

## Enteric Diseases Reference Unit

### BACKGROUND

The Enteric Diseases Reference Unit at the NICD was started in 1997, under the guidance of a pathologist and a part-time technologist. Over the next few years, the capacity was increased through the hiring of extra staff members to its current complement, the unit developed full capacity to serotype *Salmonella enterica* and *Shigella* species and has had training Centers for Disease control, Atlanta, USA and WHO Reference Centre for *Escherichia coli* (Statens Serum Institut, Denmark). EDRU took over *Vibrio cholerae* work from the old Public Health Laboratory and has developed capacity in both phenotyping and genotyping of this group of organisms.

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 characterising 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 project comprises a vibrant and energetic team of pathologists, clinicians, scientists, technologists, clerks and surveillance officers.

The unit also comprises an enthusiastic team of senior pathologists and scientists who are actively involved in post-graduate training. 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. The senior staff members are experienced in post graduate supervision of scientists and have recently started projects with epidemiology students who are examining the extensive database.

### 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. EDRU is an active member of GERMS-SA (a more detailed explanation of GERMS-SA can be viewed under the National Microbiological Surveillance Unit).

These isolates will be characterised at no charge to the laboratory of origin, irrespective of whether the laboratory functions in a private capacity or has a public role. This includes those isolates that may represent carriage of an enteric bacterial pathogen, rather than disease due to that pathogen.

- 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.
- Although EDRU does not normally do such work for other African countries, the support of the unit may be requested should one of the neighbouring countries require it.
- EDRU currently receives specimens from over 4000 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.
- Regular reports on the isolates received are extracted from the database for the purposes of information sharing.
- Where relevant, molecular methods may be used to establish strain relatedness in outbreaks.

#### **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 are currently evaluating these new rapid tests with the assistance of the Nelspruit laboratory.

#### **ANALYSIS OF A TEMPORAL CLUSTER OF SHIGELLA BOYDII ISOLATES IN MPUMALANGA, SOUTH AFRICA, NOVEMBER TO DECEMBER 2007**

Shigellosis is a global human health problem. The disease is most prevalent in developing countries with

poor access to safe potable water and sanitation. *Shigella boydii* is of particular epidemiological importance in developing nations such as African and Asian countries. In the present study, we report on the analysis of a temporal cluster of 29 *S. boydii* serotype 2 strains, isolated in the Mpumalanga Province of South Africa (SA) over the period of November to December 2007. Bacteria were identified as *S. boydii* using standard microbiological identification techniques and serotyped using commercially available antisera. Susceptibility testing to antimicrobial agents was determined by the Etest. Genotypic relatedness of strains was investigated by pulsed-field gel electrophoresis (PFGE) analysis of digested genomic DNA. The cluster of 29 isolates revealed comparable antimicrobial susceptibility profiles, while dendrogram analysis of PFGE patterns showed that the cluster of isolates grouped together and could clearly be differentiated from a random selection of unrelated *S. boydii* serotype 2 strains. Our data has strongly suggested that this cluster of isolates may share a common ancestry. However, this cannot be substantiated by epidemiological data because a detailed epidemiological investigation was not conducted. We have documented the first cluster of *S. boydii* infection in SA. Due to the lack of adequate epidemiological investigation, we cannot emphatically state that an outbreak had occurred. However, we do hypothesize that this was an outbreak for which a waterborne source cannot be excluded. This study has highlighted the urgent need for timely and appropriate systems of epidemiological investigation of all suspected outbreaks of disease in developing countries.

#### **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 waterborne 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 *SfiI* or *NotI* 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 BioNumerics (version 5.1) software. The software creates dendrograms of the patterns which reveals percentage similarity of strains. These methods have been used to investigate groups of strains from different regions of Southern Africa.

