and 43.7% of ESBLs respectively. The antimicrobial discs for DDD testing are regular strength discs which are available in routine clinical microbiology laboratories and can be used with AUG disc in the centre of the sensitivity testing plate. In the present study CAZ, CTX, CXM, FEP and FOX discs, with an AUG disc in the centre of the plate, detected all ESBL-producing isolates of *S*. Isangi. The best choice of discs for general use is however, problematical while the distance between discs requires standardization.

The DDD method is convenient, easy to perform and cost-effective but may lack sensitivity because of the importance of optimal disc spacing, the inability of clavulanate to inhibit all ESBLs, the inability of the test to detect ESBLs in strains also producing chromosomal cephalosporinases, and the loss of CV disc potency during storage (Thompson and Sanders, 1992; Moland and Thomson, 1994). The performance of appropriate control tests is critical to the sensitivity of this method (Moland and Thompson, 1994). Some authors have proposed a disc edge-to-edge distance of 15mm

as having greater sensitivity than the aforementioned distance of 25-30mm (Coudron *et al*., 1997), although 20mm centre-to-centre distance appeared to be adequate in this study.

An interesting addition to the methodology of ESBL detection to improve its sensitivity was recently described by Jiang *et al*., (2006). They demonstrated high reliability for the DDD and combined disc tests [(in detecting ESBLs in multidrug-resistant *Pseudomonas aeruginosa* (*P. aeruginosa*)] when the activities of the efflux pumps were inhibited by the use of Phe-Arg-naphthylamide dihydrochloride (MC-207,110) (see section 1.5.3) and cloxacillin-containing plates. MC-207,110 was previously suggested by Baucheron *et al*., (2002) for use in combination with fluoroquinolones to treat infections with *Salmonella* strains with high-level resistance to fluoroquinolones. In a similar study by Poirel *et al.,* (2003), the use of cloxacillin-containing plates inhibited cephalosporinase activity that enhanced the ability of the DDD test to detect ESBLs in *Acinetobacter baumanni*. The enhanced sensitivity of cloxacillin-containing plates was also demonstrated in *P. aeruginosa* by Aubert *et al*., (2004).

3.6.2 MAST ID™ ESBL disc method

The MAST ID™ ESBL disc method follows the CLSI guidelines approach (CLSI, 2006) and proved to be easy to perform and yielded good results. In contrast to the DDD test findings where CTX detected 68.9% isolates as ESBL-producers, only 5 ESBL positive isolates were detected in the MAST ID™ ESBL test with this cephalosporin. This finding is in accordance with the MIC data recorded in Chapter 2 where the CTX Etest detected only 59 (21.7%) isolates when a CLSI breakpoint of 8µg/ml was used. Using the CLSI screening criteria for ESBL producers, however, CTX at the concentration of 1µg/ml would have detected all isolates as candidates for ESBL production, requiring confirmation by MIC testing in the presence and absence of 4µg/ml clavulanic acid.

CAZ and CPD were the most effective agents in the MAST ID™ ESBL test and detected ESBL in 93% and 77.4% of isolates respectively. In this study, the MAST ID™ ESBL

disc method was easy to perform and interpret and is recommended as an inexpensive alternative to other methods for the confirmation of ESBL production.

M'Zali *et al*., (2000) evaluated the MAST ID™ ESBL disc method using 100 epidemiologically distinct ESBL and non-ESBL de-repressed Class 1 β-lactamase-producing strains and reference strains; they achieved detection of 93% of ESBL producers when CAZ and CAZ + CV together with CTX and CTX + CV were used. The use of CAZ and CTX discs alone and complemented with CV, when evaluated separately, achieved 86 and 66% sensitivity, respectively.

A potential spin of the MAST ID™ ESBL disc method is that it lends itself, according to the DDD test methodology, to further screening for ESBL expression by placing discs in alternating positions between cephalosporin and cephalosporin + CV (Figure 3.2). The disc-diffusion phenomenon can be visualized within the MAST ID™ ESBL disc method as the CV diffuses towards the disc containing the cephalosporin. The discs would still have to be spaced approximately 20mm from centre-to-centre to observe the synergy effect between the cephalosporin and the disc containing CV.

3.6.3 Etest® ESBL strips

When TZ/TZL Etest® ESBL strips were tested, no readings had to be extrapolated or phantom zones observed compared to 86 extrapolated readings with CT/CTL and two with PM/PML. Phantom zones and deformation of ellipses were observed in 83.8%, 0% and 77.1% with CTL, TZL and PML respectively. The results showed a similar trend to that reported by Stürenburg *et al*., (2004) who found the frequencies of phantom zones and deformation with CTL, TZL and PML to be 61%, 20% and 85%, respectively when testing *Enterobacter* species. The Etest® ESBL strips were found to be more sensitive than the double-disc test for ESBL identification, however, the use was somewhat compromised by non-determinable results with CT/CTL (6.2%), PM/PML (4.5%) and TZ/TZL (24.9%). According to our results, the PML strip was

more suitable for ESBL detection in *S*. Isangi than CTL and TZL, as was previously described by Stürenburg *et al*., (2004).

In comparative studies with clinical isolates, the Etest® ESBL strip was shown to be equally or more sensitive than the disc methods and was more convenient to use (M'Zali *et al*., 2000; Cormican *et al*., 1996; Sanders *et al*., 1994). Similar to previous reports, the present study also encountered problems with TZ/TZL and CT/CTL for the detection of ESBL production as compared to the PM/PML strip (Stürenburg *et al*., 2004). The Etest® ESBL strips required an extra agar plate to be inoculated to observe the augmentation phenomenon and therefore cannot be easily incorporated into a routine antibiogram testing system. It is also comparatively expensive.

All three ESBL screening tests showed relatively high sensitivity for detection of ESBLs when CAZ and/or CPD were used. Although previous studies using ESBL Etests to detect ESBL-producing *Salmonella* proved reliable and convenient, the results of this study showed that the two disc screening methods that were evaluated were more cost effective and equally reliable as the Etest® ESBL strips. Cefepime was a useful marker in this study to detect 'hidden' resistance and synergy was observed between this agent and amoxycillin/clavulanic acid in 99.2% of isolates. It served to highlight that we should not restrict the screening of ESBLs by using only third-generation cephalosporins.

Chapter 4

CHARACTERIZATION OF EXTENDED-SPECTRUM β-LACTAMASES BY POLYMERASE CHAIN REACTION (PCR)

4.1 INTRODUCTION

There are currently 160 TEM-type, 100 SHV-type and about 40 CTX-M β-lactamases described. In the early days of studying ESBLs, determination of the isoelectric point played an important role in the identification of these β-lactamases. Many β-lactamases however, have identical isoelectric points which limit its usefulness. It retains, however, its place as important in the characterization of β-lactamases.

An alternative to the phenotypic isoelectric point testing of β-lactamases and testing for substrate profiles is analysis at the genotypic level. Various methods have been reported to detect point mutations in *bla* genes which includes sequence-specific oligonucleotide probe hybridization (Fasching *et al.*, 1991), sequencing of the target *bla* genes (Granjeaud *et al.*, 1999), restriction-fragment length polymorphism (RFLP) determination (Lukinmaa *et al.*, 2004), single strand conformational polymorphism (SSCP) analysis (Nair *et al*., 2002; M'Zali *et al*., 1998), and allele-specific PCR in combination with RFLP (Giraud *et al.*, 1999). The most common molecular method employed today is PCR with oligonucleotide primers that are specific for a β-lactamase gene.

4.2 MOLECULAR METHODS FOR ESBL DETECTION

Two years after its first description by Mullis and Faloona (1987), and only 15 years ago, the first diagnostic application of PCR was published by Saiki *et al*., (1988). New developments in labeling technology in recent years have expanded the applicability of PCR and many variations of PCR have been applied to the typing of ESBL-producing organisms. Two of these are randomly amplified polymorphic DNA, which is also known as arbitrarily primed PCR, and PCR based on repetitive chromosomal

sequences. Because of ease of performance, arbitrarily primed PCR has become a popular method used to evaluate the genetic relatedness of ESBL-producing strains. Neither of these two PCR approaches, however, discriminates among different variants of TEM or SHV as they do not target the genes encoding these β-lactamases directly.

One of the first molecular methods targeting the *bla* genes was oligotyping, developed by Ouellette *et al*., (1988) to discriminate between TEM-1 and TEM-2 β-lactamases. This was improved by Mabilat and Courvalin (1990) who developed additional probes to detect mutations within the bla_{TEM} gene at six positions. PCR can be sensitized further by subjecting the amplified products to restriction endonucleases and separating the fragments by electrophoresis. Point mutations are indicated by the size of fragments generated. This method is called restriction fragment length polymorphism analysis (PCR-RFLP).

PCR-RFLP has also been employed to identify SHV variants (Nüesch-Inderbinen *et al*., 1997). The PCR product is digested with *Nhe*I which detects the G to A nucleotide change. This method detects the mutation at position 238, but does not indicate which SHV-type ESBL is present. Restriction site insertion PCR is a recently developed technique to detect mutations of the SHV genes to identify ESBLs. This method uses amplification primers designed with one to three base mismatches near the 3' end of the nucleotide sequence to engineer a desired restriction site. Chanawong *et al*., (2001) demonstrated that the combination of PCR-RFLP and PCR-SSCP techniques can be readily applied to the study of 17 different SHV β-lactamases.

Another technique, the ligase chain reaction (LCR), an alternative to PCR, has been used to distinguish SHV variants and can detect single base pair changes (Kim and Lee, 2000). The LCR product is detected by an enzymatic reaction using NADPH-alkaline phosphatase. It was found to be a simple and rapid method to define seven of the SHV variants. Randegger and Haechler (2001) developed a real-time PCR-based method for the detection of SHV mutations. Real-time PCR using the Roche LightCycler monitors PCR products on line as they form during thermocycles in air heated capillary tubes

with fluorescently labeled hybridization probes. The rapid PCR amplification cycles are followed by melting curve analysis, based on the principle that each dsDNA product has its own highly specific melting point. Melting point analysis performed in the LightCycler is able to differentiate SHV variants and discriminated between non-ESBLs and ESBLs.

PCR has been applied successfully to characterize CTX-M β-lactamases, but detection of all members from the five groups requires multiple PCRs with group-specific primers or consensus primers which only amplifies a few CTX-M alleles (Canton *et al*., 2002). Recently a rapid and accurate multiplex PCR assay (CTX-Mplex PCR) for the amplification of all CTX-M genes and the differentiation of the five groups have been reported (Xu *et al*., 2005).

Nucleotide sequencing is a well-known technique and the standard for determination of the specific β-lactamase gene present and is almost universally performed by dideoxy sequencing (Sanger *et al.*, 1977). More recent techniques have moved in the direction of DNA arrays and DNA chips, based on the principle of hybridization which allows for mass screening of sequences (Chan *et al*., 2003; Pelludat *et al*., 2005). Extended-spectrum β-lactamases contain one to four amino acid substitutions (commonly at residues 104, 164, and 237-240, either as individual mutations or in combination) near the active site and are derived either from the TEM or SHV β-lactamases. The advantages and disadvantages of each method are outlined in Table 4.1.

Table 4.1 Comparison of molecular techniques for ESBL detection.

Test	Advantages	Disadvantages	Reference(s)
DNA probes	Specific for gene	Labor intensive, Cannot detect variants of TEM or SHV or distinguish between ESBL and non-ESBL.	(Arlet and Philippon,1991)
***PCR**	Specific for gene, easy to perform.	Cannot detect variants of TEM or SHV or distinguish between ESBL and non-ESBL.	(Mabilat and Courvalin, 1990)
Oligotyping	Detect specific TEM variants.	Needs specific oligonucleo-tide probes and cannot detect the new variants, labour intensive.	(Ouellette *et al*., 1988)
PCR-RFLP	Easy to perform, detect specific nucleotide changes.	Nucleotide changes must result in altered restriction site to detect.	(Nüesch-Inderbinen and Hächler, 1997)
PCR-SSCP	Distinguish between a number of SHV variants.	Needs special electrophoresis conditions.	(M'Zali *et al*., 1998)
LCR	Distinguish between a number of SHV variants.	Needs large number of oligonucleotide primers.	(Kim and Lee, 2000)
***Nucleotide Sequencing**	Gold Standard, detects all variants.	Labor intensive, can be technically challenging and difficult to interpret.	(Bradford, 1999)

This table has been adapted from Bradford, 2001.
*Method used in this study.

4.3 MATERIALS AND METHODS

4.3.1 Bacterial strains for β-lactamase characterization

4.3.1.1 Control Strains

The following control strains were used in molecular β-lactamase characterization tests:

Positive control strain for SHV:

- *Klebsiella pneumoniae* ATCC 700603 (SHV-18 β-lactamase producing strain)

Positive control strains for TEM:

- CHB 20274 (TEM-63 β-lactamase producing *Salmonella* Isangi strain)
- CHB 20338 (TEM-131 β-lactamase producing *Salmonella* Isangi strain)

Positive control strain for CTX-M:

- *Escherichia coli* (CTX-M-14) obtained from Dr Linda Meyer, Department of Medical Microbiology at the University of Pretoria, Johannesburg, South Africa

4.3.1.2 Test Isolates

One hundred and one of the total collection of 279 isolates of *S*. Isangi were selected for the study of β-lactamase mediated resistance. This was done according to the number of isolates per hospitals and where possible, representing each month for the period of 2000 to 2002. The total number of *S.* Isangi, as well as the total number of non-typhoidal salmonellae isolates (per province) received at EDRU between 2000 and 2002 is listed in Table 4.2. No *S.* Isangi isolates were received from Mpumalanga, Free State, Northern Cape or KwaZulu Natal provinces during the 3-year period.

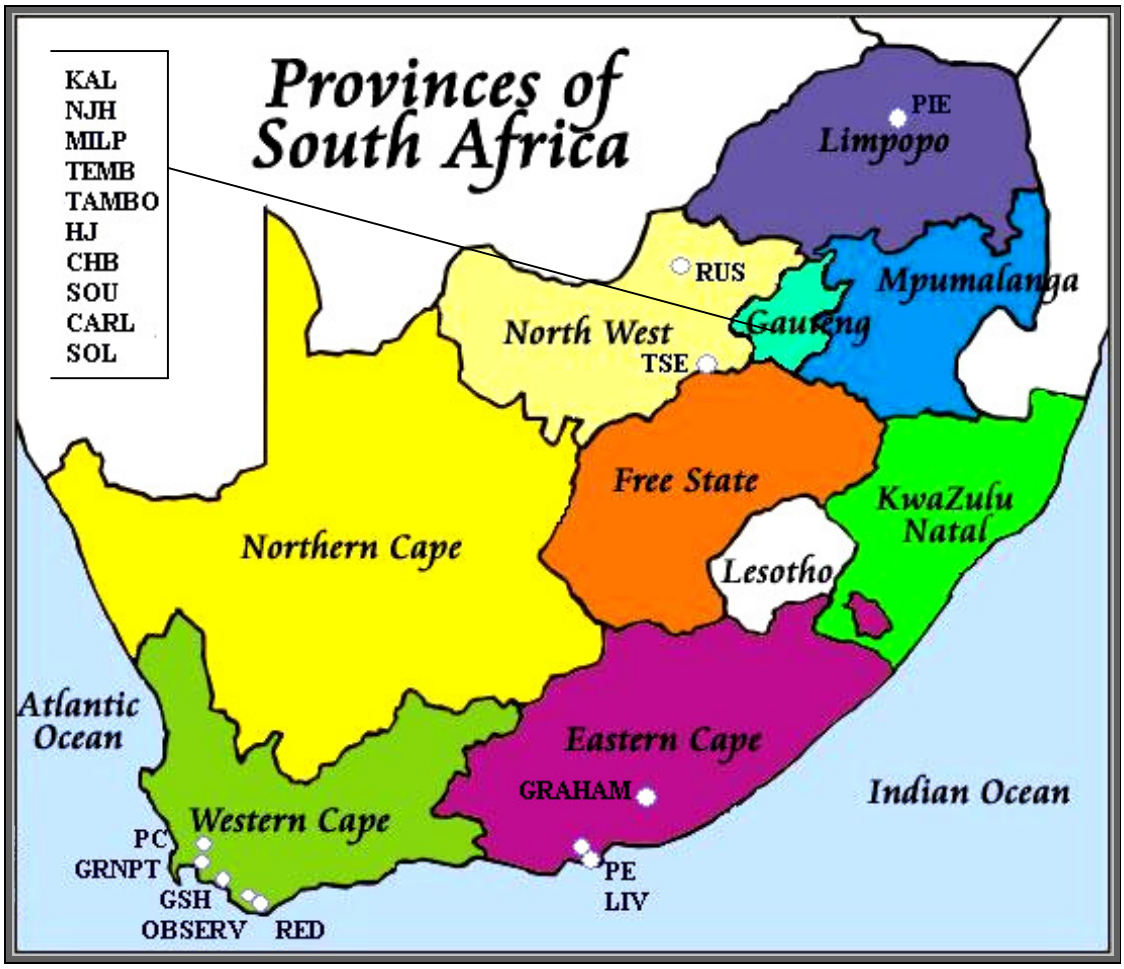


Figure 4.1 Geographical distribution of *Salmonella* Isangi isolates identified between 2000 and 2002 in the provinces in South Africa.

Key: PE, Port Elizabeth Hospital; GRAHAM, Grahamstown Hospital; LIV, Livingstone Hospital; CARL, Carletonville Hospital; HJ, Helen Joseph Hospital; TAMBO, Tambo Memorial Hospital; NJH, New Johannesburg Hospital; CHB, Chris Hani Baragwanath Hospital; KAL, Kalafong Hospital; MILP, Milpark Hospital; SOU, South Rand Hospital; TEMB,Tembisa Hospital; SOL, Soldin le Roux private pathology practice; PIE, Pietersburg Hospital; TSE, Tsepong Hospital; RUS, Rustenburg Hospital; GRNPT, Green Point Hospital; GSH, Groote Schuur Hospital; OBSERV, Observatory Hospital; PC, Pathcare private laboratory practice; RED, Red Cross Childrens Hospital.

Table 4.2 Number of non-typhoidal *Salmonella* (NTS) and percentage of *Salmonella* Isangi isolates submitted from provinces in South Africa, 2000-2002.*

Province	Population size†		% *S.* Isangi (Total non-typhoidal salmonellae)		
	x 10^6	(%)	2000	2001	2002
Gauteng	8,8	(19,0%)	18.4 (141)	15.8 (342)	32.6 (239)
Eastern Cape	6.4	(15,2%)	2.1 (48)	0 (100)	59.5 (89)
Western Cape	4,5	(9,8%)	0 (79)	1.6 (237)	16.9 (254)
North West	3,6	(8,2%)	0 (5)	5.2 (19)	16.6 (12)
Limpopo	5,2	(11,9%)	50 (6)	25.7 (35)	4.3 (23)
KwaZulu Natal	9,4	(20,7%)	0 (0)	0 (28)	0 (0)
Free State	2,7	(6,3%)	0 (8)	0 (100)	0 (40)
Mpumalanga	3,1	(7,0%)	0 (8)	0 (19)	0 (0)
Northern Cape	0.8	(1,9%)	No isolates received until the year 2003		

† Provincial share (%) estimates of total population for 2001 (http://www.statssa.gov.za).
* NHLS laboratories were requested to submit all salmonella isolates to the EDRU as part of a national *Salmonella* surveillance program (KwaZulu Natal Province is only due to join the NHLS during 2006-2007).

4.3.2 Detection of *bla*$_{TEM}$, *bla*$_{SHV}$ and *bla*$_{CTX-M}$ genes by polymerase chain reaction (PCR)

4.3.2.1 Genomic DNA extraction

A colony of an overnight culture on 5% blood agar (DMP) of the test isolate was homogenized in 400µl of sterile water and boiled for 10min to lyse the bacterial cell walls. The boiled suspensions were centrifuged (13,000rpm for 1min) to pellet the cell debris. The supernatant was transferred to a sterile eppendorf tube and stored at -20°C to be used as the DNA template.

4.3.2.2 Amplification of *bla*$_{TEM}$, *bla*$_{SHV}$ and *bla*$_{CTX-M}$ genes by PCR

PCR amplification was performed using the supernatant from the genomic DNA extraction step. The primer sets to detect TEM- and SHV-type β-lactamases (Essack *et al.*, 2001) and universal (consensus) primers which correspond to conserved regions of CTX-M-type genes (Liebana *et al*., 2006) are shown in Table 4.3. Reaction mixtures

(30µl) contained 50mM KCI, 1.5mM $MgCl_2$, 0.5µM of each primer, 100µM of each deoxynucleotide triphosphate (dNTP) (Sigma-Aldrich, South Africa), 1unit of Red*Taq* polymerase (Sigma-Aldrich) and 1µl of DNA.

The DNA amplification was performed in an iCycler thermal cycler (Bio-Rad, Marnes-la-Coquette, France) with the following parameters: an initial denaturation step (94°C, 1min), followed by 30 cycles of denaturation (94°C, 1min), annealing (45°C for bla_{TEM}, 50°C for bla_{SHV} and 62°C for bla_{CTX-M} for 1min) and extension (72°C, 1min), and a single final extension of 10min at 72°C. Ten microlitres of the reaction mixture containing the PCR product, was analyzed by electrophoresis in 1% (w/v) agarose gel in 1x TAE buffer (0.04M Tris-acetate, 0.002M EDTA [pH(8.5)]) stained with ethidium bromide.

4.3.2.3 Isolation and detection of the amplified PCR product

The QIAquick PCR purification Kit (Qiagen GmbH, Hilden, Germany) was used as per manufacturer's instructions to purify the amplified DNA fragments (amplicons) from PCR by combining spin-column technology with selective binding properties of a silica-gel membrane.

Yields of the cleaned PCR product (2µl) were determined by electrophoresis in 1% (w/v) agarose using the 1kb molecular weight marker VI (Roche Diagnostics, GmbH, Mannheim, Germany) as standard. The cleaned PCR products were stored at -20°C for sequencing.

Table 4.3 Oligonucleotide primers used for amplification and sequencing of *bla* genes.

Primer ([a])	Sequence (5'-3')	Nucleotide positions	Amplicon size (bp)
***bla*$_{TEM}$:**			840
Amplification and sequencing:			
TEM-1 (F)	ATGAGTATTCAACATTTCCGTG	1-22	
TEM-4 (R)	TTACCAATGCTTAATCAGTGAG	861-840	
Sequencing:			
TEM-2 (R)	TTCTGTGACTGGTGAGTACT	324-305	
TEM-3 (R)	GAGTAAGTAGTTCGCCAGTT	595-576	
***bla*$_{SHV}$:**			846
Amplification and sequencing:			
SHV-1 (F)	ATGCGTTATATTCGCCTGTG	1-20	
SHV-3 (R)	GTTAGCGTTGCCAGTGCTCG	865-846	
Sequencing:			
SHV-2 (R)	CGTTTCCCAGCGGTCAAGG	489-471	
***bla*$_{CTX-M}$:**			585
Amplification:			
CTX-M (F)	CGATGTGCAGTACCAGTAA	271-289	
CTX-M (R)	TTAGTGACCAGAATCAGCGG	837-856	

[a] (F), forward primers; (R), reverse primers. Primer sets obtained from Inqaba Biotechnologies, Pretoria, South Africa.

