



Microbiology Division

Enteric Diseases Reference Unit

BACKGROUND

EDRU collects data on patients presenting throughout South Africa with both invasive and non-invasive disease caused by *Salmonella* species (including *Salmonella* Typhi), *Shigella* species, *Vibrio cholerae* and diarrhoeagenic *Escherichia coli*. In order to make these data representative and reflective of disease burden in each province in the country, we actively motivate all diagnostic laboratories throughout the country to voluntarily submit limited demographic details and isolates to us centrally. In exchange, we offer serogrouping and serotyping results free of charge (urgent results need to be requested telephonically), regular feedback (quarterly reports by province sent to every laboratory participating) and aggregated numbers are published in the NICD Bulletin. We actively contact laboratories to assess numbers of missed cases.

In addition to serogrouping and serotyping, E-tests are used to determine the minimum inhibitory concentration (MIC) of each isolate to antimicrobial agents, according to CLSI guidelines. We also perform genotypic characterization of isolates, should this be required, such as in outbreak situations. The molecular epidemiology of these bacterial pathogens is continually being elucidated, specifically that of outbreak or epidemic-prone pathogens such as *Salmonella* Typhi, *Shigella dysenteriae* type 1 and *Vibrio cholerae*. A multiplex polymerase chain reaction is used to elucidate the presence of toxin genes in diarrhoeagenic *E. coli*. Our unit is developing its molecular research laboratory involved with characterizing the molecular basis for antimicrobial resistance in these pathogens and has plans to further characterize the mechanism of disease due to these pathogens at a molecular and cellular level.

Together with collaborators from the CDC in the USA, a number of sites in the country are performing "enhanced" surveillance, where additional clinical data on all patients is being collected, by trained surveillance officers (registered nursing sisters), representing almost all the provinces.

The staff has a specialized programme for the training of microbiology registrars, and over a two-week period, registrars are exposed to a range of biochemical, serotyping and molecular techniques in the identification of bacterial enteric pathogens.

ACTIVITIES, HIGHLIGHTS AND ACHIEVEMENTS

SURVEILLANCE ACTIVITIES

EDRU currently has the responsibility for surveillance and characterisation of bacterial enteric disease in South Africa; specifically, EDRU collects all human isolates from diagnostic microbiology laboratories in South Africa for surveillance (Table 1), from sites listed in the same table. EDRU is an active member of GERMS-SA (a more detailed explanation of GERMS-SA can be viewed under the National Microbiological Surveillance Unit).

- The case definition for these pathogens for all surveillance done by EDRU includes those isolates from body sites as specified below, in both in-patients and out-patients. Specifically this includes those individuals who sought treatment at a hospital or clinic, such as outpatients who have positive stool cultures or rectal swabs, but are not admitted or discharged from casualty. In this instance carriers may be included because they add to the burden of treatment, if not the burden of disease and may represent sub-clinical cases e.g. cholera.
- The case definition for enhanced surveillance isolates includes only those *Shigella* and *Salmonella enterica* isolates that are from normally sterile body sites in "in-patients" only that is the patient should have been admitted to the hospital or enhanced surveillance site, as currently defined by the Enhanced Surveillance core, or there should have been the intention to admit, to include those patients who may expire in casualty, as established from the bed letter. This also allows for changes in the ES site, either to include new South African sites or to exclude sites which may be viewed as no longer appropriate for the study.
- EDRU currently receives specimens from over 3000 human cases per annum, according to the definition above. In addition the unit undertakes to serotype *Salmonella*, *Shigella* and diarrhoeagenic *E. coli* (DEC) isolates for commercial purposes and has in the past performed a multiplex polymerase chain reaction (PCR) to diagnose DEC from veterinary specimens.
- Where relevant, molecular methods may be used to establish strain relatedness in outbreaks.

Table 1: Isolates for referral to EDRU, NICD, showing how these isolates are currently characterized.

Culture	Organism	Biochemical identification	Serotyping	Antimicrobial susceptibility testing	Genotyping
All body sites	<i>Aeromonas</i>	v	x	v	v
All body sites	<i>Salmonella</i> species	v	v	v	v
All body sites	<i>Shigella</i> species	v	v	v	v
All body sites	<i>Vibrio cholerae</i> O1 and non-O1	v	v	v	v
All body sites	Non cholera vibrios	v	v	v	v
All body sites	<i>Yersinia enterocolitica</i> Diarrhoeagenic	v	x	v	v
Stool/ rectal swab	<i>Escherichia coli</i> Suspect EHEC/E coli	v	v	v	v
All body sites	O157/STEC				

EVALUATION OF THE RAPID DIAGNOSTIC TESTS TUBEX® TF AND TYPHIDOT® IN A TYPHOID ENDEMIC AREA IN SOUTH AFRICA

Isolation of *Salmonella enterica* serotype Typhi is the gold standard for confirming a case of typhoid fever. Emerging drug resistance among circulating *Salmonella* Typhi strains has greatly complicated the treatment of typhoid fever, and heightened the need for rapid accurate diagnosis in outbreaks and the appropriate and selective use of antimicrobial agents.