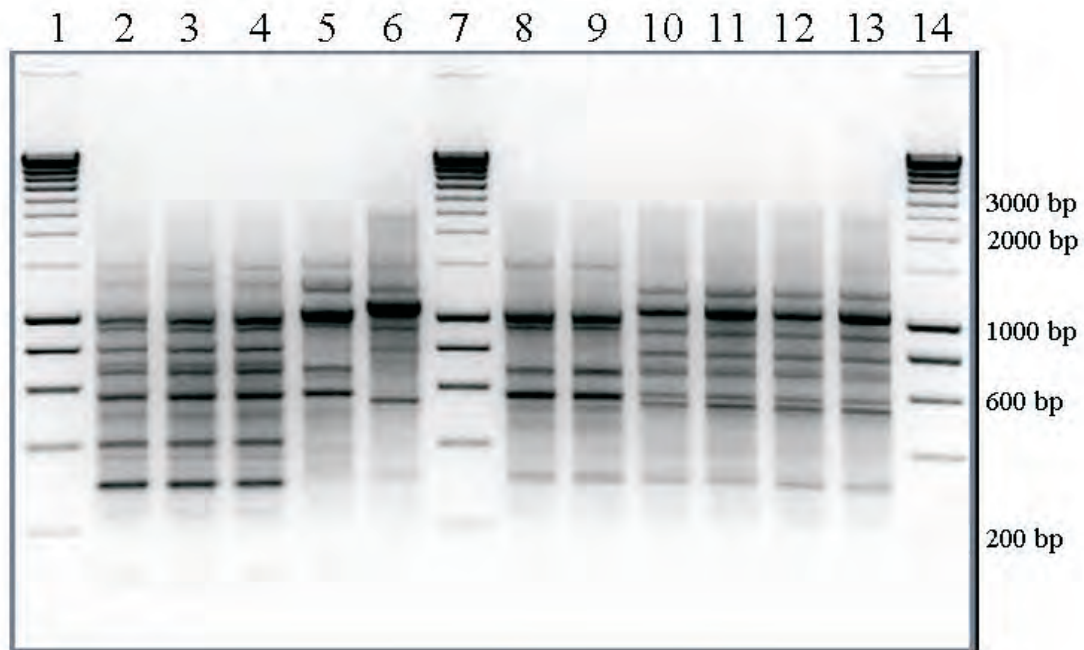


Figure 1: REP-PCR fingerprint patterns of *Vibrio cholerae* strains

#### CHARACTERIZATION OF CHOLERA AMONGST ILLEGAL MINERS IN THE MPUMALANGA PROVINCE OF SOUTH AFRICA, MAY TO JULY 2008

Outbreaks of cholera occur periodically in South Africa and have the potential to cause substantial morbidity and mortality. The laboratory plays a critical role in providing early confirmation of *Vibrio cholerae* O1 as an aetiological agent in outbreaks of watery diarrhoea. In May 2008, an outbreak of cholera was identified amongst illegal miners in Ehlanzeni district, Mpumalanga Province following the notification of a single laboratory-confirmed case of *V. cholerae* O1 on 29 May 2008. During the period May to July 2008, a total of 31 *V. cholerae* O1 isolates were received by the Enteric Diseases Reference Unit. Bacteria were confirmed as *V. cholerae* O1 serotype Ogawa, using standard microbiological techniques. Additional PCR tests determined that all strains were of the El Tor biotype and were positive for production of the cholera enterotoxin (*ctxA* positive). Pulsed-field gel electrophoresis (PFGE) analysis determined that all strains were highly related and clustered at 95% similarity following dendrogram analysis of PFGE patterns. Susceptibility testing to antimicrobial agents was determined by the Etest. All strains were shown to be multidrug resistant, with resistance to ampicillin, augmentin, ceftriaxone, ceftazidime, trimethoprim, sulfamethoxazole, tetracycline, chloramphenicol, kanamycin, streptomycin, nitrofurantoin and nalidixic acid. Most isolates also showed extended-spectrum  $\beta$ -lactamase (ESBL) activity. In view of the multidrug resistance of this outbreak strain, the choice of antibiotics for patient treatment would be extremely challenging.