4.3.3 Nucleotide sequencing of *bla* genes

The sequencing primer sets (Inqaba Biotechnologies, Pretoria, South Africa) are shown in Table 4.3. Numbering follows the scheme of Ambler *et al*., (1991). Purified genes were sequenced by Inqaba Biotechnical Industries (PTY) Ltd (Pretoria, South Africa), using the BigDye version 3.1 dye terminator cycle sequencing kit (Applied Biosystems) and the SpectruMedix SCE2410 genetic analysis system (SpectruMedix LLC, Pennsylvania, USA). Data collection and sequencing analysis was performed using CHROMAS v1.43 (http://www.trishul.sci.gu.edu.au/~conor/chromas.html) sequencing analysis software.

4.4 RESULTS

4.4.1 Polymerase chain reaction

The collection of 101/273 isolates selected for β-lactamases characterization that tested positive in the three ESBL tests (Chapter 3) had positive PCR reactions for *bla*$_{TEM}$. Two isolates (2/273), both from NJH, were positive for *bla*$_{SHV}$ and *bla*$_{TEM}$ simultaneously (16490 and 16970). Figure 4.2 shows a 1% (w/v) agarose gel depicting the amplified *bla*$_{TEM}$ and *bla*$_{SHV}$ products, using the primer sets in Table 4.3.

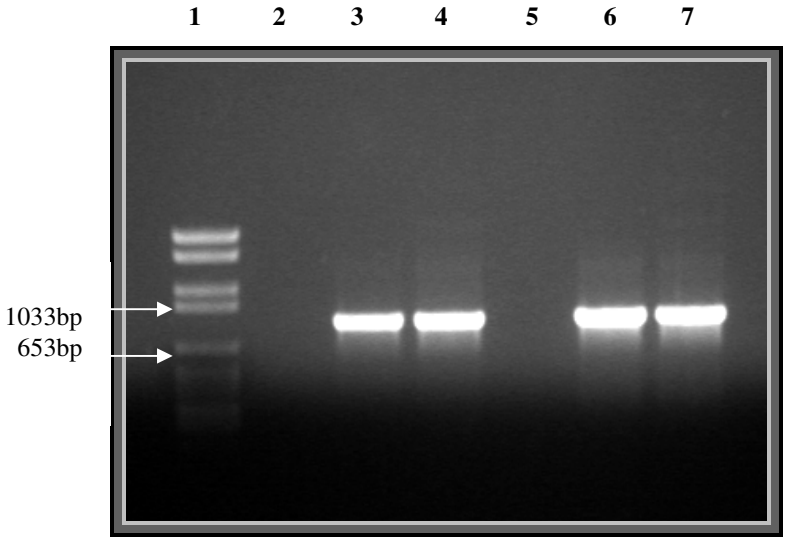


Figure 4.2 A 1% (w/v) agarose gel depicting PCR amplified *bla*$_{TEM}$ and *bla*$_{SHV}$ ESBL genes. Lane 1, molecular weight marker VI; 2, negative TEM control; 3, positive TEM control (isolate 20274); 4, isolate 16970; 5, SHV negative control; 6, *Klebsiella pneumoniae* ATCC 700603; 7, isolate 16970.

All 101 isolates from the sample population were negative for CTX-M-type β-lactamases by PCR. Figure 4.3 shows a 1% (w/v) agarose gel depicting the amplified *bla*$_{CTX-M}$ product of the *Escherichia coli* CTX-M-14-producing strains, using the primer sets in Table 4.3.

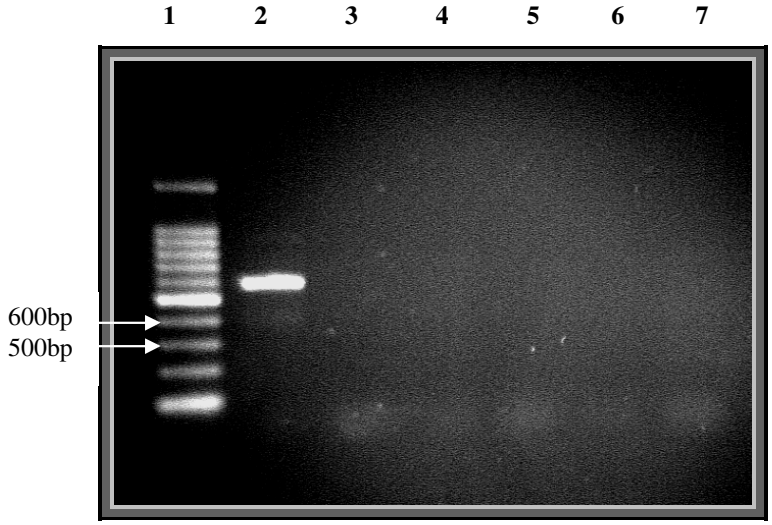


Figure 4.3 A 1% (w/v) agarose gel depicting PCR amplified *bla*$_{CTX-M}$ ESBL genes. Lane 1, molecular weight marker 100bp plus (Qiagen); 2, CTX-M-14 positive isolate (585bp); 3, CTX-M negative control; 4-7, *Salmonella* Isangi isolates.

4.4.2 Nucleotide sequencing

Sequences of PCR amplified *bla*$_{TEM}$ and *bla*$_{SHV}$ ESBL genes were confirmed using three primers to amplify parts of sequences that overlapped one another. TEM-63, SHV-5 and TEM-1 were the known ESBLs identified. TEM-1 was identified in only one isolate and was from the New Johannesburg Hospital (NJH, 14340).

TEM-63 was detected in 92/101 (91%) isolates tested for β-lactamase production. A novel TEM sequence, TEM-131, was found in seven isolates (GenBank accession number AY436361). The sequence of TEM-63 has four amino acid changes compared with the sequence of TEM-1, while TEM-131 has an additional change compared to TEM-63 (Table 4.4). Isolate 16970 carried TEM-63 together with SHV-5, while isolate 16490 carried the novel TEM-131 together with SHV-5. Figure 4.4 shows the nucleotide and amino acid changes identified in TEM-63 and TEM-131 with respect to the TEM-1 gene and Figure 4.5 shows the nucleotide and amino acid changes identified in SHV-5 with respect to the SHV-1 gene.

Table 4.4 Nucleotide and amino acid changes of the TEM-63, TEM-131 and SHV-5 β-lactamases as compared to TEM-1.

β-lactamase gene	Nucleotide position [a]	Nucleotide change	Amino acid position [b]	Amino acid change [c]
TEM-63	263-265	**C**TT→ **T**TT	21	Leu (L) → Phe (F)
	436	C→ T	-	-
	512-514	**G**AG→**A**AG	104	Glu (E)→ Lys (K)
	692-694	**C**GT→ **A**GT	164	Arg (R)→ Ser (S)
	746-748	A**T**G→ A**C**G	182	Met (M)→ Thr (T)
TEM-131	263-265	**C**TT→ **T**TT	21	Leu (L)→ Phe (F)
	346	A→ G	-	-
	512-514	**G**AG→ **A**AG	104	Glu (E)→ Lys (K)
	604	T→ G	-	-
	682	T→ C	-	-
	692-694	**C**GT→ **A**GT	164	Arg (R)→ Ser (S)
	746-748	A**T**G→ A**C**G	182	Met (M)→ Thr (T)
	911-913	**G**CC→ **A**CC	237	Ala (A)→ Thr (T)
SHV-5	700-702	**G**GC→**A**GC	238	Gly (G)→ Ser (S)
	703-705	**G**AG→**A**AG	240	Glu (E)→ Lys (K)

[a] Nucleotide numbering according to AGT codon start of previously reported TEM and SHV sequences.
[b] Amino acid numbering according to Ambler *et al*., (1981).
[c] A, alanine; E, glutamic acid; F, phenylalanine; G, glycine; K, lysine; L, leucine; M, methionine; R, arginine; S, serine; T, threonine
Nucleotide change are indicated in bold red

Figure 4.4 Nucleotide and amino acid changes identified in TEM-63 and TEM-131 with respect to the TEM-1 gene.

Figure 4.4 ***continued***

Nucleotide and amino acid changes identified in TEM-63 and TEM-131 with respect to the TEM-1 gene.

Figure 4.5 Nucleotide and amino acid changes identified in SHV-5 with respect to the SHV-1 gene.

Table 4.5 Minimal inhibitory concentrations (MICs) of TEM-1, TEM-131, TEM-131 + SHV-5 and TEM-63 + SHV-5 *Salmonella* Isangi isolates.

Isolate number	Hospital	ESBL-type		Etest MIC (ug/ml)								
		TEM	SHV	TZ	CT	FEP	CIP	TS	TET	GM	IMI	NA
14340	NJH	1		**>32**	1	3	0.016	0.125	**96**	0.38	0.25	6
14798	TAMBO	131		**>32**	**256**	**24**	0.012	**>32**	**48**	**128**	0.19	16
15582	NJH	131		**>32**	**256**	**256**	0.012	**>32**	**64**	**96**	0.38	16
16115	CHB	131		**>32**	**16**	8	0.016	**>32**	**128**	**128**	0.38	**24**
16390	GRNPT	131		**>32**	3	**32**	0.094	**>32**	**64**	**128**	0.19	**>256**
18305	TAMBO	131		**>32**	**16**	**12**	0.012	**>32**	**64**	**>256**	0.25	16
16576	CHB	131		**>32**	**16**	6	0.064	**>32**	**>256**	**96**	0.19	8
16490	NJH	131	5	**>32**	**>256**	**>256**	0.012	**>32**	**48**	**>256**	0.19	4
16970	NJH	63	5	**>32**	**256**	**256**	0.094	**>32**	**128**	**96**	0.19	**>256**

NJH, New Johannesburg hospital; CHB, Chris Hani Baragwanath hospital; GRNPT, Greenpoint hospital; TAMBO, Tambo Memorial hospital;
TZ, ceftazidime; CT, cefotaxime; FEP, cefepime; CIP, ciprofloxacin; TS, cotrimoxazole; TET, tetracycline; GM, gentamicin; IMI, imipenem; NA, nalidixic acid.
MICs in bold indicate resistance (including intermediately resistant)

4.5 DISCUSSION

Initially, ESBLs emerged gradually as a result of the introduction of new β-lactam antibiotics, but the number and variety has increased and disseminated at an unparalleled rate (Medeiros, 1997).

4.5.1 The hospital setting in South Africa

Hospitals where bacteria have greater levels of resistance are more often teaching institutions that provide tertiary care to a population at greater risk of serious diseases. The New Johannesburg Hospital (NJH) and Chris Hani Baragwanath (CHB) Hospital are the two largest teaching hospitals in Johannesburg. NJH, the main teaching hospital for the University of the Witwatersrand, has approximately 1300 beds with a nurse-to-patient ratio of 0.83 and an in-patient volume of ~70 000 patients per annum. CHB, one of the largest hospitals in the world, with 3200 beds (only 2865 beds currently used due to financial constraints), serves mostly the African community of 3.5 million people in Soweto (~213 981 admissions and 652 526 outpatients annually) in the surrounding area. More than 2000 patients check into the hospital daily and nearly half of them are HIV positive.

The patients served by these institutions are mobile and often come from poor socio-economic backgrounds, inevitably associated with poor personal hygiene. In these institutions there is a risk of cross-infection outbreaks and the sheer diversity of ESBLs isolated is a reflection of the multiplicity of the population visiting these hospitals. An ESBL gene-carrying plasmid, an ESBL-producing strain and even an ESBL outbreak may persist in a medical centre over a prolonged period. In ICUs, by means of cross-infection, the same bacterial organism may settle in different patients who were being treated with different β-lactams. This may create a fluctuating selective environment and it is thus possible that the isolates of the same resistance types and subtypes proliferated by nosocomial transmission within and between wards.

4.5.2 Extended-spectrum β-lactamases

NJH showed the most diversity in ESBL producers. TEM-1, TEM-63, TEM-131 and SHV-5 were isolated from this hospital. Two isolates that harbored two types of β-lactamases simultaneously (TEM-63 + SHV-5 and TEM-131 + SHV-5) were also isolated at NJH.

TEM-63 appeared indigenous to HJ, PE, PIE, RED, RUS and TEMB hospitals and was predominantly isolated in 2000 and 2001. The novel TEM-131 was isolated from CHB, GRNPT, TAMBO and NJH hospitals and was only detected in isolates from 2002, but was absent in 2000 and 2001. The temporal relationship between TEM-63 and TEM-131 suggests that antibiotic pressure probably played a role in the emergence of this new β-lactamase. TEM-131 essentially differs from TEM-63 by one mutation.

Ceftazidime MICs were greatly elevated (>32µg/ml) for both TEM-63 and TEM-131 producing strains (Table 4.5). Of potential interest is that we observed somewhat higher cefotaxime MICs for TEM-131-producing transformant strains compared to TEM-63-producing transformant strains (Kruger *et al*., 2004). In this study only five TEM-63-producing isolates had cefotaxime MICs >256µg/ml. TEM-63 seems to be unique to the African continent and has been reported from South Africa (Pitout *et al*., 1998; Essack *et al.*, 2001; Hanson *et al*., 2001; Paterson *et al.*, 2003; Kruger *et al*., 2004), Tanzania (Blomberg *et al*., 2005) and recently in Malawi (Gray *et al*., 2006). TEM-63 is only slightly more resistant to ceftazidime and aztreonam than TEM-1 and TEM-2. Both TEM-63 and TEM-131 have an isoelectric point of 5.6 (Essack *et al*., 2001; Kruger *et al*., 2004).

TEM-63 has two mutations (Gly238Ser and Arg164Ser/His) and also showed the Met182Thr mutation which is observed in inhibitor resistant TEMs (IRTs), all SHV β-lactamases and 16 other TEM-types. The Ala237Thr mutation of TEM-131 has also been found in seven other TEM-genes (TEM-5, TEM-24, TEM-86, TEM-114, TEM-121, TEM-130 and TEM-136). The plasticity of this binding site can cause modulation

of the activity against various β-lactams and could select bacterial cells harbouring extended-spectrum TEM-type β-lactamases (Healey *et al*., 1989).

Residue 237 has been shown to be part of the hydrogen-bond network that stabilizes initial binding of the substrate in the active site (Strynadka, *et al*., 1992). This mutation shows improved catalytic efficiency for both ceftazidime and aztreonam. The mutation at position 237 in TEMs modulates β-lactamase activity by reducing it against certain β-lactams (ceftazidime and aztreonam) while increasing it towards others (ceftazidime and cephalothin) (Blázquez *et al*., 1998). Blázquez *et al.,* (1998) proposed that ESBLs evolve under the constant pressure of β-lactam antibiotics and postulate that such conditions may be responsible for the selection of TEM enzymes carrying the modulating substitution at position 237.

Spectrum-extending mutations are the most important (position 164 in TEMs, 179 in SHVs and 238 in both) because they enlarge the β-lactam binding site, making enough room for enzyme interactions with compounds possessing bulky oxyimino side-chains. Other substitutions (104 in TEMs and 240 in TEMs and SHVs) enhance β-lactamase interactions with oxyimino side-chains of specific compounds (ceftazidime and aztreonam) (Knox, 1995). Single amino acid substitutions (e.g. position 21 in TEMs), which are considered to be neutral (Table 4.4 and Figure 4.4), were inherited by TEM and SHV ESBL genes from different variants of their parental penicillinase genes and comprise specific markers of different genealogic lineages of ESBL genes (Leflon-Guibot *et al*., 2000). Analysis of these mutations is very useful in evolutionary as well as epidemiologic investigations within particular ESBL families.

The present study led to the first report of TEM-63 in *Salmonella* strains worldwide (Kruger *et al*., 2004), although it was previously reported in *Klebsiella*, *Proteus* and *Enterobacter* in South Africa (Hanson *et al*., 2002). It is not certain whether the origin of $bla_{\text{TEM-63}}$ and $bla_{\text{TEM-131}}$ was in *Salmonella* spp. or whether it originated in other organisms and was then transferred by horizontal gene transfer to *Salmonella* spp. TEM-131 was also reported in *S*. Typhimurium and *S.* Muenchen (Kruger *et al*., 2004).

SHV-5 has previously been found in South Africa (Essack *et al*., 2001) and in almost every country in which ESBLs have been studied at the molecular level.

4.5.3 Emergence of CTX-M type β-lactamases

The emergence of the cefotaxime-hydrolyzing β-lactamase (CTX-M) among *Salmonella* species is of concern as it has uniquely potent hydrolytic activity against cefotaxime and may be related to the increased usage of extended-spectrum cephalosporins in recent years. Organisms producing CTX-M display higher levels of resistance to CTX and ceftriaxone than CAZ (Bonnet, 2004). Ceftazidime MICs of these organisms are sometimes in the susceptible range and, because many laboratories use this agent alone as an indicator of ESBL production, many CTX-M producing isolates may be missed by routine susceptibility testing.

In a previous report (Kruger *et al*., 2004) where salmonellae were collected within the same time-frame as the present study (2000-2002), no isolates among the seven *Salmonella* serotypes were found to carry a CTX-M-type β-lactamase. Although only one third (101/279) of the isolates were screened for β-lactamase production, no isolates were found to carry a CTX-M-type β-lactamase in this study. It is however possible that some might have been missed. The apparently slow spread of CTX-M in *S*. Isangi is in contrast to the rising significance of CTX-M-type ESBLs in *Klebsiella* and other genera (Paterson *et al*., 2003) and has been found previously in other non-typhoidal *Salmonella* spp. (Bradford *et al*., 1998). A recent report described CTX-M-37 in *S*. Isangi from a paediatric ward of a tertiary hospital in Durban, South Africa (Govinden *et al*., 2006) while nine CTX-M producing *S.* Isangi isolates were reported from Bulgaria (Asseva *et al*., 2006). Although the isolates are from the same period as the present study, it might indicate that CTX-M-37 resistance emerged in Durban as a result of antibiotic selective pressure which is likely to be strong due to the high HIV/AIDS prevalence in the province. *S*. Isangi was found to be associated with this disease at the Chris Hani Baragwanath Hospital (CHB) (Wadula *et al*., 2006).

Chapter 5

STRAIN DIFFERENTIATION OF *Salmonella* Isangi BY PLASMID TYPING

5.1 DISSEMINATION OF RESISTANCE

ESBL-producing *Enterobacteriaceae* has the tendency to spread resistance by clonal strain transmission or conjugative plasmid transfer. Horizontal gene transfer (HGT) encodes antimicrobial resistance among ecologically related strains or species and occurs via transmissible genetic structures such as plasmids, transposons, or lysogenic bacteriophages (Cantón *et al*., 2003). Dissemination of plasmids carrying resistance genes acquired by conjugation has been thought to be an important mechanism responsible for the increase of resistance rates in gram-negative bacilli (Dionisio *et al.*, 2002).

In the study by Avramova *et al*., (1979), *S*. Isangi was found to carry naturally occurring R plasmids, rRB1 (31.5kb) and rRB2 (13.95kb), which were stable class II plasmids, composed of a transfer factor and a resistance plasmid. The plasmid rRB2 was found to mediate resistance to cephalothin. Studies have shown that R plasmids can behave in a variety of ways and considerable reassortment (Richmond and Wiedemann, 1974) and dissociation of the plasmid DNA can be demonstrated. The R-factor in its ecological situation has become entrenched in many hospital and even community settings due to its properties which enable plasmids to spread very effectively into the environment.

Epidemics of MDR organisms are frequently due to the dissemination of a single clone containing an MDR plasmid or to the dissemination of an MDR plasmid among different clones. A predominant well-adapted clone may first disseminate and then act as an efficient donor to other bacterial clones which are not as efficient at dissemination and spread. Examples of such transmission patterns involving ESBL-producing *Enterobacteriaceae* have been outlined by Cantón *et al.,* in 2003.

It is likely that all 'plasmid-mediated' β-lactamases have chromosomal origins, although the source organisms for many types remain unknown (Livermore, 1995). ESBLs are mostly encoded by large plasmids (up to 100kb and even more) that are transferable from strain to strain and between bacterial species (Livermore, 1995; Jacoby and Medeiros, 1991). ESBL production is usually encoded on transmissible plasmids together with a range of aminoglycoside-modifying enzymes and, therefore, most of these cephalosporin-resistant salmonellas are also resistant to aminoglycosides. The increasing use of newer cephalosporins may select for novel types of plasmid-mediated cephalosporinases.

5.2 THE ROLE OF PLASMID TYPING

Plasmid profiles often offer a high level of sensitivity in distinguishing between strains with specific antimicrobial resistance patterns. The same resistance pattern can however be encoded by unrelated plasmids (O'Brien *et al*., 1982), and even the same antimicrobial modification enzymatic activity has shown to be encoded by two different DNA sequences (Lee *et al*., 1987).

Plasmid typing for epidemiological purposes involves characterization of carried plasmids in terms of the numbers per strain and their molecular weights (Mayer, 1988). The resultant plasmid profile can be used for strain differentiation within serovars and within phage types. It is necessary to determine the plasmid banding patterns in isolates with the intention of determining whether single, similar, or specific plasmid(s) were prevalent among isolates expressing similar β-lactamases or carrying similar β-lactamase genes.

Previous studies have found plasmid profiling to be of use in intraserotype differentiation (Crichton *et al*., 1996; Schmidt *et al*., 1982; Threllfall *et al*., 1986) and has been successfully applied in the analysis of outbreaks of nosocomial infections (Schaberg *et al*., 1981), although many of these were performed prior to the widespread

implementation of the more advanced methods of PFGE, ribotyping and variable number tandem repeat (VNTR)-based genotyping.

Virulence determinants in enteric bacteria that are responsible for pathogenicity are often encoded by large plasmids in low-copy numbers that have a 7.8kb region known as the *Salmonella* plasmid virulence region (*spv*). Virulence plasmids of 50kb to 285kb in size, called serovars-specific plasmids, have been found in only a few serovars of *S. enterica* subspecies *enterica* (Guiney *et al*., 1994; Chu *et al*., 1999). The frequency of virulence plasmids in bacteraemic infections (isolates from blood) and extra-intestinal infections e.g. arthritis, meningitis (isolates from joints, cerebrospinal fluid) when compared to those of diarrhoeal diseases (isolated from faeces) has been variably reported as 76% versus 42% and 98% versus 68% (Fierer *et al*., 1992; Montenegro *et al*., 1991). The presence of these virulence plasmids in host-adapted serovars suggests that virulence plasmid acquisition may have expanded the host range of *Salmonella*.

Multidrug-resistance, also mediated by large plasmids carrying resistance genes, has been described by Guerra *et al*., in 2001. Plasmids encoding drug resistance have been isolated from salmonella strains and range in size from less than one megaDalton (MDa) to 180MDa (271.8kb) (Ou *et al*., 1990). The isolation of small and large plasmids has been successfully achieved by alkaline lysis, followed by an optional step of protein extraction with phenol: chloroform (Kado and Liu, 1981; Yuan *et al*., 1998). Plasmid extraction methods suitable for the analysis of a large number of salmonellae strains are based on the resistance of covalently closed plasmid DNA to degradation at high temperatures, or in solutions of high alkalinity (Birnboim and Doly, 1976; Kado and Liu, 1981; Sambrook *et al*., 1989).