Alternative practices in South Africa include the modified Widal test, which has not been evaluated. This test may cross-react with other organisms resulting in a high false positive and high false negative rate in early disease.

TUBEX TF and Typhidot are new rapid diagnostic tests that have not been evaluated in Africa. These new rapid tests are currently being evaluated with the assistance of the Nelspruit laboratory.

MULTIPLEX PCR FOR THE IDENTIFICATION OF HUMAN DIARRHOEAGENIC *ESCHERICHIA COLI* (DEC) AND THE GENES TARGETED

Enteric bacteria are the major etiological agents of sporadic and epidemic diarrhoea in both children and adults. The discovery of etiological agents of diarrhoea is important with respect to implementing suitable control strategies as well as therapeutic initiatives. The bacterial pathogen most commonly related with endemic type of diarrhoea in developing countries is diarrhoeagenic *Escherichia coli* (DEC). DEC is classified based on their virulence traits, unique clinical features and serotypes. The primary method of analysis involves a multiplex polymerase chain reaction (mPCR)

assay that simultaneously detects the primary virulence genes associated with each pathotype of DEC. Multiple primer pairs comprising specific forward and reverse oligonucleotides have been designed to target and amplify specific virulence genes situated on chromosomal and plasmid DNA. The genes amplified include the *eae* (codes for the intimin protein), *bfp* (codes for the bundle-forming pilus), *stx1* (codes for the shiga-toxin 1 protein), *stx2* (codes for the shiga-toxin 2 protein), *elt* (codes for the heat-labile enterotoxin), *est* (codes for the heat-stable enterotoxin), *ipa* (codes for the invasion protein), *aat* (codes for the transporter protein), *daaC* (codes for the accessory protein for production of F1845 fimbriae) and the *16SrRNA* (serves as an internal amplification control). To extend further identification of *E. coli* serogroups O157 and O111, primer pairs have been designed to amplify the *hlyA*, *uidA*, *rfaE* and *wbdI* genes.

MOLECULAR CHARACTERIZATION OF *SALMONELLA ENTERICA* SEROTYPE TYPHI

Salmonella enterica serotype Typhi (*Salmonella* Typhi) is responsible for typhoid fever infections and is a leading cause of morbidity and mortality amongst children and adults in developing countries. Infections normally result in bacteraemia and are characterized by frequent fevers, headaches, malaise, abdominal cramps, constipation and diarrhoea. Strain subtyping by molecular methods is an important tool for surveillance and outbreak analyses. Pulsed-field gel electrophoresis (PFGE) with restriction enzyme *XbaI* is our primary molecular method for subtyping *Salmonella* Typhi. DNA fingerprint patterns that are produced are analyzed with GelCompar II (version 4.6) software. This software generates dendrograms of the different patterns which in turn display percentage similarity of strains. Additionally, *Salmonella* Typhi strains are

further analyzed by multiple-locus variable number tandem repeats analysis (MLVA). This subtyping technique incorporates amplification and fragment size analysis of polymorphic regions of bacterial DNA containing variable numbers of tandemly repeated sequences by capillary electrophoresis. In September to December 2005, there was an outbreak of typhoid fever in the town of Delmas, Mpumalanga, a town 100 km from Johannesburg, in which over 600 cases were reported. Previously in 1993 this town had also experienced an outbreak of typhoid fever where over 1000 cases had been reported. We have recently completed an investigation of the molecular epidemiology of the strains from the 1993 and 2005 outbreaks using PFGE and automated MLVA. A probable link between the 2 outbreaks has been established. Last year another typhoid case was reported from the Delmas area, of which this strain showed an identical MLVA allele profile to a previous identified profile from 1993/2005 strains. This suggests that a strain of *Salmonella* Typhi that was involved in the 1993/2005 outbreaks is still circulating in the area. Now that we have established automated MLVA in our laboratory, we can rapidly investigate the molecular epidemiology of any future typhoid fever outbreaks in South Africa.

MOLECULAR CHARACTERIZATION OF *VIBRIO CHOLERAE*

Cholera is an infection caused by the bacteria *Vibrio cholerae* O1 or *V. cholerae* O139. Cholera remains a serious and significant bacterial disease in developing countries, including many African countries. Cholera is acquired by ingestion and is predominately a water-borne disease. Molecular characterization determines the genetic relatedness of *V. cholerae* strains. We are able to perform this analysis on strains with both epidemic potential (serogroups O1 and O139) and non-epidemic potential (serogroups other than O1 and O139). Our primary method of analysis involves pulsed-field gel electrophoresis (PFGE) analysis of digested genomic DNA incorporating either *Sfi*I or *Not*I digestion. In addition, non-epidemic strains are further investigated using repetitive element sequence-based PCR (REP-PCR) fingerprinting. DNA fingerprint patterns are analyzed and compared using the GelCompar II (version 4.6) software. The software creates dendrograms of the patterns which reveal percentage similarity of strains.