#### MULTIPLEX PCR ASSAY FOR IDENTIFICATION OF HUMAN DIARRHOEAGENIC *ESCHERICHIA COLI* IN SOUTH AFRICA

Enteric bacteria are the major aetiological 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 the multiplex PCR assay that simultaneously detects the primary virulence genes associated with each pathotype of DEC. Our multiplex PCR assay detects for the presence of enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), shiga-toxin producing *E. coli* (STEC) [which includes the enterohemorrhagic *E. coli* (EHEC)], enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAggEC) and diffusely adherent *E. coli* (DAEC). Multiple PCR primer pairs have been designed to target and amplify specific virulence genes situated on chromosomal and plasmid DNA. The genes targeted include the: *eae* (codes for the intimin outer membrane 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 an invasion protein), *aat* (codes for a transporter protein) and *daaC* (codes for an accessory protein involved in production of F1845 fimbriae). To extend further identification of *E. coli* serogroups O157 and O111, primer pairs have also

been designed to amplify the *hlyA*, *uidA*, *rfbE* and *wbdI* genes. For the year 2008, the Enteric Diseases Reference Unit processed 671 isolates of *E. coli*, of which 296 were non-virulent (negative for all virulence genes), 186 were EPEC, 70 were EAgtEC, 64 were DAEC, 32 were ETEC, 12 were EIEC, 9 were EHEC and 2 were STEC. Serogroups O119 and O111 were the most commonly (15%) identified serogroups of *E. coli*, of which these serogroups were mostly represented by EPEC.

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

The molecular epidemiology and mechanism of quinolone resistance of South African human isolates of *Salmonella* Typhi for the period 2003-2007, *Salmonella* Enteritidis, *Salmonella* Isangi and *Salmonella* Typhimurium for the period 2004-2006, received by the Enteric Diseases Reference Unit (EDRU) of the National Institute for Communicable Diseases was investigated. Molecular epidemiology was investigated using pulsed-field gel electrophoresis (PFGE) analysis for all four serotypes, as well as multiple-locus variable-number tandem-repeats analysis (MLVA) for *Salmonella* Typhi and *Salmonella* Typhimurium. Three probable mechanisms for quinolone resistance were investigated which included: amino acid mutations in the quinolone resistance determining regions (QRDRs) of DNA gyrase (*gyrA*/*gyrB*) and topoisomerase IV (*parC*/*parE*), active efflux of antibiotic out the bacterial cell and plasmid-mediated resistance encoded by *qnr* genes. For the period 2003-2007, 498 human isolates of *Salmonella* Typhi were received by the EDRU, of which 27 were resistant to nalidixic acid (MICs,  $\geq 32$   $\mu\text{g/ml}$ ). Only 19 *Salmonella* Typhi quinolone-resistant isolates were available for analysis. For the period 2004-2006, 329 human isolates of *Salmonella* Enteritidis, 1005 human isolates of *Salmonella* Isangi and 2624 human isolates of *Salmonella* Typhimurium were received by the EDRU. Of these isolates, 119 *Salmonella* Enteritidis, 143 *Salmonella* Isangi and 532 *Salmonella* Typhimurium were invasive, nalidixic acid-resistant. Only 116 *Salmonella* Enteritidis, 137 *Salmonella* Isangi and 516 *Salmonella* Typhimurium invasive, nalidixic acid-resistant isolates were available for analysis. For each respective serotype the isolates were genetically diverse as they could be differentiated into many PFGE types, suggesting that quinolone-resistant strains have emerged independently of one another for all four serotypes. The use of MLVA for *Salmonella* Typhi and *Salmonella* Typhimurium also illustrated the genetic diversity of the isolates by differentiating the isolates in various MLVA types. The investigation into the contributory mechanisms of resistance showed that an over-active efflux system in combination with mutations in both *gyrA* and *parC* play a major role in facilitating quinolone resistance in *Salmonella* Typhi, *Salmonella* Enteritidis and *Salmonella* Isangi. These very same mechanisms were also found to be responsible for the

quinolone resistance in the majority of the *Salmonella* Typhimurium isolates along with the rarely isolated mechanism of resistance, a *qnr* plasmid. This is the first report of any kind identifying the presence of *qnr* genes in South African *Salmonella* isolates. Our study also highlights the need for further work to establish the link amongst the various mechanisms of resistance as their interactions remains unclear.