In this chapter, plasmid profiles of 50 *S*. Isangi isolates will be analyzed. A plasmid profile typing system based on plasmid size and pattern was defined and plasmid profiles of isolates compared with their respective pulsed-field gel electrophoresis pattern, as described in detail in Chapter 6.

5.3 MATERIALS AND METHODS

5.3.1 Bacterial strains for plasmid typing

5.3.1.1 Control strain and molecular weight marker

- *Escherichia coli* strain 39R861 (NCTC 50192), carrying plasmids (RAI-1, X, Sa, NTP168) of sizes 148.5kb (A), 63.8kb (B), 36.2kb (C) and 7kb (D) (Leung *et al.,* 1997).
- Molecular weight standard with sizes 16.2kb, 14.2kb, 12.2kb, 10.1kb, 8.1kb, 7kb, 6kb, and 5kb (supercoiled DNA ladder D5292, Sigma).

5.3.1.2 Test isolates

Due to financial and time constraints plasmid profiling was performed on only 50 *S.* Isangi isolates. These were representative of the different hospitals of the study and each ESBL-type (TEM-63, TEM-131, SHV-5, and TEM + SHV) that was characterized in Chapter 4.

5.3.2 Preparation of cultures

The *S.* Isangi isolates were plated onto 5% blood agar plates (Diagnostic Media Products [DMP] Johannesburg, South Africa) and incubated overnight at 37°C. A single colony was inoculated into 5ml Luria-Bertani (LB) broth (Oxoid), containing 2µg/ml ceftazidime, and grown overnight with vigorous shaking (250rpm) at 37°C. The cells (1,5ml) were centrifuged at 10,000rpm for 5min and the supernatant discarded. The bacterial pellet was stored at -20°C or subjected for further testing.

5.3.3 Isolation of plasmid DNA

Total plasmid DNA was extracted using the plasmid DNA mini-preparation method with modifications by Kotchoni *et al.,* (2003). Bacteria were lysed under alkaline conditions, and the lysate was subsequently neutralized and adjusted to high-salt

binding conditions. The entire plasmid isolation procedure was performed at room temperature which was a crucial parameter for the isolation of the large plasmids in this study. A short description of the method follows:

i) The bacterial pellet from 5.3.2 was resuspended in 200µl of Solution I (Appendix Two) and incubated at room temperature for 5min.
ii) 400µl of freshly prepared Solution II (Appendix Two) was added and mixed well by inverting gently 4 to 6 times. Do not vortex.
iii) 200µl of solution III (Appendix Two) was then added and mixed gently (either by pipetting up and down or inverting swiftly) and incubated on ice for 5min.
iv) The mixture was centrifuged at 10000g for 5min at room temperature and the supernatant carefully transferred into new Eppendorf tubes.
v) 600µl isopropanol (i.e. 0.6ml isopropanol for 1ml of supernatant from step iv) was added and mixed by inverting four to six times before being incubated at room temperature for 10min.
vi) Centrifuging, as described in step iv) was repeated and the supernatant discarded.
vii) The pellet was washed with 400µl of 70% (v/v) ethanol (Merck Chemicals, Wadeville, South Africa) and centrifuged at 10000g for 3min at room temperature.
viii) The supernatant was removed and the pellet air-dried (~10-20min).
ix) The pellet was resuspended in 30µl to 50µl of 10mM Tris-HCL (pH 8.0) or sterile distilled water.

5.3.4 Detection of plasmid DNA

The plasmid extract (15µl) was mixed with 3µl loading buffer and resolved through a 0.6% (w/v) agarose gel. Electrophoresis was performed at 70V for four hours in 1 x TBE buffer. The gel was stained for 20-30min with ethidium bromide by diluting 40µl of ethidium bromide stock solution (10mg/ml) with 400ml of reagent grade water, followed by destaining in 400ml reagent grade water for 60-90min.

A supercoiled DNA ladder (Sigma-Aldrich, Co, South Africa) and the plasmid-containing strain *Escherichia coli* NCTC 50192 were used to estimate plasmid sizes. The image was captured on the Gel Doc 1000 documentation system (Bio-Rad Laboratories, California, USA) and saved as an uncompressed TIFF image (*.tif). Plasmid sizes were determined using the Genetools v3.06 software program (SynGene, Cambridge, England).

5.3.5 Designation of plasmid profiles

The nomenclature used for plasmid profiling is based on the number and size of the plasmids present in a strain. Traditionally capital letters have been used for the designation of respective plasmid profiles (Threlfall *et al*., 1995) but various authors have used different designations e.g. capital letters followed by numbers (Borrego *et al*., 1992), numbers only (Rodrique *et al*., 1992) or roman figures (Morris *et al*., 1992). For the purpose of this study, plasmids were designated to one of the two plasmid profile groups (A or B). Strains that contained only large plasmids (105kb to 166kb) were assigned to profile A and different combinations of plasmid sizes were designated A1-A7. Profile B represented isolates that contained smaller plasmids (6kb to 44kb) in addition to the larger plasmids and plasmid types within category B were designated B1-B7.

5.4 RESULTS

5.4.1 Isolation of large plasmids carried by *Salmonella* Isangi

The method commonly used in research laboratories for the isolation of plasmids employs a cold room method (-4°C) (Birnboim and Doily, 1979; Kado and Liu, 1981; Sambrook *et al.*, 1989) that performed poorly for the isolation of large plasmids of *S.* Isangi isolates in this study. A recent method by Kotchoni *et al*, (2003), which was designed for plasmid isolation in tropical climates (28°C), proved to be considerably better for large plasmid isolation from *S.* Isangi (Figure 5.1). Figure 5.1 shows the results of the cold room method versus that of Kotchoni *et al*., (2003) while the

different types of β-lactamases isolated in this study is represented simultaneously on the agarose gel. The plasmid sizes as well as the hospital where the particular ESBL-carrying *S.* Isangi was isolated are shown in Table 5.1.

Isolates of plasmid profile A1 (lane 11), A3, A5 and B7 all belonged to different PFGE clusters (the two cluster B isolates showed 90% similarity between them and 88.2% similarity with sub-cluster B5). The isolate represented in lane 10 was not typed by PFGE and PFGE fingerprint similarity with the isolate in lane 11 could therefore not be established.

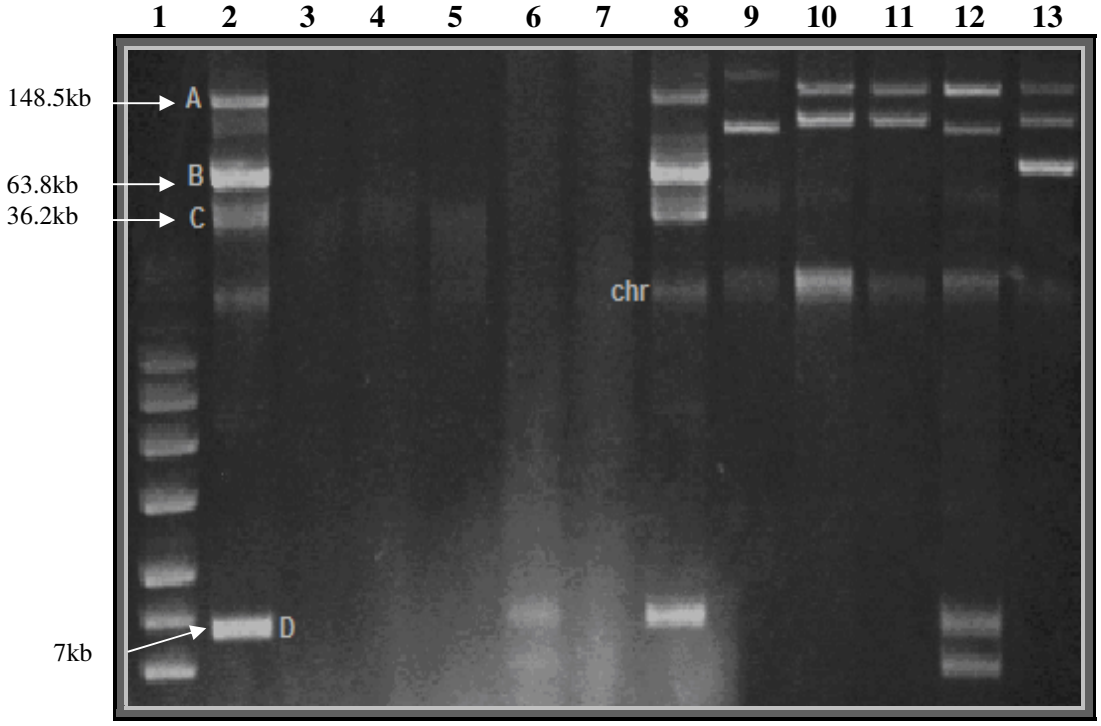


Figure 5.1 Plasmid banding patterns of isolates expressing different β-lactamases. Lanes 1, supercoiled molecular weight marker; 2 and 8, *E. coli* NCTC 50192 (A-D); 3 to 7, plasmids isolated with the cold room method (-4°C); 9 to 13, same isolates as in lanes 3-7, isolated at room temperature (28°C).

Table 5.1 Summary of plasmids isolated from *S.* Isangi in Figure 5.1 (For PFGE typing see Chapter 6)

Lane	Plasmid size (kb)	Plasmid profile	Hospital	Isolate number	ESBL gene	PFGE cluster
9	195, 105	A5	NJH	14340	TEM-1	B
10	166, 116	A1	CHB	16527	TEM-63	Not typed
11	166, 116	A1	GRNPT	16390	TEM-131	B
12	166, 105, 7.4, 6	B7	NJH	16970	TEM-63, SHV-5	A
13	166, 116, 67	A3	NJH	16490	TEM-131, SHV-5	B5

NJH, New Johannesburg Hospital; CHB, Chris Hani Baragwanath Hospital; GRNPT, Greenpoint Hospital

5.4.2 Correlation between plasmid profile, β-lactamase and PFGE types

Possible correlation between the plasmid banding patterns on the electropherograms and the expression of specific β-lactamases, or, the carriage of specific β-lactamase genes was determined. Figure 5.2 shows the plasmid banding patterns of *S.* Isangi isolates from two hospitals, Helen Joseph (HJ) and Tembisa (TEMB) hospitals. All eight of these isolates were found to express TEM-63, but showed two different plasmid profiles, unique to each hospital. Of the total of 50 *S.* Isangi isolates (from all hospitals), all but seven harboured a 166kb plasmid which was thought to be the virulence plasmid. Twelve different plasmid profiles were detected among 50 *S.* Isangi isolates from all hospitals. Similar plasmid banding patterns could not be inferred to carry identical β-lactamase genes.

The plasmid profile types from TEMB showed 100% correlation with PFGE typing while two of the four isolates from HJ corresponded to sub-cluster B5 at 100% similarity. The other two HJ isolates also belonged to the major cluster B at similarities of >95%.

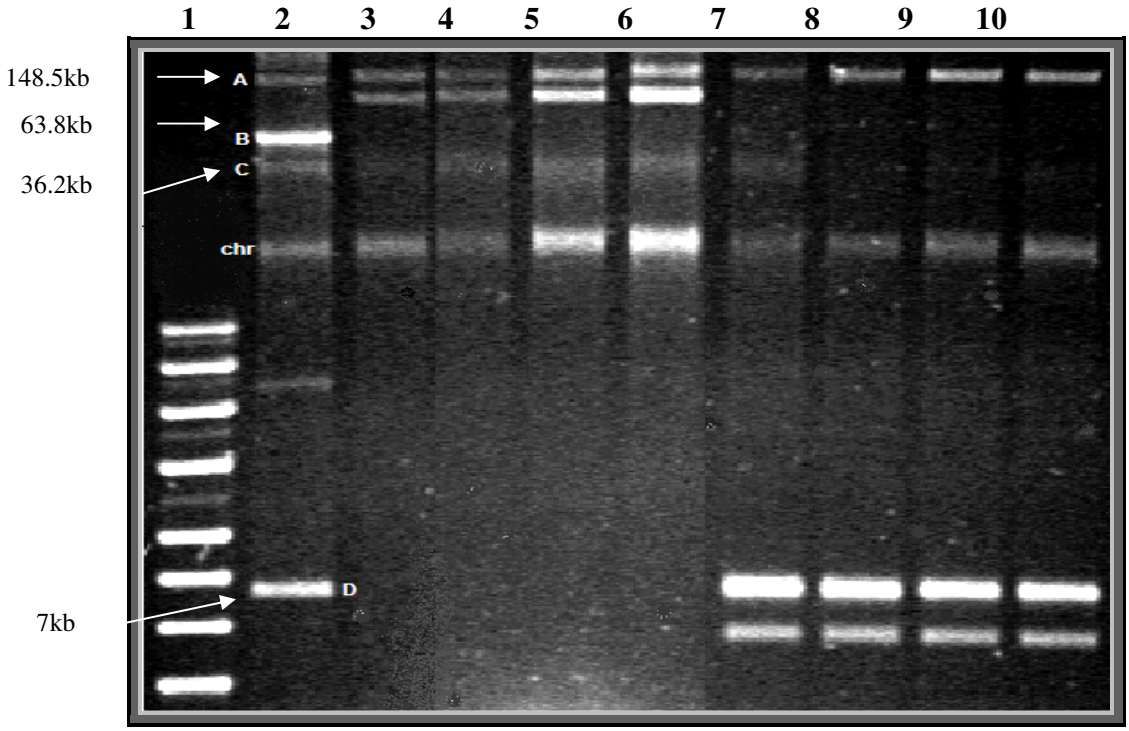


Figure 5.2 Plasmid banding patterns of isolates from Helen Joseph (Lanes 3 to 6) and Tembisa hospitals (Lanes 7 to 10) (see Table 5.2). Lane 1, supercoiled molecular weight marker (Sigma-Aldrich, Co, South Africa); Lane 2, *E. coli* NCTC 50192. A = 148.5kb plasmid; B = 63.8kb plasmid; C = 36.2kb plasmid; D = 7kb plasmid; chr = chromosomal DNA

Table 5.2 Summary of plasmids isolated from *S*. Isangi in Figure 5.2 (For PFGE typing see Chapter 6)

Lane	Plasmid size (kb)	Plasmid profile	Hospital	ESBL gene	PFGE cluster
3-6	166, 116	A1	HJ	TEM-63	B [a]
7-10	166, 7.4, 6	B1	TEMB	TEM-63	C [b]

[a] Two isolates at 100% similarity in sub-cluster B5 and 2 in major cluster B with 95.5% and 97.3% similarity respectively compared to sub-cluster B5
[b] Four isolates in cluster C at 100% similarity
HJ, Helen Joseph Hospital, TEMB, Tembisa Hospital

5.4.3 Plasmid profile and PFGE data from *S.* Isangi isolated from different hospitals

S. Isangi isolates from nine different hospitals resulted in diverse plasmid banding patterns (Figure 5.3 and Table 5.3). The plasmid banding patterns seemed to be identical in certain institutions and diverse in others and was not serotype, hospital or

ESBL-type specific. Banding patterns from Milpark, Pathcare and Observatory showed identical plasmid profiles which were interesting as they are from two different provinces (Gauteng and the Western Cape). The same could be seen with the isolates from South Rand and Tsepong (Gauteng and North West), although these provinces are geographically not as distant from each other as Gauteng and the Western Cape.

PFGE-based typing showed moderate correlation with the findings of plasmid profiling. The two isolates of plasmid profile A4 showed 90% similarity between their PFGE patterns, while three plasmid profile B1 isolates showed 96.6% similarity on PFGE typing (major cluster A), two being identical (A3 sub-cluster). The isolates in lanes 8, 9 and 12 (Figure 5.3) belonged to plasmid profile B1 with two of these being members of PFGE sub-cluster A3 (100% similarity). The isolate in PFGE sub-cluster A3 in lane 11, however has a plasmid profile type B3.

The plasmid banding patterns within each hospital could not infer a plasmid profile unique to the institution (Figure 5.4 and Table 5.4). Isolates (12) from Tambo Memorial Hospital showed seven different plasmid profiles, consisting of profile type A and B, while six isolates were grouped into profile type B (four B1 and two B5). All six isolates from profiles B1 and B5 expressed TEM-63 β-lactamases. The two isolates which carried the TEM-131 β-lactamase had different plasmid profiles (A1 and A2), although they were from the same plasmid profile type.

Of the 12 isolates typed by plasmid profiling and PFGE from TAMBO, eight shared a plasmid profile type (four within B1 and two each within A1 and A5). Of the four plasmid-profile identical isolates, three were identical on PFGE typing (sub-cluster A2) while the fourth (sub-cluster A4) showed a 92.7% similarity with the other three isolates. The two A1 and two B5 isolates typed in PFGE cluster B and A respectively and showed PFGE similarities of 92.7% and 90.5%. The isolates with A2, A6, A7 and B6 plasmid profiles all belonged to the major PFGE cluster B and showed similarities within a range of 70.1% to 94.2% between the four isolates.

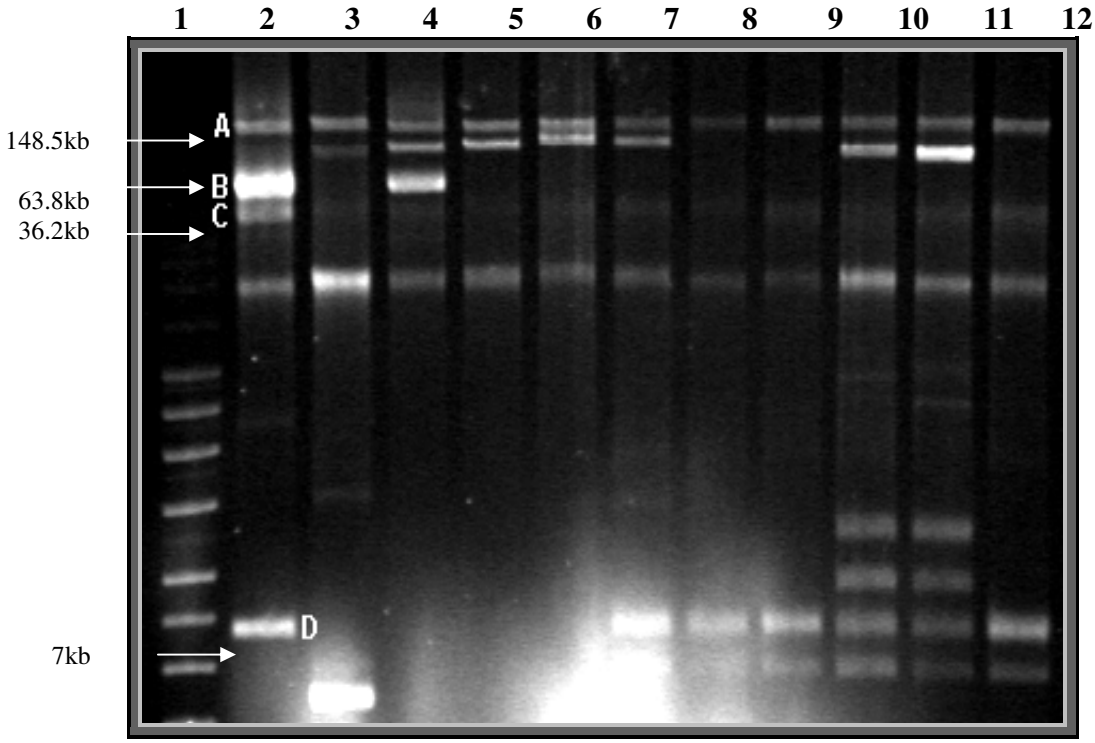


Figure 5.3 Plasmid banding patterns of isolates from ten hospitals. Lanes 1, supercoiled molecular weight marker; 2, *E. coli* NCTC 50192 (A-D); 3-12 (see Table 5.3)

Table 5.3 Summary of plasmids isolated from *S.* Isangi in Figure 5.3 (For PFGE typing see Chapter 6)

Lane	Plasmid size (kb)	Plasmid profile	Isolate number	Hospital	ESBL	PFGE cluster
3	166, 93, 5	B4	2186	CARL	TEM-63	A4
4	148, 106, 67	A3	2456	RUS	TEM-63	B
5	156, 120	A4	7343	SOU	TEM-63	B1[a]
6	156, 125	A4	11450	TSE	TEM-63	B[a]
7	156, 120, 7.4, 6	B2	12751	GRAHAM	TEM-63	A
8	166, 7.4, 6	B1	15193	PC	TEM-63	A3[b]
9	166, 7.4, 6	B1	15714	OBSERV	TEM-63	A[b]
10	166, 120, 11, 9, 7.4, 6	B3	16079	RUS	TEM-63	A2[c]
11	166, 116, 11, 9, 7.4, 6	B3	15972	SOL	TEM-63	A3[c]
12	166, 7.4, 6	B1	16121	MILP	TEM-63	A3[c]

[a] Isolates showed 90% similarity between them; [b] Isolates showed 96% similarity between them; [c] Isolates A2 and A3 showed 92.7% similarity and two A3 isolates 100% similarity on PFGE typing; CARL, Carletonville; RUS, Rustenburg; SOU, South Rand; TSE, Tsepong; GRAHAM, Grahamstown; PC, Pathcare; OBSERV, Observatory; SOL, Soldin le Roux; MILP, Milpark.