These methods have been used to investigate groups of strains from different regions of Southern Africa. Through December 2006 to February 2007, Namibia experienced their first outbreak of cholera. We determined that this outbreak was caused by *V. cholerae* O1 serotype Inaba biotype El Tor. The outbreak strains revealed identical PFGE patterns which determined that the outbreak was caused by a single strain of *V. cholerae*. Through March to April 2007, we investigated 11 strains of *V. cholerae* (non-O1, non-O139 serotypes) isolated in the Northern Cape province of South Africa. The strains included ten water

isolates and one blood culture isolate from a patient who died as a result of cardiac arrest ascribed to septic shock and multiple organ failure. PFGE and REP-PCR analysis showed a diversity of five clones amongst the 11 strains. The genotype of the human strain was identical to the genotype of strains sourced at the Vaalharts Weir (on the Vaal River) suggesting that this weir was the source of the patients' infecting strain (Figure 1).

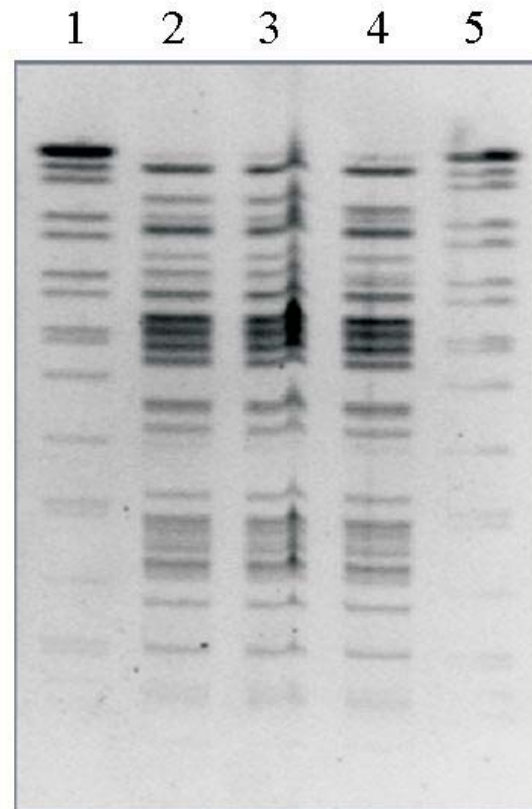


Figure 1: Pulsed field gel electrophoresis of the *Vibrio cholerae* non-O1 isolates from the Northern Cape showing identical fingerprints between a patient's isolate (lane 2) and environmental isolates from the Vaalharts Weir (lanes 3 and 4).

MOLECULAR IDENTIFICATION OF FLAGELLAR (H ANTIGENS) IN *SALMONELLA* SPECIES

Conventional serotyping relies on the phenotypic expression of bacterial properties. Conventional serotyping (H antigens) in *Salmonella enterica* is dependent on the expression of flagellar genes and subsequent manufacture of active flagella. Bacterial strains may fulfill all the genetic requirements (structural genes) for a particular serotype, yet sometimes may not express the necessary genes, resulting in an incomplete serotype determination. Molecular serotyping offers an alternative to conventional phenotypic serotyping and its accompanying gene expression problems. Molecular serotyping examines at the level of the genomic DNA of a bacterial cell, and will determine what flagella structural gene(s) is present and so define the H-antigen potential of a bacterial cell. In South Africa, *Salmonella* Typhimurium (O:4,5 H:i;1,2)

and *Salmonella* Isangi (O:6,7 H:d;1,5) account for 67% of all serotypes of *S. enterica* isolated from humans. We have implemented a multiplex PCR method to assist in the detection of H antigens associated with these 2 serotypes. *S. enterica* possesses two structural genes (*fliC* and *fliB*) for flagellin, the component protein for flagellar filaments. Expression of these genes defines the H antigens of the bacterium. The *fliC* gene encodes flagellin protein of phase 1 antigenic type H:i and H:d for *Salmonella* Typhimurium and *Salmonella* Isangi, respectively. The *fliB* gene encodes phase 2 antigenic type H:1,2 and H:1,5 for *Salmonella* Typhimurium and *Salmonella* Isangi, respectively. Our multiplex PCR targets specific DNA sequences associated with the above antigenic types. Validation of the method included an analysis of 40 strains from the year 2006 which were incomplete in their serotype identification, yet their O antigen serogrouping was highly suggestive of either serotype Typhimurium (O:4,5) or serotype Isangi (O:6,7). PCR analysis of the 40 strains resulted in complete serotyping for 27 of the strains.