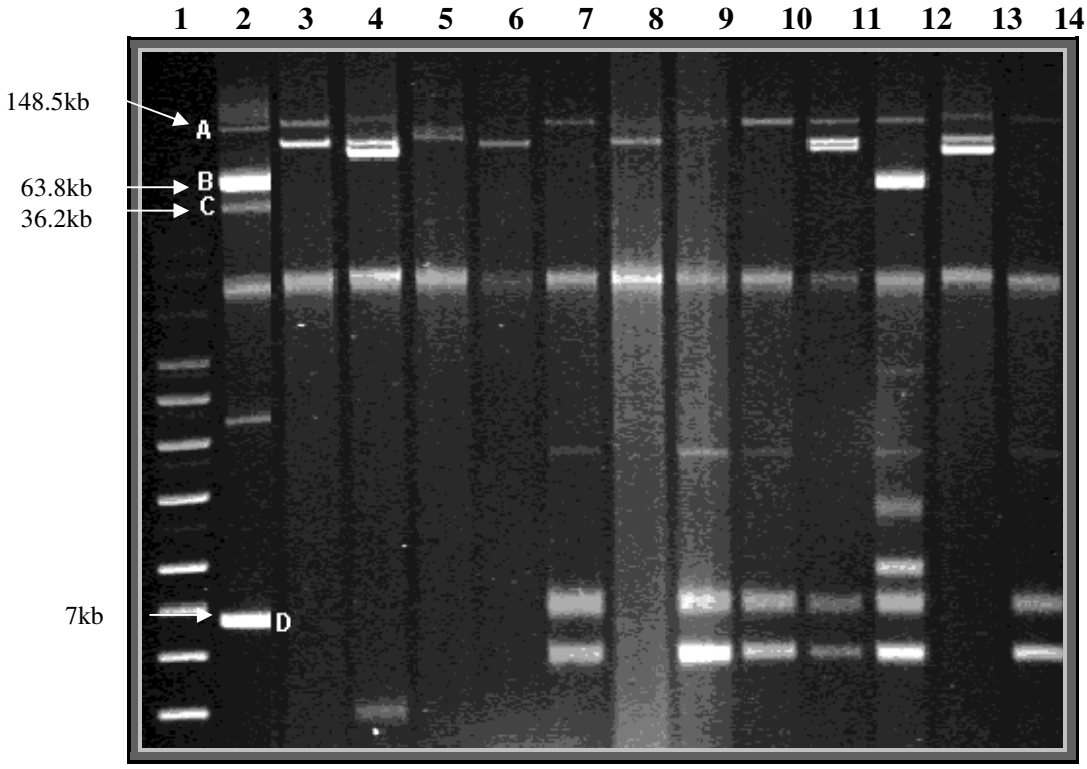


Figure 5.4 Plasmid banding patterns of isolates from Tambo Memorial hospital. Lanes 1, supercoiled molecular weight marker; 2, *E. coli* NCTC 50192 (A-D); lanes 3-14 (see Table 5.4)

Table 5.4 Summary of plasmids isolated from *S.* Isangi in Figure 5.4 (For PFGE typing see Chapter 6)

Lane	Plasmid size (kb)	Plasmid profile	Isolate number	Hospital	ESBL	PFGE cluster
3	166, 116	A1	6784	TAMBO	TEM-63	B [a]
4	166, 116, 110, 5.4	B6	6902	TAMBO	TEM-63	B [a]
5	148, 130	A6	8605	TAMBO	TEM-63	B [b]
6	116	A7	11146	TAMBO	TEM-63	B [b]
7	166, 7.4, 6	B1	13064	TAMBO	TEM-63	A4 [c]
8	166, 116	A1	14798	TAMBO	TEM-131	B [b]
9	166, 7.4, 6	B1	15255	TAMBO	TEM-63	A2 [d]
10	166, 7.4, 6	B1	16161	TAMBO	TEM-63	A2 [d]
11	166, 116, 110, 7.4, 6	B5	16602	TAMBO	TEM-63	A [e]
12	166, 67, 11, 9, 7.4, 6	B5	17776	TAMBO	TEM-63	A2 [d]
13	166, 116, 110	A2	18305	TAMBO	TEM-131	B [b]
14	166, 7.4, 6	B1	18629	TAMBO	TEM-63	A2 [d]

TAMBO, Tambo Memorial Hospital; [a] Isolates are 97% similar on PFGE
[b] These isolates in Cluster B showed 94.2% similarity
[c] Sub-cluster A4 shared 92% similarity with sub-cluster A2 (these 2 isolates belonged to plasmid profile B1)
[d] These plasmid profile B1 isolates showed 100% similarity, all belonging to PFGE sub-cluster A2
[e] This isolate showed 93.2% similarity with sub-cluster A2

The six ESBL-negative isolates did not show evidence of the large plasmid/s and had different plasmid profiles when compared to the ESBL-positive isolates (Figure 5.5).

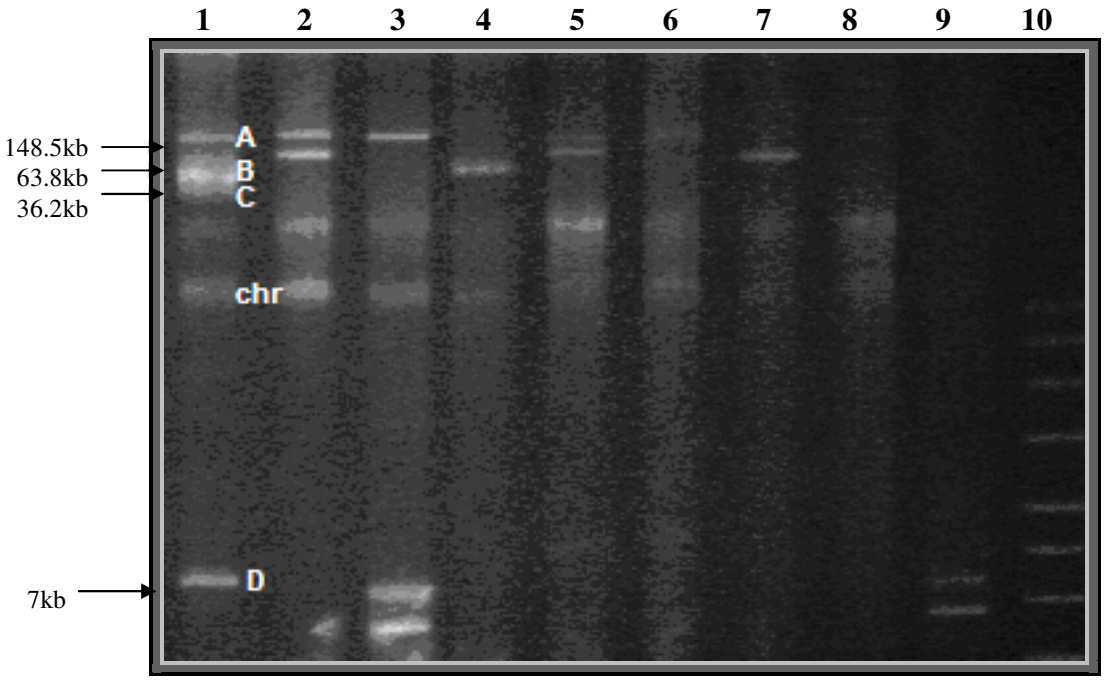


Figure 5.5 Plasmid banding patterns of the six ESBL-negative isolates (bands not clearly visible). Lane 1 (A-D), *E. coli* NCTC 50192; Lanes 2 and 3, TEM-63; Lanes 4-9 (Table 5.5); Lane 10, supercoiled molecular weight marker.

5.5 DISCUSSION

Strain typing is epidemiologically important as it greatly facilitates the identification of outbreaks. Plasmid profiling allows the differentiation between the spread of resistant plasmids and strain dissemination (Gori *et al*., 1996). Plasmid profiles often offer a higher level of sensitivity in distinguishing between strains with similar antibiotic resistance patterns. It also aids in determining whether the resistant isolates are due to the dissemination of former epidemic strains or the introduction of different strains (Branger *et al*., 1998). The presence of ESBL-encoding genes on transposons allows unrestrained transfer of enzymes, as genes may disseminate between plasmids and chromosomes within and between isolates, strains and species (Bush, 1997). The same antibiotic resistance pattern can be encoded by unrelated plasmids (O'Brien *et al*., 1982).

Twelve distinct plasmid profile types were observed among the 50 *S.* Isangi isolates tested, with one to six plasmids present per isolate. Plasmid banding patterns, even in isolates expressing identical beta-lactamases, were diverse while identical plasmid profiles were found in some institutions (Figure 5.1) and diverse profiles were present in others (Figure 5.4) and were ESBL-type specific. Plasmids determining extended-spectrum enzymes or carrying multiple antibiotic resistance genes are relatively large and range in size from 50kb to 300kb (Jacoby and Sutton, 1991; Hadfield, *et al*., 1985; Tacket *et al*., 1985). Plasmids carried by all, except 7 of the *S.* Isangi isolates in the present study were approximately 166kb in size. Although not specifically investigated, resistance in *S*. Isangi strains, isolated in this study, is likely to be due to resistance genes that are carried on the larger plasmids, as previously described in non-typhoidal *Salmonella* in Kenya (Kariuki *et al*., 2005).

The conventional plasmid isolation method, performed at -4°C, that was also used in the present study, proved to be more successful with the quality control strain *E. coli* NCTC 50192 than with the *S*. Isangi isolates. It is possible that the large plasmids from *S*. Isangi were more likely to lose their integrity during the isolation process. This may be due to entrapment of the large plasmids in cell debris or that they were more prone to mechanical shearing. Instability and loss of large plasmids on sub-culture is well recognized (Borrego *et al*., 1992). It is not clear why the method permitting performance at 28°C proved to be more successful in demonstrating large plasmids in *S.* Isangi isolates.

Antibiotic resistance typing can be used in conjunction with serotyping, biotyping, phage typing and genetic characterization of resistance plasmids for epidemiological purposes. Antibiotic resistance patterns by itself cannot be regarded as an acceptable primary method for discrimination within serovars as a result of the fluidity of resistance plasmids and transposons. Plasmid profiling is most useful for epidemiological studies that are limited temporally and geographically.

The relationship between plasmid profile types and PFGE typing is complex. The finding of plasmid profile types in different PFGE clusters e.g. plasmid profile A1 was represented in PFGE type D (Table 5.1), B5 (4 isolates in Table 5.2) and in two distantly-related (90.1% similarity) PFGE cluster B isolates (Table 5.3) provides evidence of genetic instability of *S*. Isangi clones which may affect both plasmid profiling and PFGE typing. A major factor in this regard would be the mobility of plasmids through horizontal conjugative spread.

In the case of the 34 isolates featuring in settings described in Tables 5.1-5.4 and Figures 5.1-5.4, 11 plasmid profile types (32.4%) showed 100% correlation with PFGE types. Plasmid profile types belonging to the A series (A1-A7) correlated 93.8% with major PFGE cluster B (15/16) while 14 out of 18 plasmid profile types of the B series (B1-B7) belonged to the major PFGE cluster A. No obvious explanation for this finding can be offered except to point out that the plasmid profile A series harbours large plasmids and PFGE cluster B featured prominently in 2000 with very few isolates belonging to cluster A, while the latter cluster predominated in 2002 and correlated with the plasmid profile B series containing both small and large plasmids.

A comprehensive comparison between plasmid profiling and PFGE typing was not attempted in this study as only 50 isolates were processed for plasmid profiling. It is clear however that plasmid profiling could add to the discrimination potential of PFGE typing and would be useful in specific temporal-related settings. This was found to be the case by Malorny *et al*., (2001) in a study involving *S*. Typhimurium (DT104) isolates. The authors found that combining plasmid profile typing with PFGE macro-restriction typing increases discrimination between isolates. Such an approach is however cumbersome and is not widely practiced.

Chapter 6

STRAIN DIFFERENTIATION OF *Salmonella* Isangi BY PULSED-FIELD GEL ELECTROPHORESIS (PFGE)

6.1 INTRODUCTION

Detailed strain identification using modern technology provides a powerful tool for, and adds a new dimension to, the successful epidemiological investigation of *Salmonella enterica* outbreaks. A number of molecular typing methods have been employed to improve the differentiation of salmonella strains below the level of serotypes. Molecular typing enables the tracking of the dissemination of specific clones and thus facilitates understanding endemic contamination to the level of micro-epidemics (Blanc, 2004).

Although modern molecular typing methods were used in the present study, a limitation was incomplete epidemiologically-relevant information available to the laboratory, especially relating to the time when clinical samples were collected for bacteriological investigation. Such information could have assisted in the assessment of whether transmission was likely to have been nosocomially or community acquired. Wadula *et al*., (2006) however, based on clinical and epidemiological information on isolates from CHB characterized in the present study, postulated extensive hospital transmission of *S*. Isangi during this period. Transmission patterns as evidenced by PFGE-based cluster formation will be considered to be predominantly hospital-associated without reference to nosocomial or community acquired origins of the isolates.

6.2 METHODS FOR STRAIN TYPING

DNA-related techniques for the differentiation of strains include plasmid profiling (Weill *et al*., 2004a, 2004b; Liebana *et al*., 2001b, 2004), ribotyping (Esteban *et al*., 1993; Guerra *et al*., 1997), IS*200* fingerprinting (Ezquerra *et al*., 1993; Beuzon *et al*., 2004), PCR ribotyping (Lagatolla *et al*., 1996), amplified fragment length

polymorphism (AFLP) (Aarts *et al*., 1998; Nair *et al*., 2000), random amplified polymorphic DNA (RAPD) analysis (Shangkuan and Lin, 1998) and pulsed-field gel electrophoresis (PFGE) (Lukinmaa *et al*., 2004; Mhand *et al*., 1999; Winokur, 2003; Thong *et al*., 1998).

Multilocus sequence typing (MLST) is a relatively new high-resolution typing system developed for evolutionary and epidemiological studies (Kotetishvili *et al*., 2002). It involves the nucleotide sequencing of approximately 400bp regions of at least seven genes (usually housekeeping genes). MLST is a tool for studying evolution and global epidemiology of the salmonellae and has been used in conjunction with PFGE and antimicrobial susceptibility typing for *Salmonella enterica* serotype Newport isolates from humans, food animals and retail foods in the United States (Harbottle *et al*., 2006). MLST of *Salmonella* housekeeping genes provide a satisfactory level of discrimination for diverse isolates of *Salmonella*, but may not be suitable for closely related isolates within a serotype, due to sequence identity of their housekeeping genes.

A more recent typing method, based on the number of repetitive sequences found in particular regions (loci) of the genome, have been developed for several bacterial species including serotypes of *Salmonella enterica* (Ramisse *et al*., 2004; Lindstedt *et al*., 2004; 2005). These regions are referred to as variable-number tandem repeat sequence (VNTR) loci and the typing method is referred to as VNTR-based typing or more specifically multi-locus variable nucleotide tandem repeat analysis (MLVA).

In a study involving 106 isolates of *S*. Typhimurium from the Norwegian Institute of Public Health, MLVA produced clusters linking isolates from birds with those of humans and in different clusters linking isolates from humans, to pigs and hedgehogs. The authors found the methods to be fast, robust and easy to use (Lindstedt *et al*., 2004).

6.3 TYPING BY PULSED-FIELD GEL ELECTROPHORESIS

Although novel typing methods, such as MLVA and MLST, have come to the fore and have important niches in the typing of microorganisms, PFGE is still considered the 'gold standard' of molecular typing methods for several bacterial species (Struelens *et al*., 1998a, 1998b; Tenover *et al*., 1995). The method was first described in 1984 as a tool for examining the chromosomal DNA of eukaryotic organisms (Schwartz and Cantor, 1984). It involves the use of rare-cutter (e.g. *Xba*I, *Spe*I, *Not*I) restriction enzymes to generate and compare a limited number (10 to 20) of high-molecular-weight (10 to 800 kilo bases) DNA restriction fragments. PFGE has been widely used for *Salmonella* DNA fingerprinting (Bender *et al*., 2001; Lukinmaa *et al*., 1999; Lyytikainen *et al*., 2000; Valdezate *et al*., 2000) and is a crucial tool, comparable or superior to other techniques, for public health surveillance and disease prevention efforts for *Salmonella* species (Fakhr *et al*., 2005; Tamada *et al*., 2001).

PFGE analysis of genomic macro-restriction patterns is a discriminatory strain typing technique and has allowed the delineation of clonal diversity (Gori *et al*., 1996). PFGE is also a suitable reference method for the typing most nosocomial bacterial pathogens. One of the factors that have limited the use of PFGE is the time involved in completing the analysis. While the procedural steps are straightforward, the time needed to complete the procedure can be 2 to 3 days. This can reduce the laboratory's ability to analyze large numbers of samples timeously in outbreak situations. PFGE employs >80% of the chromosome to produce banding patterns, making it one of the most discriminating methods available today. Computerized gel analysis allows the comparison of band profiles presented in different gels and the construction of databases that could be useful in the development of library typing systems of microorganisms (Struelens *et al*., 1998b). Tenover *et al*., (1995) have proposed a system for standardizing the interpretation of PFGE patterns in relation to determining strain relatedness.

PFGE has become a standard technique among public health agencies due to its discriminatory power and reproducibility between different laboratories. The PulseNet 'One-Day (24-28h) Standardized Laboratory Protocol for Molecular Sub-typing of *Escherichia coli* 0157:H7 by Pulsed Field Gel Electrophoresis (PFGE)' was used to analyze strain relatedness. The protocol was adjusted for *Salmonella* species (Swaminathan *et al*, 2001; Gautom, 1997).

6.4 MATERIALS AND METHODS

6.4.1 Bacterial strains for PFGE

PFGE was performed on 265 isolates. Of these, one culture was recovered from poultry in KZN while all others, including five of the six antimicrobial-susceptible strains, were clinical isolates from patients entered into the study. Fourteen of these isolates became either contaminated or non-viable during the freezing/thawing/storage/reculturing stages of performing this method. Only one isolate per infection-episode was entered into the study. Of the total number of *S.* Isangi cultures received at EDRU, the numbers of isolates entered into the study by hospital and province, between 2000 and 2002 are listed in Table 6.1. No *S.* Isangi isolates were received from Mpumalanga, Free State, Northern Cape or KwaZulu Natal provinces. One of the ESBL-negative isolates is not shown in the dendrograms (18909 from GRNPT) as it was non-viable on repeated sub-culture at the time of performing PFGE.

6.4.2 Preparation of plugs from agar cultures

Overnight cultures of isolates were homogenized in 2ml of cell suspension buffer (CSB) (100mM Tris, 100mM EDTA, pH8.0) and adjusted to a turbidity of 0.48-0.52 concentration using the Microscan Turbidity Meter (Dade, California, USA). Proteinase K (20µl of 20mg/ml stock) was added to 400µl of the adjusted cell suspension and mixed gently. Equal volumes (400µl) of 1% SeaKem Gold® (Cambrex Bio Science, Rockland, USA): 1% sodium dodecyl sulphate (SDS) agarose and

bacterial cell suspension were mixed and immediately dispensed into appropriate well(s) of reusable plug molds and allowed to solidify (~10mins).

Table 6.1 Clinical isolates of *S*. Isangi collected and tested by PFGE between 2000 and 2002 from five of the nine provinces in South Africa

Province	**Institution/Hospital**	**Total *S*. Isangi**	
		Collected	**PFGE tested**
Eastern Cape (EC)	Port Elizabeth Hospital (PE)	31	31
	Grahamstown Hospital (GRAHAM)	1	1
	Livingstone Hospital (LIV)	1[(1)]	1[(1)]
Gauteng (GA)	Carletonville Hospital (CARL)	1	1
	Helen Joseph Hospital (HJ)	7	7
	Tambo Memorial Hospital (TAMBO)	37[(1)]	37[(1)]
	New Johannesburg Hospital (NJH)	20	20
	Chris Hani Baragwanath Hospital (CHB)	83	79
	Kalafong Hospital (KAL)	1	1
	Milpark Hospital (MILP)	2	2
	South Rand Hospital (SOU)	1	1
	Tembisa Hospital (TEMB)	6	6
	Soldin le Roux private pathology practice (SOL)	1	1
Limpopo (LP)	Pietersburg Hospital (PIE)	11[(1)]	11[(1)]
North West (NW)	Tsepong Hospital (TSE)	1	1
	Rustenburg Hospital (RUS)	3	3
Western Cape (WC)	Green Point Hospital (GRNPT)	25[(2)]	21[(1)]
	Groote Schuur Hospital (GSH)	16	16
	Observatory Hospital (OBSERV)	1	1
	Pathcare private laboratory practice (PC)	1	1
	Red Cross Childrens Hospital (RED)	29[(1)]	22[(1)]
		279	**264**

[(1)], [(2)] Number of ESBL-negative isolates collected from the hospital.
PE, Port Elizabeth Hospital; GRAHAM, Grahamstown Hospital; LIV, Livingstone Hospital; CARL, Carletonville Hospital; HJ, Helen Joseph Hospital; TAMBO, Tambo Memorial Hospital; NJH, New Johannesburg Hospital; CHB, Chris Hani Baragwanath Hospital; KAL, Kalafong Hospital; MILP, Milpark Hospital; SOU, South Rand Hospital; TEMB, Tembisa Hospital; SOL, Soldin le Roux Private pathology practice; PIE, Pietersburg Hospital; TSE, Tsepong Hospital; RUS, Rustenburg Hospital; GRNPT, Green Point Hospital; GSH, Groote Schuur Hospital; OBSERV, Observatory Hospital; PC, Pathcare private laboratory practice; RED, Red Cross Childrens Hospital.; EC, Eastern Cape; GA, Gauteng; LP, Limpopo; NW, North West; WC, Western Cape

6.4.3 Lysis of cells in agarose plugs

The agarose plugs were removed from the moulds and transferred to an appropriately labeled tube containing 5ml cell lysis buffer (CLB) (50mM Tris, 50mM EDTA pH 8.0 and 1% sarcosyl) and 0.1mg/ml proteinase K (25µl of 20mg/ml stock). The tubes containing the agarose plugs were incubated overnight in a 54°C shaker water bath with constant agitation (175-200 rpm).

6.4.4 Washing of agarose plugs

The tubes were removed from the water bath and the lysis buffer poured off. The plugs were washed in sterile reagent grade water (preheated to 54°C). The water was decanted and followed by three washes with sterile TE (10mM Tris, 1mM EDTA pH 8.0) buffer at 50°C, alternating with three water washes.

6.4.5 Restriction digestion of DNA

The plug was removed from the TE buffer and thin (2-3mm) slices of the agarose plugs were cut and added to a new tube containing restriction enzyme buffer (H-buffer, Roche Diagnostics, GmbH, Mannheim, Germany). The plugs were incubated at 37°C in a water bath for 10min. The H-buffer was removed and 50U/sample *Xba*I (Roche Diagnostics GmbH, Mannheim, Germany) restriction enzyme added and incubated overnight at 37°C in a shaking water bath.

Methods and constituents for reagents and solutions are given in Appendix Three.

6.4.6 Casting, loading and running of agarose gel

The H-buffer was removed from the tubes and TBE (tris-borate EDTA) buffer was added to the plugs and incubated for 5min at room temperature. The plugs were removed from the TBE buffer, loaded onto the bottom of the comb teeth and allowed to air dry for 5-10min. DNA size standard, Lambda ladder (Bio-Rad Laboratories,

California, USA) was also included on all gels. The comb was positioned and 1% SeaKem Gold® agarose, prepared in 0.5M TBE buffer (100ml), was poured into the gel mould. The gel was allowed to set (± 1hour).

The gel was loaded into the electrophoresis chamber of a contour-clamped homogenous electric field (CHEF DR III) apparatus (Bio-Rad Laboratories) containing 0.5 x TBE buffer (± 2L) cooled to 14 ℃ and subjected to the following running conditions:

Initial A time 2.2s, Final A time 63.8s, Start ratio 1.0, Voltage 200V, Run time 18-20h (CHEF DR III) or 20-22h (CHEF DR II).

6.4.7 Data capturing and analysis

Once electrophoresis was completed, the gel was stained for 20-30min with ethidium bromide by diluting 40µl of ethidium bromide stock solution (10mg/ml) with 400ml of reagent grade water, followed by destaining in 400ml reagent grade water for 60-90min. The image was captured on the Gel Doc 1000 documentation system (Bio-Rad) and saved as an uncompressed TIFF image (*.tif) for analysis with the GelCompar™ v4.1 software program (Applied Maths, Kontrijk, Belgium) (Garaizer *et al*., 2000). A dendrogram of PFGE profiles was generated and similarity was determined using the Dice coefficient. Clustering was based on the unweighted pair group method using arithmetic averages (UPGMA) algorithm with an optimization of 1% and a band position tolerance of 1.5%. This method considers only the presence or absence of a band. A dendrogram was generated to examine relatedness of PFGE profiles for all 265 *S*. Isangi isolates (Figure 6.2).

Consensus guidelines for correlating variations in restriction profiles with epidemiological relatedness were interpreted according to the criteria published by Tenover *et al*., (1995; 1997). Clusters were defined at three similarity levels; isolates with 100% similarity, ≥97% similarity and ≥80% similarity on the dendrogram.

6.5 RESULTS

6.5.1 Analysis of pulsed-field gel electrophoresis patterns

A total number of 279 *S.* Isangi isolates were typed during the three-year study period (2000-2002). Of these isolates, 273 were from patients that had single infection episodes. Two patients experienced two episodes each (two isolates) while a third (two isolates) was likely to have had two infection episodes. PFGE of *Xba*I-digested chromosomal DNA from 265 isolates gave stable and reproducible patterns (Figure 6.1) and revealed a total of 103 different PFGE banding patterns (XPs), which grouped into four major clusters (A to D) and two minor clusters (1 and 2) with ≥80% pattern similarity (Figure 6.2). Clusters A to D are demonstrated in partial dendrograms (Figures 6.3 to 6.6) and the banding patterns of isolates were described at similarities of ≥97% and 100%. The red dotted lines indicate the 80% similarity coefficient mark and the blue lines the 97% similarity level. Of the 265 isolates analyzed, 20.4% (54/265) were collected during 2000, 13.6% (36/265) from 2001 and 66% (175/265) from 2002.

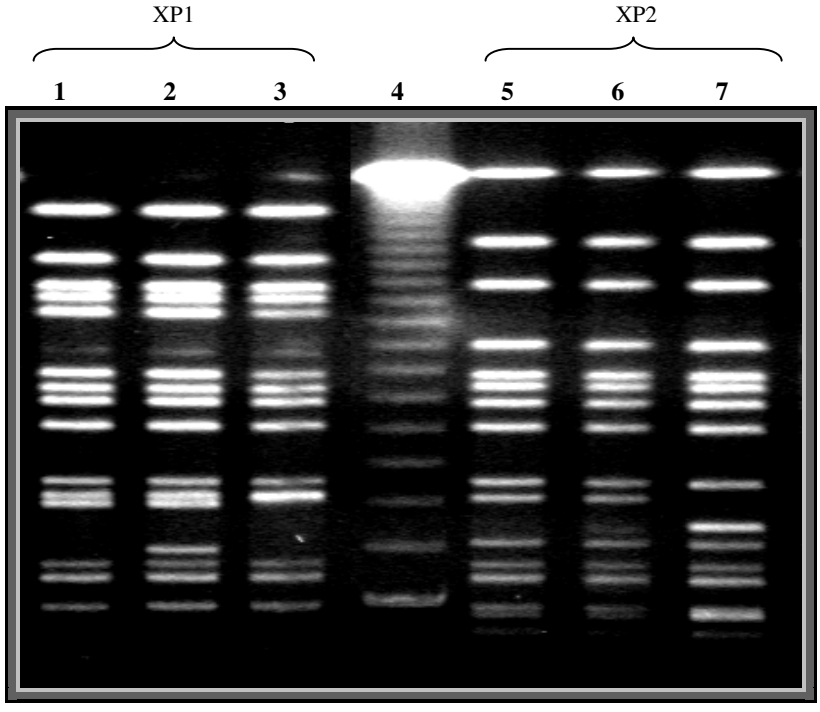


Figure 6.1 PFGE patterns of *Xba*I digested genomic DNA of *S.* Isangi depicting the 2 types of banding patterns (XP1 and XP2). Lanes 1-3, XP1; lane 4, Lambda DNA size standard (Bio-Rad); lanes 5-7, XP2, XP - PFGE banding pattern.

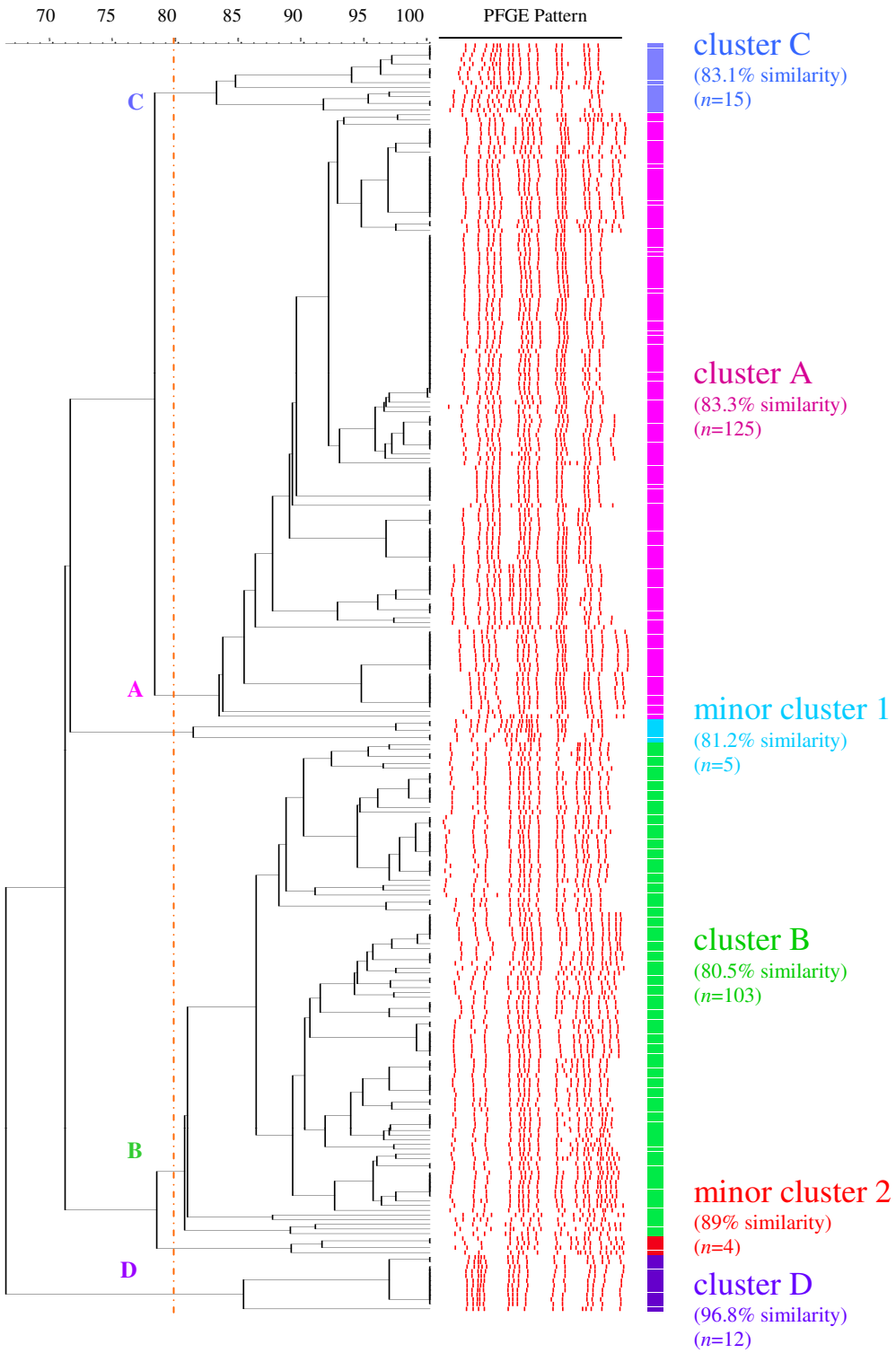


Figure 6.2 Dendrogram representing the four major clusters A to D, (shown in different colours) of the total (265) *S*. Isangi isolates produced by PFGE with *Xba*I restriction. The percentage in brackets is the similarity coefficient for isolates in the particular cluster and *n* the number of isolates in each cluster.

6.5.2 Clusters produced by PFGE with *Xba*I restriction

Major clusters were described at ≥80% and sub-clusters at two similarity levels: ≥97% similarity corresponding to less than three band differences between isolates within the sub-cluster that are considered as 'closely related' and 100% similarity that shows identical banding patterns between isolates (Tenover *et al*., 1995; 1997). The ≥97% similarity cut-off point correlated well with epidemiologically linked isolates at CHB and other hospitals (see Figures 6.3 to 6.6).

In order to compare transmission rates between clusters over time this index was calculated according to the formula used by Small *et al*., (1994) by dividing the total number of isolates in clusters, minus the number of clusters under consideration (to remove index cases from clusters), divided by the total number of typed isolates. As the submission of cultures was not actively controlled, reliable measurement of the extent and rates of transmission was not possible. However, based on the assumption that similar conditions were likely to apply to the groups under comparison, transmission rates were calculated to describe broad trends only. Because of the limitations of the approach, the term 'transmission rate' will be printed in the text in inverted commas, where appropriate.

Cluster A

This cluster of 125 isolates represents 47.5% of the 265 isolates tested (Figure 6.3). The largest PFGE pattern (XP) was identified in this cluster and contained isolates from seven hospitals in three provinces. Cluster A consisted of 31 different XPs that displayed ≥83.3% similarity. Of these isolates 46% (58/126) were from Gauteng, 39.7% (50/126) from the Western Cape, 12.7% (16/126) from Eastern Cape and 1.6% (2/126) from North West Province. On analysis of the temporal aspects of the cluster, the majority of isolates (92.9%; 117/126) were from 2002, 6.3% (8/126) from 2001 and one isolate from 2000. This cluster was represented in sixteen (76%) of the 21 hospitals that participated in the surveillance programme.

Using the ≥97% similarity cut-off similarity point, 116 of the 126 isolates in cluster A were divided into 12 sub-clusters. Isolates in these sub-clusters tended to group according to the hospital they were isolated from or hospitals that are located within a short distance of each other, indicating possible transmission of strains within and between these hospitals. Four sub-clusters at this similarity level stand out: The largest contained 32 isolates from six hospitals [RED (15); GRNPT (8); NJH (3); Milpark (2); CHB (1); PE (1)] and two private laboratories in GA (1) and WC (1) respectively; a 14-membered sub-cluster with isolates from TEMB (8); NJH (3); RUS (2) and GRNPT (1); and two sub-clusters in two different hospitals, CHB (9) and GSH (8). The first two isolates in the large 32-membered sub-cluster came from NJH in 2001 while all the other isolates in this cluster were isolated during 2002 (Figure 6.3).

Isolates in cluster A that showed 100% similarity are represented in 13 sub-clusters, nine of which consisted of ≥5 isolates (A1-A9) (Table 6.2 and Table 6.3). A few of the sub-clusters comprised large numbers of isolates from a single hospital. One isolate from Carletonville Hospital (2186) represented a patient with previous hospitalization at NJH and shared 100% similarity with isolates at NJH from the following year (2001).

Isolate number 3571 which was included for comparative reasons, was isolated from poultry in June 2001 from KwaZulu Natal and shared a relatively low 83.5% similarity with the rest of the isolates in cluster A, suggests poultry as a possible but not very likely source of *S*. Isangi in cluster A.

Of the 101 isolates that were characterized by ESBL gene probing by PCR, 47 fell within this cluster A. Of the 47 isolates, 46 carried TEM-63, while one carried both TEM-63 and SHV-5 (NJH, 16970). Although an isolate from Livingstone Hospital (6832) did not carry an ESBL, it showed 97% similarity with isolates from GRNPT. These two hospitals are geographically distant from each other, although from two neighbouring provinces.

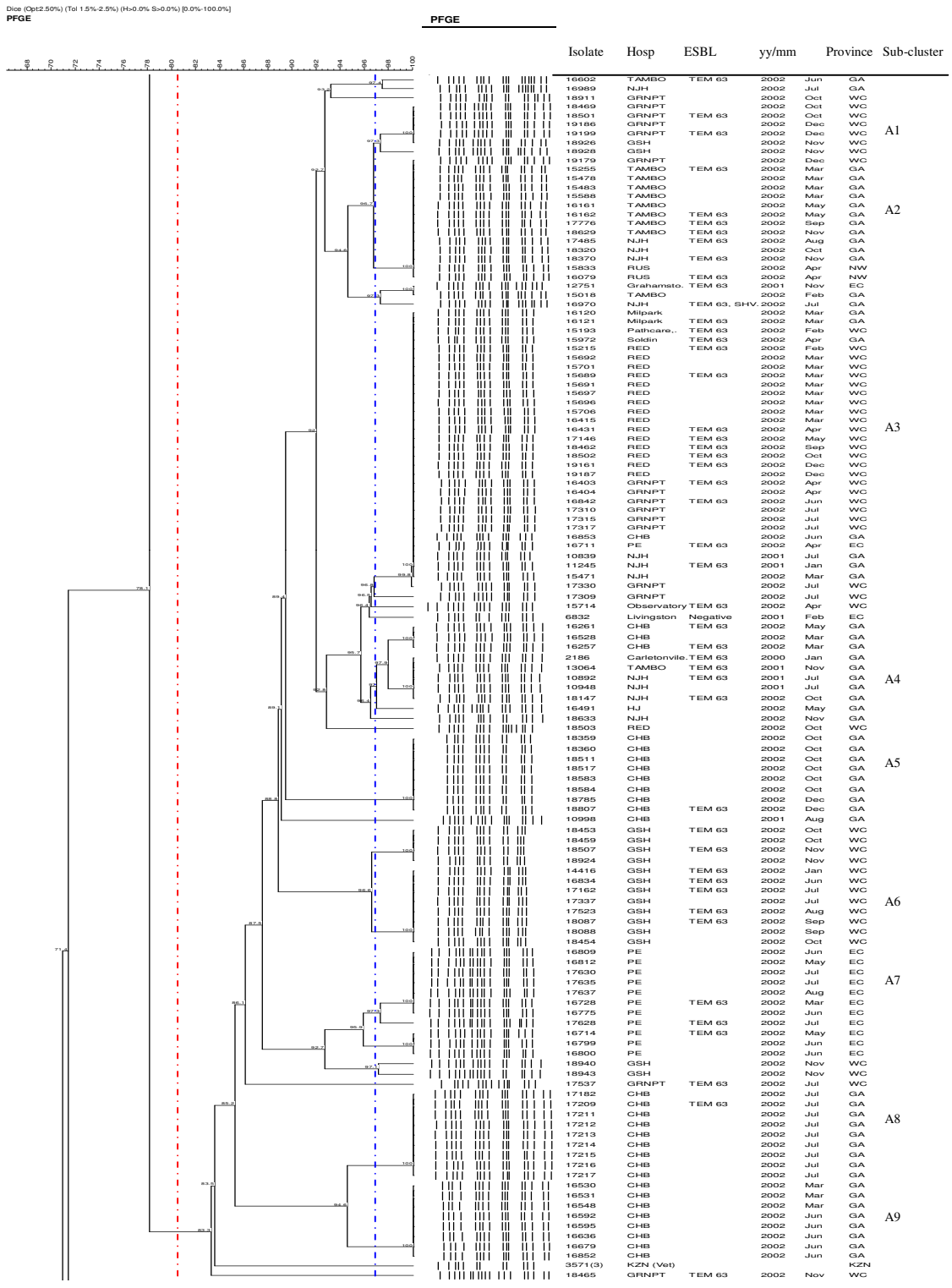


Figure 6.3 Partial dendrogram depicting cluster A (*n*=125) (pink colour in Figure 6.2). The red dotted line indicates an 80% similarity coefficient mark and the blue line 97%. For hospital and province abbreviations, see Table 6.1.

Table 6.2 Description of large clusters (≥5 isolates) at 100% similarity

Sub-cluster (chronological ranking) [a]	Number of isolates in cluster	Time relationship			Number of hospitals involved
		Duration (months)	Period (year/months)	Isolates (per month)	
A1 (13)	5	3	'02 O-D	2,1,2	2 (GRNPT, GSH)
A2 (8)	14	10	'01 N '02 Mr-D	1 4,2,2,0,0,1,1,1,1,1	4 (TAMBO RUS, NJH, GRNPT)
A3 (6)	30	24	'01 Ja-Jl '02 F-D	1,0,0,0,0,0,1 2,11,4,1,2,4,0,1,1,0,2	5 (NJH, MILP, RED, GRNPT, PE)
A4 (1)	5	34	'00 Ja 1 '01 Jl-N '02 O	 2,0,0,0,1 1	3 (CARL, NJH, TAMBO)
A5 (13)	8	3	'02 O-D	6,0,2	1 (CHB)
A6 (7)	8	10	'01 Au '02 Ja-N	1 1,0,0,0,0,1,2,0,2,1	1 (GSH)
A7 (10)	7	6	'02 Mr-Au	1,0,1,2,2,1	1 (PE)
A8 (12)	9	1	'02 Jl	9	1 (CHB)
A9 (10)	8	4	'02 Mr-Jn	3,0,0,5	1 (CHB)
B1 (3)	6	8	'00 My-D	1,0,0,0,4,0,0,1	2 (SOU, CHB)
B2 (4)	6	4	'00 Au-N	1,3,1,1	1 (CHB)
B3 (11)	6	8	'02 Ap-N	5,0,0,0,0,0,0,1	2 (CHB, TAMBO)
B4 (2)	5	30	'00 Mr-N '01 Ja '02 S	2,0,0,0,0,0,0,0,1 1 1	2 (PIE, TAMBO)
B5 (5)	6	23	'00 D '01 Mr-Ap '02 My-O	1 1,1 1,0,0,0,0,1	2 (HJ, NJH)
D1 (9)	9	7	'02 F-Au	1,1,0,0,1,2,4	2 (GRNPT, PE)

[a] The chronological ranking of the sub-clusters as they occurred is given in brackets. Ja, January; F, February; Mr, March; Ap, April; My, May; Jn, June; Jl, July; Au, August; S, September; O, October; N, November; D, December. No clusters of ≥5 isolates at 100% similarity were present in cluster C or in the two minor clusters.

Table 6.3 Comparison of clusters with isolates at 100% similarity

Cluster/	Total			TR[a](%)	Number of isolates in cluster/year			
Year	Isolates	Clusters	Isolates in clusters		<5	≥5	Range	Median
A	126	13	106	73.8	4	9	2-30	8
B	103	19	68	47.6	14	5	2-6	4
C	15	3	9	40.0	3	0	2-4	3
D	12	2	11	75.0	1	1	2-9	9
2000	54	9	33	44.4	6	3	2-6	5
2001	36	8	23	41.7	7	1	2-7	3
2002	175	23	136	64.6	13	10	2-30	8

[a] TR: The transmission rate was calculated using the following formula (Small *et al*., 1994):

$$\frac{[\text{Total number of isolates in clusters ('Isolates in clusters')}] - [\text{The number of clusters ('Total clusters')*}]}{[\text{Total number of isolates typed ('Total isolates')}]}$$

*The number of clusters represents removal of an index case for calculation of transmission

Cluster B

Cluster B was the second largest cluster representing 38.9% (103/265) of the total number of isolates, of which the isolates shared ≥80.5% similarity with one another (Figure 6.4). Cluster B was extremely heterogeneous and accommodated smaller sub-clusters compared with those of the other three major clusters (see Table 6.2 and Table 6.3). Ten (47.6%) of the 21 hospitals were represented in cluster B, with 98% of the isolates (101/103) originating from the northern provinces of South Africa. The 103 isolates in cluster B included 85.4% (88/103) from Gauteng (CHB, NJH, KAL, SOU, TAMBO and HJ), 10.7% (11/103) from Limpopo (PIE) and 1.9% (2/103) from North West Province (RUS and TSE). Temporal analysis of cluster B showed the majority of isolates (47.6%; 49/103) were from 2000, 26.2% (27/103) from 2001 and 26.2% from 2002.

When the similarity cut-off point of ≥97% was applied, 17 small clusters comprising 84 isolates showed close relatedness. Isolates in cluster B, as with cluster A, grouped according to the hospital from which they were recovered and showed evidence of possible transmission within the particular hospitals themselves.

Isolates showing 100% similarity were represented in five clusters (B1-B5) (Table 6.2 and Table 6.3) that are smaller than those seen in cluster A. Two isolates from RED hospital were from Western Cape province and one of these (14066) did not carry an ESBL gene.

Of the 101 isolates tested for the presence of an ESBL gene, 43 fell within this cluster. One isolate expressed TEM-1 (14340), five the newly described TEM-131, 37 carried TEM-63, while one isolate expressed both TEM-131 and SHV-5 (16490). The isolate from South Rand Hospital (7343) represented a patient with previous hospitalization at CHB and showed 100% similarity with isolates from CHB, collected at the same time. TEM-63 was the common ESBL type in cluster B and was detected in 37/46 isolates screened for the presence of an ESBL.

Cluster C

Cluster C (Figure 6.5) contained 15 of the total 265 (5.7%) isolates and showed ≥83.2% similarity to one another. Isolates from Gauteng (TEMB), Western Cape (RED) and the Eastern Cape (PE) provinces were distributed among nine different XPs and were from 2000 and 2002. The four isolates from 2000 were all from TEMB in Gauteng, comprising two isolates with identical XPs in 2000 while one isolate from 2000 at this hospital showed an identical XP in another cluster with two isolates from 2002. The fourth isolate from 2000 shared a 93.8% similarity with the latter and former clusters. In contrast, nine of the 11 isolates from 2002 were in the Eastern Cape (five isolates) and the Western Cape (four isolates, all from RED) provinces.

Using the ≥97% cut-off similarity, isolates were classified into 3 clusters, comprising 12 of the 15 isolates in cluster C.

Compared with the other major clusters, no large sub-clusters (≥5 isolates) were present (see Table 6.3). Like cluster B, this cluster displayed considerable heterogeneity.