MOLECULAR EPIDEMIOLOGY AND MECHANISM OF RESISTANCE OF INVASIVE QUINOLONE-RESISTANT SOUTH AFRICAN ISOLATES OF SALMONELLA ENTERICA, 2004-2006

The objectives of this study are: (1) to investigate the clonal structure of invasive quinolone-resistant *S. enterica* serotypes Typhimurium, Typhi, Isangi, and Enteritidis; (2) to determine the molecular basis for quinolone resistance in the above *Salmonella* serotypes (3) to determine the optimum concentration of antibiotic required to treat quinolone-susceptible and -resistant *S. enterica* serotypes Typhimurium, Typhi, Isangi, and Enteritidis.

To achieve these objectives the study will incorporate the use of PFGE, with *Xba*I restriction enzyme digestion of DNA, as well as MLVA analysis, to determine strain relatedness; PCR and sequencing to target possible mutations in the gyrase genes *gyrA* and *gyrB* and the topoisomerase IV genes *parC* and *parE*, resulting in fluoroquinolone antibiotic resistance; agar dilution MIC's with and without the addition of an efflux-pump inhibitor, as well as time-kill studies, to determine the optimal MIC for South African *Salmonella* isolates.

THE MOLECULAR EPIDEMIOLOGY OF INVASIVE SALMONELLA ENTERICA SEROVAR TYPHIMURIUM ISOLATES

Salmonella is a zoonotic disease responsible for food-borne enteric infections in both humans and in animals. In this study, the molecular epidemiology of invasive *Salmonella enterica* serovar Typhimurium isolates from humans was investigated. These isolates were collected from human patients and chicken products in the Gauteng province, for the years 2006 and 2007, respectively. A total of 411 invasive human isolates were

received at NICD in the Gauteng province for the year 2006. Twenty-seven chicken samples were received at NICD from informal outlets during the year 2007. Pulsed-field gel electrophoresis (PFGE) was the genotypic method used to generate fingerprint patterns using *Xba*I. GelCompar II software was used to analyse fingerprint patterns to identify similar fingerprint patterns. Fingerprint patterns that had a ninety percent and greater similarity value fell in the same cluster. Chicken samples were tested for *Salmonella* Typhimurium using selective broths, selective media, conventional stool sugars and specific anti-sera. Cluster analysis identified twenty-four clusters amongst the human isolates, three of which were major clusters. The majority of the *Salmonella* Typhimurium isolates were isolated from male patients. Results show that a large percentage of isolates came from patients with a positive HIV-status. Twelve of the 27 chicken samples were positive for *Salmonella*.

CAPACITY BUILDING

POST-GRADUATE STUDENTS

Ananta Nanoo (MSc Epidemiology)
Masters Dissertation: The impact of cotrimoxazole usage in HIV on cotrimoxazole resistance in non-typhoidal *Salmonella*.

Rugola Mtandu (MSc Epidemiology)
Masters Dissertation: The impact of HIV on clinical-microbiologic features and mortality among patients with invasive non typhoidal *Salmonella* infection in South Africa (submitted).

Nevashan Govender (MSc)
Masters Dissertation: Molecular Epidemiology and Mechanism of Resistance of Invasive Quinolone-Resistant South African Isolates of *Salmonella enterica*, 2004-2006.

Sarika Dwarika (MSc)
Masters Dissertation: Molecular epidemiology of invasive isolates of *Salmonella enterica* serovar Typhimurium in Gauteng, South Africa, 2005-2007.

REGISTRARS

EDRU offers both short and long courses to microbiology registrars from South African universities in specialised techniques that are relevant for the identification of enteric pathogens.

FELTP

EDRU has assisted in the training supervision of FELTP students.

SITE VISITS AND TRAINING

EDRU staff received training in the rapid diagnosis of typhoid fever on 26 and 27 March 2007 (Figure 2).



Figure 2: Training EDRU staff in the use of the rapid diagnostic tests for typhoid fever.

Dr Karen Keddy and Ms Arvinda Sooka visited Rob Ferreira Laboratory, Nelspruit from 28 to 30 March to initiate training in rapid diagnosis of typhoid fever for the TUBEX Typhidot study (page 5).

Dr Karen Keddy and Ms Arvinda Sooka visited the NHLS laboratories in Upington and Springbok in the Northern Cape as part of the GERMS-SA outreach programme from 14th to 17th August 2007 (Figure 3).



Figure 3: Dr KH Keddy (EDRU), Ms M Durandt (NHLS, Upington) and representatives from Northern Cape Department of Health at a lecture given by Dr Keddy on GERMS-SA surveillance activities in Upington, August 2007.

WORKSHOPS, CONGRESSES AND CONFERENCES ATTENDED