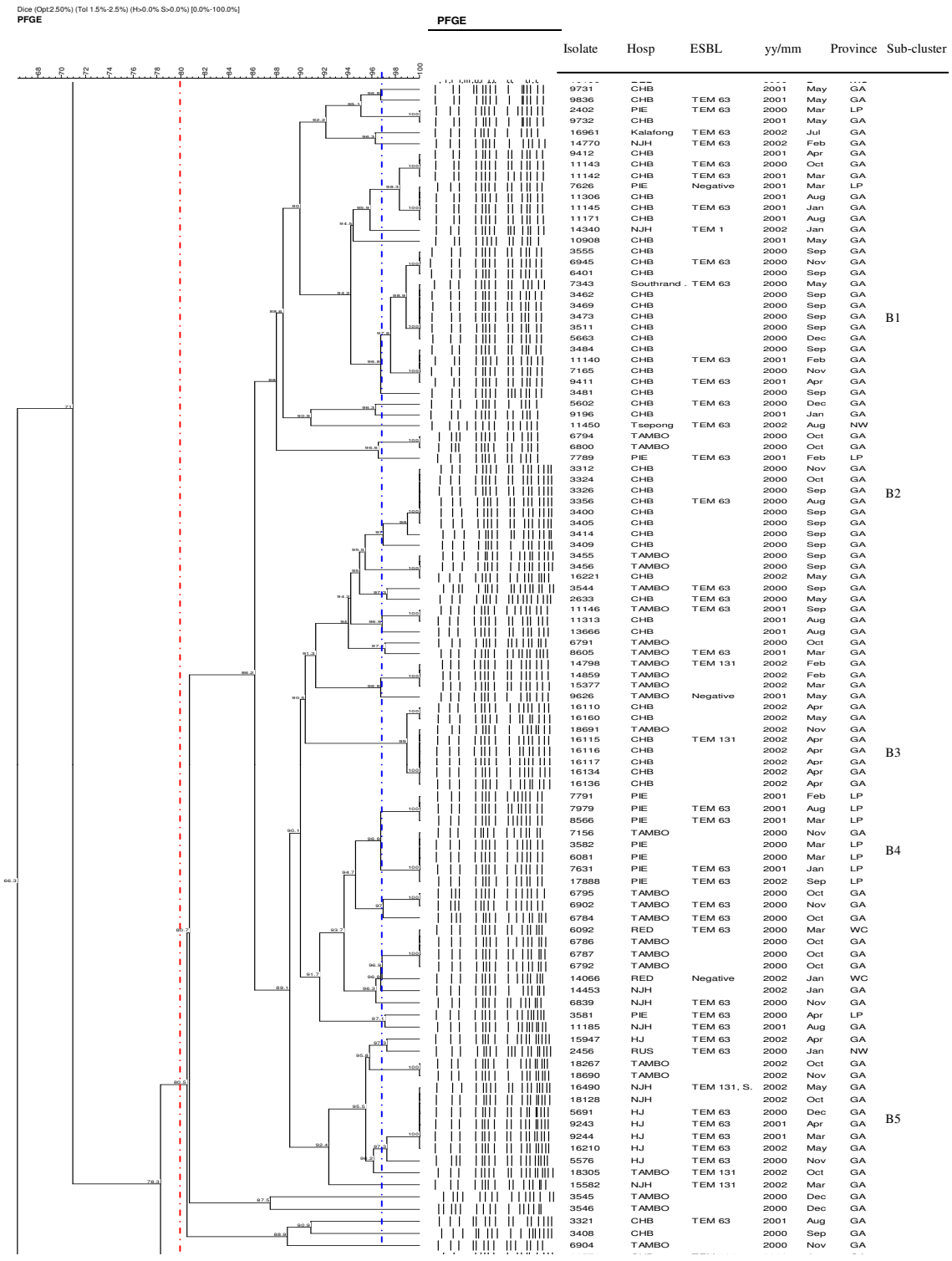


Figure 6.4 Partial dendrogram depicting cluster B (*n*=103) (green colour in Figure 6.2). The red dotted line indicates an 80% similarity coefficient mark and the blue line 97%. For hospital and province abbreviations see Table 6.1.

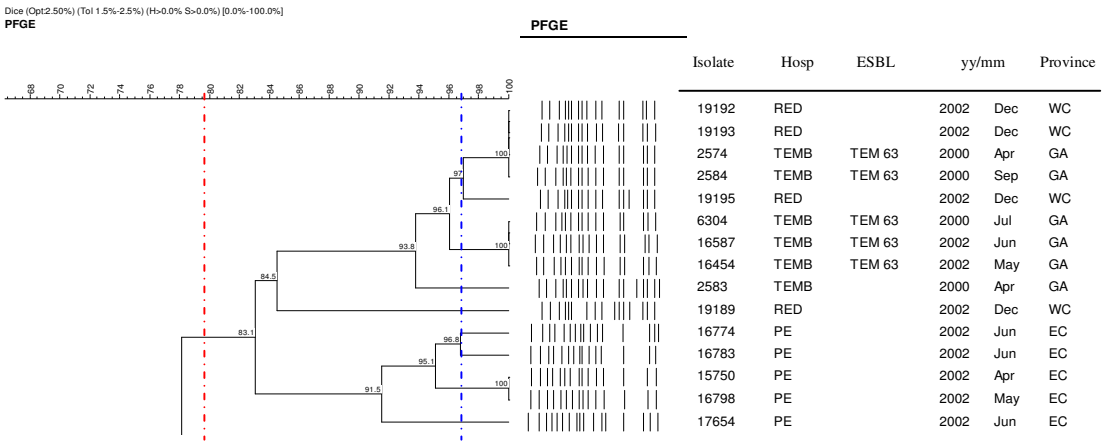


Figure 6.5 Partial dendrogram depicting cluster C (light blue colour in Figure 6.2). The red dotted line indicates an 80% similarity coefficient mark and the blue line 97%. (*n*=15)

Cluster D

Cluster D (Figure 6.6) consisted of 12 isolates (4.5%) that shared ≥85.2% similarity with one another. Isolates from the Western Cape (GRNPT) and Eastern Cape (PE) provinces were distributed among three different banding patterns revealing 11 of the isolates as closely related (96.8% similarity). Of these, nine had identical XPs, seven from PE and two from GRNPT, all isolated during 2002. The 12th isolate (GRNPT 13480) did not express an ESBL and was isolated in December 2001. When the ≥97% similarity cut-off was used, 11 of the 12 isolates were clustered together.

Like cluster A, this cluster is relatively homogeneous compared with cluster B and cluster C (see Tables 6.2 and Table 6.3) and as indicated above, all the cultures except the ESBL-negative, were from 2002.

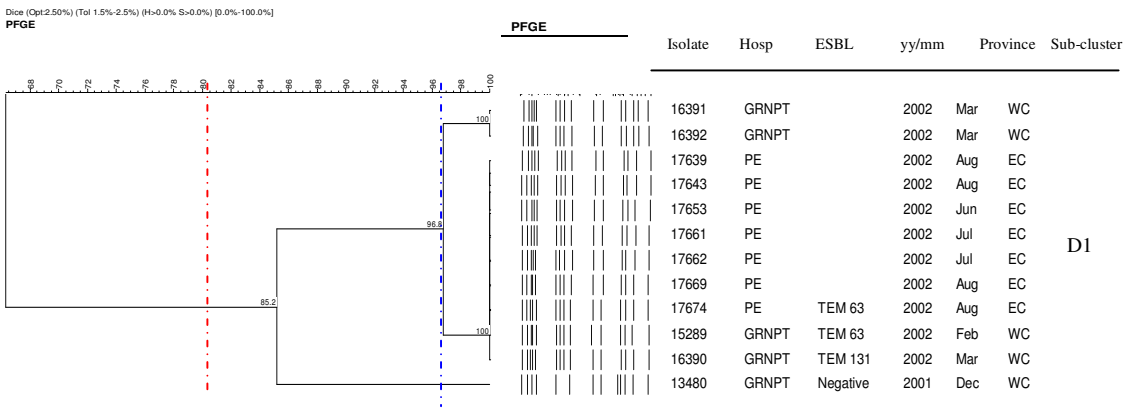


Figure 6.6 Partial dendrogram depicting cluster D (dark blue colour in Figure 6.2). The red dotted line indicates an 80% similarity coefficient mark and the blue line 97%. (*n*=12)

Minor clusters

The remaining nine isolates (3.4%) not represented in the four major clusters are from the Eastern Cape (5/265; minor cluster 1) and Gauteng (4/265; minor cluster 2) provinces. All nine isolates were from 2002, representing six different banding patterns.

Distribution of ESBL-negative isolates

Of the six ESBL-negative isolates, five were available for PFGE typing and three of these were in cluster B. Cluster information which included an ESBL-negative strain at a similarity of approximately 97% or greater is given in Table 6.4. Of the three ESBL-negative isolates in cluster B, one was from PIE (7626), one from TAMBO (9626) and the third from RED (14066). The isolate from PIE had an identical PFGE fingerprint to three isolates from CHB collected in the same year, while the RED isolate formed a cluster at 96.3% similarity with TAMBO and NJH strains, while the TAMBO susceptible strain formed a cluster at the 96.8% similarity level at that hospital. The other two ESBL-negative isolates fell into cluster A and cluster D. The sub-cluster at 96.4% similarity within cluster A was formed over a period of two years and involved hospitals throughout the country. In contrast, the ESBL-negative isolate in cluster D was unique and only formed a cluster with other members of cluster D at the 85.2% similarity level (see Table 6.4).

Table 6.4 Clustering by PFGE typing involving five ESBL-negative isolates

Hospital of ESBL-negative isolate	Time isolated	Major cluster	Clustering at ≥97% similarity		
			Cluster size	Period of cluster	Hospitals involved [a]
Livingstone	F 2001	A	33[b]	Ja 2001 – D 2002	RED 15, NJH 3, GRNPT 8, MILP 2, Other 6[c]
PIE	Mr 2001	B	7	O 2000 - Au 2001	PIE 1, CHB 6
TAMBO	My 2001	B	4[d]	Mr 2001 – Mr 2002	TAMBO 4
RED	Ja 2002	B	7	O 2000- Ja 2002	RED 2, NJH2, TAMBO 3
GRNPT	D 2001	D	1	NA	NA

NA, Not applicable; Ja, January; F, February; Mr, March; My, May; Au, August; O, October; D, December
[a] The numbers in this column denote the number of isolates from the indicated hospital
[b] Similarity of cluster at 96.8%
[c] Other: Private laboratories, CHB 1, Observatory 1, PE 1
[d] This cluster contained TEM-131 which was ≥96.8% similar to the ESBL-negative isolate

6.5.3 Patients with more than one infection episode

Of the total number of 279 *S.* Isangi isolates typed during the three-year study period only four from two patients were clearly involved in two separate infection episodes, both occurring approximately four weeks after each other.

The two isolates from the first patient showed 94.6% similarity in cluster A with one isolate in sub-cluster A8 and the other in sub-cluster A9 (see Table 6.2 and Figure 6.3). Isolates from these two sub-clusters, comprising nine and eight members respectively were all from CHB. The two cultures from separate episodes involving the second patient, hospitalized at GRNPT, were identical and shared 100% similarity with 28 other isolates from several hospitals including six isolates from GRNPT, and fifteen from RED in sub-cluster A3.

Based on incomplete information and the balance of probabilities, it is possible that a third patient could have had two infection episodes, approximately three months apart. The names attached to the two isolates were exactly the same but the ages of the babies involved were discrepant by several months. This patient had two non-identical cultures isolated from different hospitals, one from GRNPT and the other from RED. The isolate from GRNPT shared 100% similarity with another isolate from GRNPT and seven isolates from PE in sub-cluster D1 while the isolate from RED belonged to the large sub-cluster A3 (see Table 6.2, Figure 6.3 and Figure 6.6). The period between the episodes of this patient was approximately three months, and if valid, would be an example of re-infection with two different strains.

6.5.4 Analysis of PFGE patterns by year

As transmission of pathogens is linked temporally, the progression of cluster formation was investigated by examining the cluster status of the three years of the study. The distribution of the major *S.* Isangi clusters during the years 2000, 2001 and 2002 respectively is given in Figure 6.7. Cluster B, which was virtually confined to the northern provinces of South Africa, was predominant in 2000 and remained well represented during 2001 and 2002 while cluster A expanded rapidly and widely in 2002, affecting mainly Gauteng, Western Cape and Eastern Cape provinces. The relatively small cluster C and cluster D were first encountered in 2000 and 2001 respectively and both contained 11 isolates during 2002.

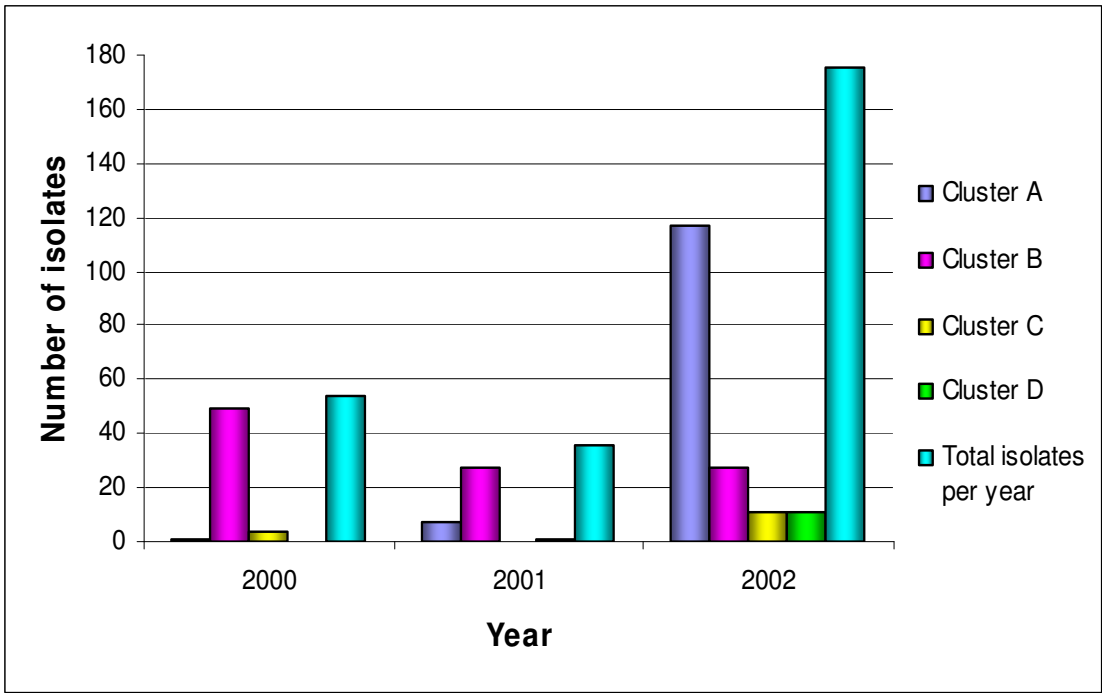


Figure 6.7 Distribution of clinical isolates from major clusters during the years 2000, 2001 and 2002 respectively.

During 2000 all cluster B isolates were from the northern provinces while in 2002 cluster A isolates/sub-clusters featured predominantly in the Western and Eastern Cape province. The 7 isolates in Cluster A for 2001 excludes the KZN isolate from poultry

2000

Clusters with 100% similarity and ≥5 isolates per cluster occurred first in the northern provinces and subsequently featured prominently in the southern provinces of South Africa (Table 6.2). Of the six early isolates that occurred during the period January to March 2000, five were from the northern provinces (Figure 6.8). Five of six isolates including one from RED belonged to cluster B (the sixth isolate was from Carletonville and belonged to cluster A). A dendrogram of the year 2000 (Figure 6.8) demonstrates four distinct clusters at ≥90% similarity level which tend to group the isolates from particular hospitals where they were isolated. There are three distinct/large clusters that showed isolates with similarities of ≥90.6%, ≥93.3%, ≥93.6% and ≥94.1%. At the provincial level, isolates came from Gauteng (48 isolates), Limpopo (4 isolates) and one isolate each from the North-West province and Western Cape.

In the cluster with ≥91% similarity there were 14 isolates, 13 from CHB while the 14th isolate was from a patient who was previously at CHB. In the ≥95.2% similarity cluster nine of the 12 isolates were from CHB and three from TAMBO. The third cluster (≥93.3% similarity) was heterogeneous and harboured 14 isolates, eight from TAMBO, and four from PIE and one each from NJH and RED hospitals.

As comprehensive information was not available on the duration of hospital stay before specimens were sent for culture, nosocomial spread of *S*. Isangi could not be defined with any degree of certainty. For the purpose of this study, however, all clusters with similarities of ≥97% were regarded as epidemiologically linked and likely to be evidence of transmission. Intra-hospital (nosocomial) spread was assumed to be highly likely when clusters were large (≥5 isolates) and showed 100% similarity.

Of the 54 isolates in 2000, 33 grouped into 9 clusters that showed 100% similarity. An example of epidemiological evidence of likely transmission of *S*. Isangi between hospitals was provided by an isolate from South Rand Hospital (7343). This isolate, which belonged to a patient with documented previous admission to CHB, showed 100% similarity to other *S*. Isangi isolates from patients at CHB.

TEM-63 was found in all 20 isolates that were analyzed for an ESBL gene. TEM-131 was not detected amongst the isolates of this year.

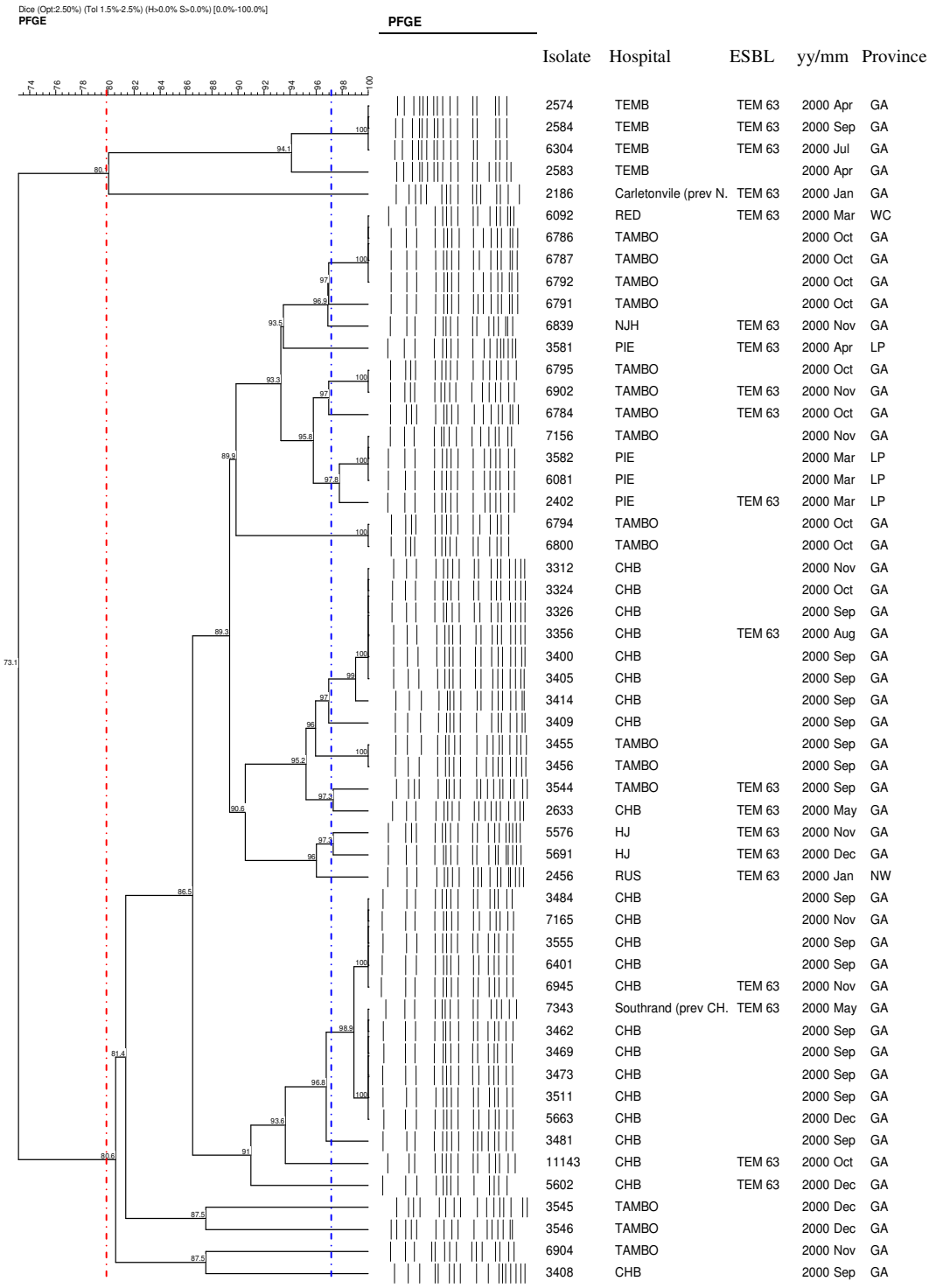


Figure 6.8 Dendrogram of PFGE with *Xba*I restriction depicting *S*. Isangi isolates from 2000. (Isolate number 2186 = Carletonville (previous admission at NJH) and isolate 7343 = South Rand (previous admission at CHB). The total number of *S*. Isangi isolates typed in this year was 54.

2001

A general decline in the number of *S*. Isangi isolates was evident for 2001 (see Figures 6.7 to 6.9), with a fall in the number of cluster B isolates and an increase in those of cluster A. Isolates were mainly from Gauteng and Limpopo provinces, with the exception of two isolates from the Eastern Cape and one from the Western Cape. Larger clusters during this year showed similarities of ≥91.9%, ≥92% and ≥92.7%. At a 100% similarity cut-off level, 23 of the 36 isolates in this year were grouped into 8 clusters and a 'transmission rate' of 41.7% (Table 6.3) was calculated. This compared with 44% for 2000, based on 33 of 54 isolates in 9 clusters. When the cut-off similarity of ≥97% was applied, 6 clusters grouped isolates according to the hospitals from which they were isolated.

TEM-63 was also the predominant ESBL isolated, as in 2000. Four of the 6 ESBL-negative isolates, from four different provinces, were recovered during this year. It is possible that they may have lost resistance determinants through curing of plasmids. Alternatively they may have been introduced from niches outside the hospital environment.

2002

The isolates from 2002 (see dendrogram in Figure 6.10) are largely represented in the dendrogram for cluster A (Figure 6.3 and Figure 6.7). When the cut-off similarity of ≥80% was applied, three large clusters of ≥80.2%, ≥81.7% and ≥80% comprising 158 of the 166 isolates in 2002 were distinguished. All five provinces that participated in the surveillance programme at the time (NW, EC, NC, GA, and LP) were represented in this year.

Using the ≥97% cut-off similarity, 160 isolates were classified into 26 clusters, also grouping according to the hospital from which they were collected. Of the total of 176 isolates in 2002, 166 were represented in the four major clusters, 117 in cluster A, 27 in cluster B and 11 each in cluster C and cluster D (see Figure 6.7), while 105 revealed 23

clusters that shared an identical banding pattern (100% similarity). Clusters with ≥5 isolates at 100% similarity were larger in 2002 compared to 2000 and 2001. The calculated 'transmission rate' (64.6%) was higher than that seen in the previous two years (Table 6.3).

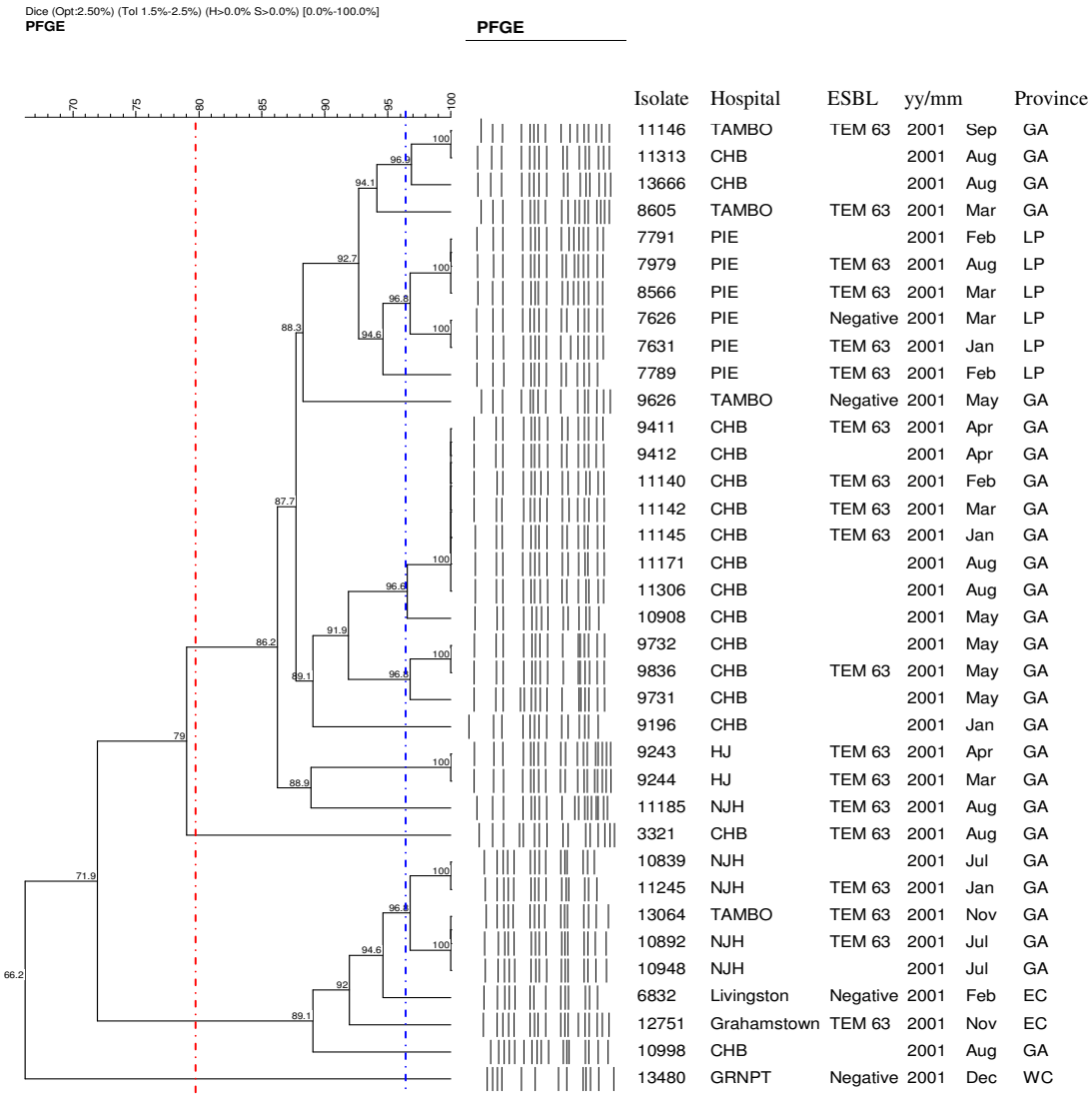


Figure 6.9 Dendrogram of PFGE with *Xba*I restriction depicting *S*. Isangi isolates from 2001. The total number of *S*. Isangi isolates typed in this year was 36. The red dotted line indicates an 80% similarity coefficient mark and the blue line 97%.

Although TEM-63 was the dominant ESBL isolated, the novel TEM, TEM-131, was detected and characterized in seven isolates from this year, while two isolates from NJH

(16970 and 16490) carried multiple β-lactamases. Only one ESBL-negative isolate was detected in 2002 (not shown on dendrogram, see Section 6.4.1).

6.5.5 Evidence of persistence ('endemicity') of clones in hospitals

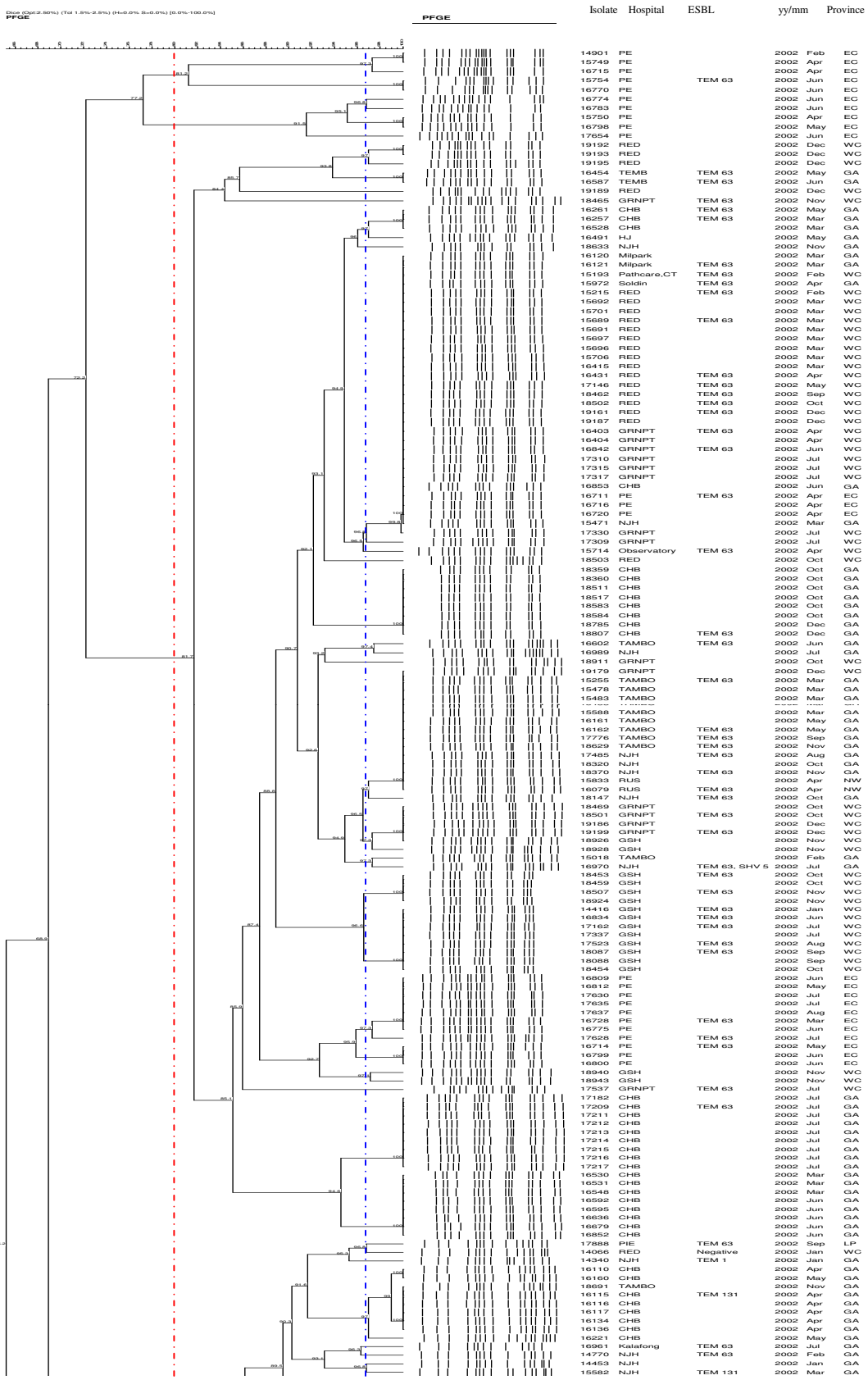
There is no specific definition of the term endemicity in quantitative terms but it generally implies the regular occurrence of a disease or pathogen over time in a specific location. Because of uncertainty about the use of the term 'endemic' in the *S*. Isangi setting, the term 'persistence' of strains/clones will be used instead. As the extent of true nosocomial transmission could not be assessed in this study, it is possible that some of the episodes caused by a particular clone or strain were acquired outside hospital.

Based on the presence of sub-clusters of ≥97% similarity in a hospital for more than 6 months seven clones in seven hospitals could be identified that for the purpose of this study could be labeled 'persistent'. Details of these clones are given in Table 6.5.

Table 6.5 Persistent ('endemic') clones in hospitals [a]

Hospital [b]	Sub-cluster	Dates	Duration in months	Number of isolates/ episodes
CHB	B1 [c]	05/00 - 04/01	11	12
NJH	A3	01/01 - 03/02	15	3
HJ	B5	12/00 - 10/02	22	5
TAMBO	A2	03/02 - 12/02	9	8
PIE	B4 [d]	03/00 - 09/02	18	7
GSH	A6	01/02 - 10/02	10	7
RED	A3	02/02 - 12/02	10	15

[a] Persistence based on prevalence of clone at ≥97% similarity for at least six months in the same hospital
[b] For names of hospitals see text
[c] Three sub-clusters, 4, 5 (sub-cluster B1) and 3 isolates each at 100% similarity with 97.6% similarity between sub-clusters
[d] Three sub-clusters, 2, 4 (sub-cluster B4) and 3 isolates each at 100% similarity with 97% similarity between sub-clusters


Dice (Opt:2.50%) (Tol 1.5%-2.5%) (H=0.0% S=0.0%) [0.0%-100.0%]
PFGE
PFGE
Isolate Hospital ESBL yy/mm Province
14901 PE 2002 Feb EC
15749 PE 2002 Apr EC
16715 PE 2002 Apr EC
15754 PE TEM 63 2002 Jun EC
16770 PE 2002 Jun EC
16774 PE 2002 Jun EC
16783 PE 2002 Jun EC
15750 PE 2002 Apr EC
16798 PE 2002 May EC
17654 PE 2002 Jun EC
19192 RED 2002 Dec WC
19193 RED 2002 Dec WC
19195 RED 2002 Dec WC
16454 TEMB TEM 63 2002 May GA
16587 TEMB TEM 63 2002 Jun GA
19189 RED 2002 Dec WC
18465 GRNPT TEM 63 2002 Nov WC
16261 CHB TEM 63 2002 May GA
16257 CHB TEM 63 2002 Mar GA
16528 CHB 2002 Mar GA
16491 HJ 2002 May GA
18633 NJH 2002 Nov GA
16120 Milpark 2002 Mar GA
16121 Milpark TEM 63 2002 Mar GA
15193 Pathcare,CT TEM 63 2002 Feb WC
15972 Soldin TEM 63 2002 Apr GA
15215 RED TEM 63 2002 Feb WC
15692 RED 2002 Mar WC
15701 RED 2002 Mar WC
15689 RED TEM 63 2002 Mar WC
15691 RED 2002 Mar WC
15697 RED 2002 Mar WC
15696 RED 2002 Mar WC
15706 RED 2002 Mar WC
16415 RED 2002 Mar WC
16431 RED TEM 63 2002 Apr WC
17146 RED TEM 63 2002 May WC
18462 RED TEM 63 2002 Sep WC
18502 RED TEM 63 2002 Oct WC
19161 RED TEM 63 2002 Dec WC
19187 RED 2002 Dec WC
16403 GRNPT TEM 63 2002 Apr WC
16404 GRNPT 2002 Apr WC
16842 GRNPT TEM 63 2002 Jun WC
17310 GRNPT 2002 Jul WC
17315 GRNPT 2002 Jul WC
17317 GRNPT 2002 Jul WC
16853 CHB 2002 Jun GA
16711 PE TEM 63 2002 Apr EC
16716 PE 2002 Apr EC
16720 PE 2002 Apr EC
15471 NJH 2002 Mar GA
17330 GRNPT 2002 Jul WC
17309 GRNPT 2002 Jul WC
15714 Observatory TEM 63 2002 Apr WC
18503 RED 2002 Oct WC
18359 CHB 2002 Oct GA
18360 CHB 2002 Oct GA
18511 CHB 2002 Oct GA
18517 CHB 2002 Oct GA
18583 CHB 2002 Oct GA
18584 CHB 2002 Oct GA
18785 CHB 2002 Dec GA
18807 CHB TEM 63 2002 Dec GA
16602 TAMBO TEM 63 2002 Jun GA
16989 NJH 2002 Jul GA
18911 GRNPT 2002 Oct WC
1919 GRNPT 2002 Dec WC
15255 TAMBO TEM 63 2002 Mar GA
15478 TAMBO 2002 Mar GA
15483 TAMBO 2002 Mar GA
15588 TAMBO 2002 Mar GA
16161 TAMBO 2002 May GA
16162 TAMBO TEM 63 2002 May GA
17776 TAMBO TEM 63 2002 Sep GA
18629 TAMBO TEM 63 2002 Nov GA
17485 NJH TEM 63 2002 Aug GA
18320 NJH 2002 Oct GA
18370 NJH TEM 63 2002 Nov GA
15833 RUS 2002 Apr NW
16079 RUS TEM 63 2002 Apr NW
18147 NJH TEM 63 2002 Oct GA
18469 GRNPT 2002 Oct WC
18501 GRNPT TEM 63 2002 Oct WC
19186 GRNPT 2002 Dec WC
19199 GRNPT TEM 63 2002 Dec WC
18926 GSH 2002 Nov WC
18928 GSH 2002 Nov WC
15018 TAMBO 2002 Feb GA
16970 NJH TEM 63, SHV 5 2002 Jul GA
18453 GSH TEM 63 2002 Oct WC
18459 GSH 2002 Oct WC
18507 GSH TEM 63 2002 Nov WC
18924 GSH 2002 Nov WC
14416 GSH TEM 63 2002 Jan WC
16834 GSH TEM 63 2002 Jun WC
17162 GSH TEM 63 2002 Jul WC
17337 GSH 2002 Jul WC
17523 GSH TEM 63 2002 Aug WC
18087 GSH TEM 63 2002 Sep WC
18088 GSH 2002 Sep WC
18454 GSH 2002 Oct WC
16809 PE 2002 Jun EC
16812 PE 2002 May EC
17630 PE 2002 Jul EC
17635 PE 2002 Jul EC
17637 PE 2002 Aug EC
16728 PE TEM 63 2002 Mar EC
16775 PE 2002 Jun EC
17628 PE TEM 63 2002 Jul EC
16714 PE TEM 63 2002 May EC
16799 PE 2002 Jun EC
16800 PE 2002 Jun EC
18940 GSH 2002 Nov WC
18943 GSH 2002 Nov WC
17537 GRNPT TEM 63 2002 Jul WC
17182 CHB 2002 Jul GA
17209 CHB TEM 63 2002 Jul GA
17211 CHB 2002 Jul GA
17212 CHB 2002 Jul GA
17213 CHB 2002 Jul GA
17214 CHB 2002 Jul GA
17215 CHB 2002 Jul GA
17216 CHB 2002 Jul GA
17217 CHB 2002 Jul GA
16530 CHB 2002 Mar GA
16531 CHB 2002 Mar GA
16548 CHB 2002 Mar GA
16592 CHB 2002 Jun GA
16595 CHB 2002 Jun GA
16636 CHB 2002 Jun GA
16679 CHB 2002 Jun GA
16852 CHB 2002 Jun GA
17888 PIE TEM 63 2002 Sep LP
14066 RED Negative 2002 Jan WC
14340 NJH TEM 1 2002 Jan GA
16110 CHB 2002 Apr GA
16160 CHB 2002 May GA
18691 TAMBO 2002 Nov GA
16115 CHB TEM 131 2002 Apr GA
16116 CHB 2002 Apr GA
16117 CHB 2002 Apr GA
16134 CHB 2002 Apr GA
16136 CHB 2002 Apr GA
16221 CHB 2002 May GA
16961 Kalafong TEM 63 2002 Jul GA
14770 NJH TEM 63 2002 Feb GA
14453 NJH 2002 Jan GA
15582 NJH TEM 131 2002 Mar GA

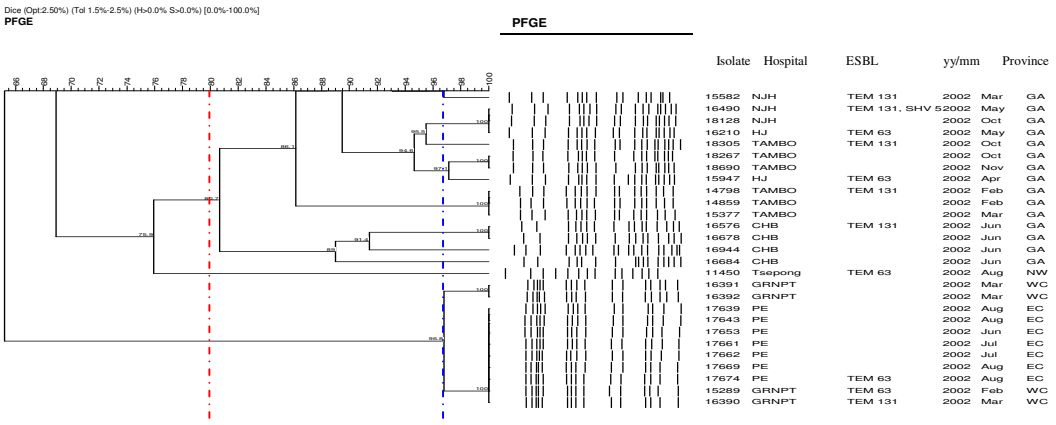


Figure 6.10 Dendrogram of PFGE with *Xba*I restriction depicting *S*. Isangi isolates from 2002. The total number of *S*. Isangi isolates typed in this year was 176. The red dotted line indicates an 80% similarity coefficient mark and the blue line 97%

6.6 DISCUSSION

A dendrogram representing PFGE XPs of 265 *S*. Isangi isolates investigated revealed four major clusters at 80% similarity. Two similarity cut-off points; ≥97% similarity (less than three band differences within the cluster) and 100% similarity (identical banding patterns between isolates) were used to describe these clusters. The ≥97% similarity cut-off point correlated well with epidemiologically linked isolates at hospitals and was used to describe clustering of isolates within the major clusters (A to D).

The lack of patient data regarding ward admission compromised accurate assessment of nosocomial transmission patterns, nevertheless, based on the premise that all or the vast majority of isolates were hospital associated, evidence of extensive transmission was obtained and many *S*. Isangi strains were found to be closely related and persistent in several hospitals for many months. Recent data from the GERMS-SA surveillance database for 2005 indicated that, of the 76 invasive laboratory-confirmed ESBL-producing NTS episodes reported 48 (63%) were diagnosed within 48hours of hospital admission, suggesting that infection was probably acquired prior to admission. An opposite trend was observed for *S*. Isangi where 57% of the episodes were diagnosed

after 48hours of admission, indicating a high rate of nosocomial acquisition (Dr. Nelesh Govender, personal communication). It is highly likely that the rate of nosocomial transmission of *S*. Isangi during the period 2000-2002 was also very high (see also Wadula *et al*., 2006) with spill-over from hospitals to the community. It is also reasonable to conclude that the majority of infection episodes in the present study was hospital associated i.e. were either nosocomial or had strong hospital links e.g. previous admission or contact with hospital cases, likely to occur in HIV/AIDS patients.

With the many HIV/AIDS patients in the study population (Wadula *et al*., 2006) one would expect multiple infection episodes due to relapse or re-infection. In the case of only two patients definitely and possibly in a third, could repeat episodes be recorded in the present study. This is likely to be an underestimate and may in part be due to the fact that the study was neither prospective nor comprehensive and that limited data was available from the hospital wards.

Many hospitals world-wide have experienced outbreaks of ESBL-producing organisms. The outbreaks are often fueled by the large number of patient transfers between units/wards within hospitals and between hospitals (Lucet *et al.,* 1999). In the present study, many of the strains were genetically related but apparently unrelated epidemiologically based on time (isolated over a period of several months). It is likely that genotypes and subtypes were carried for prolonged periods in children and also proliferated by nosocomial transmission within and between wards. Smaller clonal complexes (sub-clusters) within the major clusters (A, B and D), showing 100% similarity, were observed among all 22 hospitals (Table 6.2 and Table 6.3) throughout the three-year study period. This indicates likely recent transmission within the hospital wards. The inter-hospital and intra-hospital spread of a clone is exemplified by the isolate (7343) of a patient from SOU hospital that showed 100% similarity with five isolates from CHB.

An important limitation of the present study was the lack of relevant epidemiological data collection which did not allow for an accurate assessment of transmission patterns.

A carefully designed prospective study with relevant epidemiological and clinical information on isolates and hospitals would have given a better indication of transmission within and between hospitals, while other aspects such as faecal carriage and differentiation between relapse and re-infection in HIV/AIDS patients could have been studied.

Based on extensive nosocomial transmission recorded by Wadula *et al*., (2006) and our findings at CHB Hospital where clinicians and medical officers were actively monitoring and investigating the spread of *S*. Isangi, we calculated 'transmission rates' for each year as indices for comparison purposes only. 'Transmission rates' of 60.9%, 44% and 73.2% based on clusters in this hospital was calculated for 2000, 2001 and 2002 respectively. In 2000, a comprehensive attempt to contain the spread of ESBL-positive *S*. Isangi isolates in this hospital was made by isolating infected patients, emphasizing appropriate infection control measures and treating infected patients with ciprofloxacin for 4-6 weeks to eradicate long-term faecal carriage (Wadula et al., 2006). The lowered 'transmission rate' in the following year (44%) suggests that the control measures played a role in reducing transmission. However, following curtailment of prolonged treatment of faecal carriage of the 'transmission rate' escalated in 2002 to a higher level than that seen in the first year of the study. (This may in part be due to possible improved surveillance during 2002). Although no isolates were submitted in 2000 from NJH, the 'rates of transmission' for 2001 and 2002 were calculated as 40% and 20% respectively. Red Cross Childrens Hospital was not represented in the first two years but had a high 'transmission rate' of 75% in 2002.

Several clones persisted in the hospitals throughout the three-year study period indicating that these strains have established themselves in the hospitals in a nosocomial environment and probably disseminated by means of person-to-person spread, staff, hospital equipment, linen and the shortfall of proper infection control measures (e.g. hand washing, sharing of equipment etc.).

The majority of isolates in 2000 (with exception of one isolate from Western Cape) and 2001 (with exception of two isolates from the Eastern Cape) were from the northern provinces of South Africa while the isolates from 2002 were mostly representative of the southern provinces of South Africa (see Table 6.2). It is possible that the *S*. Isangi isolates in this study had their origin in Africa (where *S*. Isangi was initially isolated) and spread to the North West, Limpopo and Gauteng provinces in 2000 and 2001 and finally reached the southern borders of the Western and Eastern Cape provinces in 2002. The dissemination of resistance seemed to have followed the same trend through the years. TEM-63 was detected as the most common ESBL type in 2000 and 2001 and the novel TEM-131 only appeared and then proliferated in 2002 (see Table 6.2 and Figure 6.7). This process was most probably enhanced by the selective antibiotic pressure exerted on *S*. Isangi over time.

Clonal transmission within institutions or between hospitals in a geographic area is likely to be a result of patient-to-patient spread and the extensive use of antibiotics is a potent selective pressure for the emergence of the ESBL-type resistance in hospitals. Despite the improvements in the prevention and management of nosocomial infections during the past ten years, these infections continue to contribute significantly to morbidity and mortality statistics.

Chapter 8

CONCLUSION

In recent years infectious diseases have largely been driven by the HIV/AIDS pandemic, fueled by growing antibiotic resistance, inappropriate prescription of ineffective drugs and poor adherence to medication. They remain the most important immediate cause of death among children and of disability worldwide. Southern Africa accounted for >40% of deaths due to infectious diseases, more than 60% of all deaths in the region. Infectious diarrhoea makes the largest single contribution (88%) to the burden of disease associated with unsafe water, sanitation and hygiene (World Health Report, 2002) of which one third is accounted for by Africa. Ninety percent of deaths associated with this risk factor are in children.

Nosocomial outbreaks and epidemic situations due to multidrug-resistant bacteria reflecting failed infection control measures and compromising the effectiveness of antimicrobial treatment are increasingly recognized world-wide. The emergence of organisms fully resistant to fluoroquinolones and/or extended spectrum cephalosporins in typhoidal and non-typhoidal *Salmonella* serotypes makes treatment difficult and expensive. The increasing incidence of ESBLs in *Salmonella* serotypes in hospitals is particularly concerning because they have great potential to enter the microflora of farm animals and the food produced of them, resulting in further escalation of these organisms in the community. Once selected, an ESBL variant may spread by clonal dissemination of the producer strain or horizontal transmission of the ESBL gene-carrying plasmids among non-related strains.

Before 1999, *S.* Isangi was rarely isolated in South Africa. In a very short while it advanced to second place (30% of salmonella isolates received at EDRU) in the inventory of serotypes in the country. The *S*. Isangi epidemic entered into a fairly stable endemic situation with the considerable control challenges it entails. To date, reports indicate that mainly neighbouring countries of Africa as well as a few countries from

Europe and Asia have been affected by sporadic outbreaks due to this serotype (see section 1.8.1 of this dissertation).

The age distribution of patients and the documentation of extra-intestinal infection in the present study correlate with the clinical features of *S*. Isangi infection as previously described in the literature (Krubwa *et al*., 1976; Wadula *et al*., 2006). *S*. Isangi predominantly affected children, had the affinity to invade the blood stream and was multiple-antibiotic resistant. The majority of patients (83.3%; 170/204) were <2 years of age, while 25.4% accounted for extra-intestinal infections. It is likely that the proportion of extra-intestinal infections is exaggerated as the study population was predominantly from hospitals, selecting for more seriously ill patients. At the time of the present study, the HIV/AIDS rate of patients infected with ESBL-producing *S*. Isangi was 75% (Wadula *et al*., 2006).

Resistance to the extended-spectrum cephalosporins among members of the family *Enterobacteriaceae* is a world-wide problem which requires laboratory methods that will accurately detect and characterize the responsible β-lactamases in clinical isolates. The under-recognition of ESBL-mediated resistance is well described world-wide and has been due to the lack of convenient and sensitive methods for recognizing ESBL-producing strains (Cormican *et al.*, 1996). Furthermore, values for CTX or CRO for ESBLs may be below the breakpoint for resistance defined by CLSI. ESBL-producers should therefore be screened with both ceftazidime and cefotaxime as the ESBL status of all except one of the isolates in this study would have been missed if cefotaxime alone was used. Using current CLSI breakpoints for cefepime, 47 of the 273 ESBL-producing isolates would have been regarded as susceptible to cefepime and the suggestion by Szabó *et al*., (2005) that cefepime breakpoints should be reconsidered is supported by our findings. Cefepime by disc-diffusion technology was the best indicator of ESBL activity in *S.* Isangi, followed by cefotaxime and ceftazidime and confirmed that the screening of ESBLs should not be restricted to use of third generation cephalosporins only.

Although the Etest® ESBL strips detected all 273 ESBL-producers, it was found to be an expensive alternative to the double-disc diffusion and MAST ID™ ESBL tests. The MAST ID™ ESBL disc method, which is based on the CLSI-recommended approach, performed very well. An attractive feature of this method is that it is not dependent on disc spacing but the synergy effects can additionally be observed if the discs are placed in alternating positions between cephalosporin and cephalosporin + clavulanic acid discs.

Although *S.* Isangi isolates retained susceptibility to imipenem and ciprofloxacin, there was a gradual increase in the levels of ciprofloxacin resistance and a marked increase in those of nalidixic acid during the period 2000 to 2002 with MIC_{50} levels for ciprofloxacin increasing ten-fold from 0.012µg/ml in 2000 to 0.125µg/ml in 2002, while MIC_{50} levels for nalidixic acid were 4µg/ml and >256µg/ml respectively. The recognition of strains with decreased susceptibility to nalidixic acid is of concern due to the increasing number of treatment failures in invasive salmonellosis reported in association with reduced fluoroquinolone susceptibility (Threlfall and Ward, 2001).

β-Lactamase diversity was low and TEM-63 was the most common ESBL and was identified in 92% of isolates tested for ESBL production. Only two isolates were found to express two β-lactamases simultaneously (TEM-63 and SHV-5 in one and TEM-131 and SHV-5 in the other isolate). This is the first report of TEM-63 β-lactamase in salmonellae and this enzyme appears to be endemic to South Africa. It was previously reported, in addition to *S.* Isangi, also in *E. coli, K. pneumoniae* and *P. mirabilis* from southern Africa (Essack *et al.*, 2001; Paterson *et al.*, 2003; Kruger *et al.*, 2004), Malawi (Gray *et al.*, 2006) and Tanzania (Blomberg *et al.*, 2005). TEM-131, a novel TEM-enzyme, was discovered during the course of the present study in seven isolates from four institutions in two provinces in South Africa. Its recent description (Kruger *et al.*, 2004) was the first report of this β-lactamase in the literature. TEM-131 was shown to have an additional amino acid change (Ala237Thr) compared to TEM-63 and to share the same isoelectric point of 5.6 (Kruger *et al.*, 2004). Resistance seemed to have disseminated by means of hospital cross-infection as TEM-63 was detected as the most

common ESBL type in 2000 and 2001 while TEM-131 only appeared with time and proliferated in 2002. The influence of previous antibiotic therapy in patients could have been a factor contributing to the selection of the resistant strains. Conjugation experiments with the TEM-131 gene will establish its unique resistance phenotype. Such investigations fell outside the scope of the present study.

The experimental approach for the demonstration of *bla* genes in *S*. Isangi isolates adopted in this study was based on direct sequencing of PCR-amplified genes. This method however, did not detect the presence of multiple TEM- or SHV-encoding genes in the same isolate and it will be interesting to investigate this further. Due to time and financial constraints, only a representative sample (101) of the total 273 *S*. Isangi isolates collected in this study was screened for *bla* genes and expanding the sample size with more recent isolates to detect other β-lactamases in *S*. Isangi in South Africa may be rewarding.

S. Isangi isolates in this study were recovered from crowded wards in hospitals and the proliferation of isolates of the same strain types by nosocomial transmission in and between these wards, as well as between hospitals, was likely and not surprising (Wadula *et al*., 2006). Twelve distinct plasmid profile types were observed among the 50 *S*. Isangi isolates tested, the organisms carrying one to six plasmids per isolate. Large plasmids (105kb to 166kb) as well as smaller plasmids (6kb to 44kb) were isolated. The location of the ESBL resistance genes on specific plasmids was not determined in this study. Although plasmid analysis appeared to exhibit a variety of profiles, the majority contained a 166kb plasmid. Plasmid banding patterns, even in isolates harbouring identical beta-lactamase genes, were diverse. Relationships between antibiogram, plasmid profile and PFGE type are complex and only moderate correlation was found between plasmid profiling and PFGE (see Chapter 5). Apart from hospital cross-infection, dissemination of resistance probably occurred as a result of horizontal gene transfer between members of the *Enterobacteriaceae* found in the intestinal tract of patients. Such transfer of drug-resistant genes takes place between different plasmids, or between plasmids and bacterial chromosomes and is mediated by

mobile genetic elements including transposons and integrons. The multiple antimicrobial drug resistance in virtually all the isolated strains was promoted by widespread use of antibiotics and probably poor infection control. These factors inevitably were responsible for the escalation of the hospital-based epidemics of *S*. Isangi infection.

In order to better understand the dissemination of disease in the hospital environment as well as in the community, one need to differentiate between the spread of resistance by plasmid and other mobile gene cassettes and strain dissemination (Tenover *et al*., 1995). Wadula *et al*., (2006) indicated that patients contracted *S*. Isangi after admission to wards as initial cultures from some patients were negative for the isolation of *S*. Isangi and blood cultures of patients with *S*. Isangi bacteraemia became positive after admission (>48 hours). Taken in conjunction with molecular typing by PFGE of isolates in the present study convincing evidence was produced to illustrate clonal dissemination of *S*. Isangi strains. Horizontal gene transfer by addition of resistance genes to plasmids or bacterial chromosomes by integrons and/or transposons was not specifically investigated in the present study but expansion of the TEM *bla* gene complex by mutation from TEM-63 to TEM-131 was demonstrated. CTX-M type β-lactamases were not found in our isolates, unlike the CTX-M-37 β-lactamase recently described by Govender *et al*., (2006) in *S*. Isangi isolates from Durban in KZN.

All PFGE clusters together showed ≥70% similarity suggesting that all the isolates were possibly related and may share a common ancestry. Clusters of isolates generated over several months in hospitals located in different provinces were indistinguishable by PFGE, suggesting person-to-person spread, at least in the transfer of isolates between hospitals and provinces as the likely route of transmission of *S*. Isangi in this study. Person-to-person spread of *S*. Isangi was also previously reported by Wadula *et al*., (2006) and du Toit (2004) in South African settings.

An important limitation of the present study was that no clinical isolates were recovered from NC, FS, MP and especially from KZN. The latter province only joined the

National Health Laboratory Service (NHLS) in 2006 and did not participate in the surveillance program at the time. A different outbreak pattern might have been observed if KZN were included in the surveillance, because of the high HIV/AIDS prevalence rate in that province. It is likely that KZN experienced a major *S*. Isangi problem and that the organism had been introduced into KZN at an early stage.

As this study was not a comprehensive prospective investigation initiated at the onset of the introduction of *S*. Isangi into South Africa, accurate transmission patterns could not be determined. It is however likely that *S*. Isangi spread by means of single or multiple patient transfer/s between hospitals and that clusters with identical or near-identical isolates established themselves in each hospital. It was evident that isolates in certain hospitals were identical and carried the same resistance genes on common plasmids, but other hospitals showed diversity in their plasmids. Some hospitals, that were geographically unrelated, had isolates that shared the same plasmid and ESBL-types. It is possible that the emergence of *S*. Isangi described in this study might have originated from elsewhere in Africa where it was originally isolated and described as the most common *Salmonella* serotype in Kinshasa, DRC in 1970. The possibility of an African country being the source of the epidemic in South Africa is suggested by the finding that *S*. Isangi was already present in the northern provinces of South Africa (North West, Limpopo, Gauteng) in 2000 and 2001 and was only reported to the EDRU from the southern provinces (Western and Eastern Cape) in 2002. The newly discovered TEM-131 also apparently evolved late and it was only isolated in 2002, while it was absent in 2000 and 2001.

Theoretically the finding of a few drug-susceptible *S*. Isangi isolates is compatible with the concept that they may represent the original source of the South African outbreak and that drug resistance may have been acquired over time by horizontal gene transfer and/or mutations in the *bla* gene complex. However, based on the PFGE findings of the study, curing of *bla*-carrying plasmids, resulting in loss of resistance, is a much more likely explanation for the presence of the six susceptible isolates in the study.

The spread of ESBLs on the African continent has serious implications for already strained health care systems. The study emphasizes the need for surveillance and promotion of well-considered and restrictive antibiotic policies to contain further spread of these multiresistant bacteria. Indiscriminate or excessive antibiotic use, specifically of the broad-spectrum agents, should be controlled by education of clinicians and an effective policy of antibiotic use including the availability of treatment guidelines geared to retain the efficacy of antibiotics in health care systems plagued with infections and infectious diseases which are aggravated by the high HIV/AIDS prevalence. Strategies to contain further emergence and spread will have to include surveillance, adequate infection control measures and prudent use of antimicrobial agents. Outbreaks may be controlled by isolation of infected or colonized patients, antibiotic restriction, proper hygienic practices including hand washing/disinfection between patients and strict adherence to appropriate nursing procedures that reduce microbial transmission in hospital settings and hence the establishment of nosocomial pathogens resistant to multiple antibiotics. To limit the risk of nosocomial transmission to other patients and health care workers, faecal excretors of *Salmonella* should be managed with standard precautions, involving the use of barrier precautions, including the wearing of gloves (Pegues *et al*., 2005; Wadula *et al*., 2006).

Infection control practices in many hospitals in developing countries are rudimentary and often compromised by economic shortfalls and opposing traditional values. Likely sources of infection where person-to-person spread exacerbates the problem are nurseries, paediatric wards, families and homes for the elderly. Paediatric wards are particularly prone to outbreaks of salmonellosis, possibly due to the local relevance of the faecal-oral classical route of spread of *Salmonella* organisms. Hand washing has been shown to reduce the risk of transmission to extremely low levels (Pether and Scott, 1982).

The role of extra-human sources of *S*. Isangi in this study is unknown, although poultry products have been suspected. *S*. Isangi was previously associated with poultry carcasses and the processing environment in South Africa (Georgnaras and von Holy

2001). Previous studies reported *S.* Isangi from dead birds (Stefanov *et al*., 1987), poultry (Hasman *et al*., 2005; Pieskus *et al*., 2006), household wall lizards (Mascher *et al*., 1988) and a slaughterhouse for pigs (Yadava *et al*., 1986). Transmission may also occur through direct contact with animals, and contaminated water (Miller *et al*., 1995; Tauxe, 1996). Expansion of public health surveillance programmes to include animal food products, together with the use of molecular typing of isolates will aid in determining transmission patterns of MDR salmonellae in South Africa.

The study provided information on the occurrence and molecular epidemiology of multiresistant *S.* Isangi causing nosocomial infections in South Africa, and clearly demonstrated extensive transmission of *S*. Isangi in this country. I was also the first to describe the genetic basis of ESBL resistance in *S.* Isangi in South Africa. Attempts at early detection through the use of molecular technology, coupled with continued epidemiological surveillance of NTS, are important strategies in the prevention of the dissemination of *Salmonella* exhibiting drug resistance mechanisms. This project developed capacity in this emerging under-researched area and supplemented the limited data available on this organism. It is reassuring to know that continued surveillance of salmonellosis and other intestinal, as well as respiratory, infections has been instituted by GERMS-SA in South Africa and that the expertise and facilities are available at the NICD to study emerging infectious disease outbreaks in depth, including the molecular level.

"A scientist, worthy of his name above all a mathematician, experiences in his works the same impression as an artist; his pleasure is as great and of the same nature."
– Jules Henri Poincaré

Appendix One

Materials used for PCR gel electrophoresis

1.1 1% (w/v) agarose gel:

Weigh 1g of agarose into a glass container and add 100ml of 1X TAE buffer (see 1.5). Microwave or heat the mixture to dissolve agarose completely. Add 3µl of ethidium bromide (10mg/ml stock, see 1.2) solution and swirl gently to distribute evenly. Allow to cool and pour into the gel cast. Allow the agar to set for approximately 30min.

1.2 10mg/ml Ethidium Bromide solution:

Weigh 0.5g of ethidium bromide powder. Add 50ml sterile distilled water. Stir on a magnetic stirrer for several hours to ensure that the dye is completely dissolved. Wrap container in foil (or use a dark bottle) and store at 4°C.

1.3 Loading buffer:

Red*Taq* polymerase (Sigma-Aldrich) already contains a loading dye and buffer.
A separate loading buffer was not needed.

1.4 10X TAE buffer:

Weigh 48.4g Tris-base, 11.42g glacial acetic acid and 7.44g EDTA. Make up to 1000ml with sterile distilled water and store at room temperature or at 4°C.

1.5 1X TAE buffer:

Measure 100ml of the 10X TAE buffer (see 1.4) and dilute to 1000ml with sterile distilled water.

Appendix Two

Materials used in plasmid isolation

2.1 Growth of bacterial cells:

2.1.1 Luria-Bertani (LB) medium:

Weigh 5g Yeast extract, 10g Tryptone and 10g NaCl. Make up to 1000ml with sterile distilled water. Autoclave and allow to cool. Aliquot 5ml volumes into 15ml Falcon tubes and store at 4°C.

2.1.2 Ceftazidime (CAZ) antimicrobial stock solution:

Weigh 500µg of ceftazidime powder and make up to 1ml in an eppendorf tube and dissolve. Aliquot into 100µl volumes and store at -20°C.
Add 10µl of CAZ stock solution (500µg/ml) to 5ml of LB medium (Falcon tube).

2.2 10% Sodium dodecyl sulphate (SDS) solution:

Weigh 10g of SDS powder into a glass container. Dissolve in 100ml sterile distilled water.

2.3 10M NaOH:

Weigh 400g NaOH and add 800ml sterile distilled water.
Cool to room temperature and dilute to 1000ml.

2.4 25mM Tris-(hydrochloric acid) aminomethane solution pH 8.0 (Tris-HCL):

Dissolved 0.61g of Tris-HCL in 70ml sterile distilled water and adjust to pH 8.0 with 10M sodium hydroxide solution (see 2.3). The volume was brought up to 100ml with distilled water.

2.5 10mM Ethylenediamine-tetraacetic aid solution pH 8.0 (EDTA):

Dissolved 0.37g EDTA in 70ml sterile distilled water and adjust to pH 8.0 with 10M sodium hydroxide. Make up to 100ml with sterile distilled water.

2.6 Solutions I, II and III for plasmid isolation:

2.6.1 Solution I:

20mM glucose
25mM Tris-HCL pH 8.0
10mM EDTA pH 8.0

To make up 500ml of solution I:
Weigh 1.86g EDTA, 4.5g glucose (filter sterilize) and 1.97g Tris-HCL. Make up to 500ml with sterile distilled water. This solution can be autoclaved or filter sterilized and stored at 4°C.

2.6.2 Solution II (0.2M NaOH: 1% (w/v) SDS):

Measure 2ml of 10M NaOH (see 2.3) stock solution, 7ml sterile distilled water and 1ml from 10% SDS stock solution (see 2.2). This solution should be freshly prepared from stock solutions of 2M NaOH and 10% (w/v) SDS.

2.6.3 Solution III (3M Sodium acetate pH 4.8):

Dissolve 0.1g of sodium acetate in a minimal volume of water, adjust to pH 4.8 with glacial acetic acid and then adjust the volume with sterile distilled water to 250ml.

2.7 70% (v/v) ethanol:

Measure 350ml of absolute ethanol (99.9%) (v/v) and add 150ml sterile distilled water. Mix well.

2.8 1X TBE buffer:

Measure 100ml of 10 x TBE buffer (see 3.3). Make up to 1000ml with sterile distilled water.

2.9 0.6% (w/v) agarose gel:

Weigh 2.4g of agarose into a glass container and add 400ml of 1 x TBE buffer (see 2.8). Microwave or heat the mixture on a bunsen burner to dissolve agarose completely. Allow to cool and pour into gel cast. Allow agar to set for approximately 30min.

2.10 Loading buffer:

Weigh 0.25g of bromophenol blue powder (Sigma-Aldrich) and 40g sucrose (Pharmacia). Make up to 100ml with sterile distilled water and store at 4°C.

Appendix Three

Materials used for pulsed-field gel electrophoresis

3.1 1M Tris-(hydrochloric acid) aminomethane solution pH 8.0 (Tris-HCL):

Weigh 121.1g of Tris-HCL. Add 650ml sterile distilled water. Adjust pH to 8.0 with 32% hydrochloric acid (±50ml) solution. Dilute to 1000ml.

3.2 0.5M Ethylenediamine-tetraacetic acid solution pH 8.0 (EDTA):

Weigh 186.12g of EDTA powder. Add 800ml sterile distilled water.
Adjust pH to 8.0 with sodium hydroxide (NaOH) solution. Dilute to 1000ml.

10N NaOH:
Weigh 400g NaOH and add 800ml sterile distilled water.
Cool to room temperature and dilute to 1000ml.

3.3 10X TBE buffer:

Weigh 108g of Tris base and 55g of boric acid and add 40ml of 0.5M EDTA (see 3.2).
Dilute to 1000ml with sterile distilled water and dissolve well.
Store away from the light in a dark bottle or cover the bottle with foil.

3.4 0.5 x TBE buffer (Tris-Borate EDTA):

Add 100ml of 10X TBE buffer (see 3.3) to 1900ml sterile distilled water.

3.5 TE buffer (10mM Tris, 1mM EDTA), pH 8.0:

Add 10ml of 1M Tris, pH 8.0 (see 3.1) and 2ml of 0.5M EDTA pH 8.0 (see 3.2).
Dilute to 1000ml with sterile distilled water.

3.6 Cell suspension buffer (CSB) (100mM Tris, 100mM EDTA, pH 8.0):

Add 10ml of 1M Tris, pH 8.0 (see 3.1) and 20ml of 0.5M EDTA pH 8.0 (see 3.2). Dilute to 100ml with sterile distilled water.

3.7 Proteinase-K (20µl of 20mg/ml stock):

Weigh 100mg of proteinase-K powder. Add 5ml of sterile distilled water. Dissolve well.
Aliquot 500µl volumes into eppendorf tubes and store at -20°C.

3.8 1% SeaKem Gold®: 1% sodium dodecyl sulphate (SDS) agarose:

Weigh 0.5g SeaKem Gold agarose powder.
Add 47.0ml TE buffer (see 3.5) and microwave until agarose is completely dissolved.
Place flask in 55-60°C water bath for 5min.
Add 5ml of 10% SDS (see 2.2) and mix. Return to 55-60°C water bath for use or store at room temperature.

3.9 Cell lysis buffer (CLB) (50mM Tris, 50mM EDTA, pH 8.0 and 1% sarcosyl):

25ml of 1M Tris, pH 8.0 (see 3.1), 50ml of 0.5M EDTA, pH 8.0 (see 3.2), 50ml of 10% sarcosyl. Dilute to 500ml with sterile distilled water.

10% Sarcosyl:
Weigh 50g of sarcosyl powder. Carefully add sarcosyl to a small amount of water (150ml). Mix and heat to 50-60°C. Dilute to 500ml with sterile distilled water.

Appendix Four

PUBLICATION

Kruger, T., Szabo, D., Keddy, K.H., Deeley, K., Marsh, J.W., Hujer, A.M., Bonomo, R.A., Paterson, D.L. 2004. Infections with non-typhoidal *Salmonella s*pecies producing TEM-63 or a novel TEM enzyme, TEM-131, in South Africa. Antimicrobial Agents and Chemotherapy **48**:4263-4270.

Appendix Five

ETHICS CERTIFICATE

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

Division of the Deputy Registrar (Research)

COMMITTEE FOR RESEARCH ON HUMAN SUBJECTS (MEDICAL)
Ref: R14/49 Kruger

CLEARANCE CERTIFICATE **PROTOCOL NUMBER** M02-06-17

PROJECT Molecular Characterization of Multidrug Resistant Salmonella Isangi Causing Nosocomial Infections In South Africa

INVESTIGATORS T Kruger

DEPARTMENT School of Pathology, NHLS/SAIMR

DATE CONSIDERED 02-06-28

DECISION OF THE COMMITTEE *

Approved unconditionally

DATE 02-07-01 CHAIRMAN pp Mokhachane (Professor P E Cleaton-Jones)

* Guidelines for written "informed consent" attached where applicable.

c c Supervisor: Dr KH Keddy
Dept of School of Pathology, NHLS/SAIMR

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DECLARATION OF INVESTIGATOR(S)

To be completed in duplicate and ONE COPY returned to the Secretary at Room 10001, 10th Floor, Senate House, University.

I/we fully understand the conditions under which I am/we are authorized to carry out the abovementioned research and I/we guarantee to ensure compliance with these conditions. Should any departure to be contemplated from the research procedure as approved I/we undertake to resubmit the protocol to the Committee.

DATE 16 July 2002 SIGNATURE

PROTOCOL NO.: M 02-06-17

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES

REFERENCE LIST

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