**MOLECULAR EPIDEMIOLOGY OF INVASIVE ISOLATES OF *SALMONELLA ENTERICA* SEROTYPE TYPHIMURIUM IN GAUTENG, SOUTH AFRICA, 2005 TO 2008**

*Salmonella* is a frequently isolated foodborne zoonotic pathogen in humans and animals. Chicken *Salmonella* isolates were isolated from chicken specimens which were randomly purchased from retail and informal outlets from various regions in the Gauteng Province of South Africa during the time period of September 2007 through to April 2008. Chicken isolates of various serotypes were compared to equivalent serotypes of representative human isolates which were received at the Enteric Diseases reference Unit (EDRU) during the time period of January 2006 through to May 2008. One-hundred and fifty chickens were processed and 47 *Salmonella* strains were isolated. *Salmonella* Heidelberg (34%; 16/47) and *Salmonella* Infantis (34%; 16/47) were the most common *Salmonella* serotypes isolated from chickens; followed by *Salmonella* Hadar (11%; 5/47), *Salmonella* Enteritidis (11%; 5/47) and *Salmonella* Typhimurium (11%; 5/47). PFGE analysis with *XbaI* digestion and *NotI* digestion showed that chicken *Salmonella* Infantis isolates and chicken *Salmonella* Enteritidis isolates were completely different to all PFGE patterns of human *Salmonella* Infantis isolates and human *Salmonella* Enteritidis isolates, respectively. Conversely, PFGE analysis with *XbaI* digestion and *NotI* digestion showed that some PFGE patterns of chicken *Salmonella* Heidelberg isolates and chicken *Salmonella* Hadar isolates were related to some human *Salmonella* Heidelberg isolates and some human *Salmonella* Hadar isolates, respectively. PFGE analysis with *XbaI* digestion showed that 1 of the 5 chicken *Salmonella* Typhimurium isolates was a 100% identical in PFGE profile to some human *Salmonella* Typhimurium isolates but MLVA showed these isolates to be unrelated. PFGE analysis with *XbaI* digestion showed that 2 of the 5 chicken *Salmonella* Typhimurium isolates were a 100% identical by PFGE analysis and MLVA to 1 human *Salmonella* Typhimurium isolate. The current study could not confirm that there is an epidemiological relationship between chicken and human isolates; however results suggest that *Salmonella* strains with similar molecular profiles circulate in the animal and human communities, supporting the suggestion of human-to-animal transmission or animal-to-human transmission. Foodborne diseases are not historically fully investigated in South Africa and further work is required to confirm this theory.

**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 *Xba*I is our primary molecular method for subtyping *Salmonella* Typhi. DNA fingerprint patterns that are produced are analyzed with BioNumerics (version 5.1) software. This software generates dendrograms of the different patterns which in turn displays 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 of 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 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 dependant on the expression of flagella 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* possess two structural genes (*fliC* and *fliB*) for flagellin, the component protein for flagella 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 were 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.

**INTERNATIONAL VISITS & MEETINGS ATTENDED**

Miss Mimmy Ngomane travelled to Statens Serum Institut in Copenhagen, Denmark, February 24-29 2008 the WHO reference Centre for *Escherichia coli* as well as NICD suppliers of antisera and *Escherichia coli*.

Dr Karen Keddy attended the CDC GSS Strategic meeting and ICEID March 15-19 2008.

Dr Anthony Smith attended the 1<sup>st</sup> PulseNet International Strategic Meeting, Halifax, Nova Scotia, Canada June 2008.

Dr Karen Keddy attended the first Food and Waterborne Diseases meeting of European Centres for Diseases Control, Stockholm, Sweden from 1 - 2 October, 2008.

Dr Karen Keddy presented a poster at Ehrlich II, the 100<sup>th</sup> Anniversary of the Magic Bullet, in Nuremberg, Germany, from 3- 5 October 2008.

Dr Karen Keddy attended the WHO Foodborne Disease Burden Epidemiology Reference Group (FERG) meeting in Geneva 17-21 November 2008.

Dr Karen Keddy attended the BITS Life Science conference in Foshan, China from 1- 5 December 2008.

**CAPACITY BUILDING**

EDRU has assisted in the training and supervision of FELTP students.

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.

EDRU co-hosted the Principal Investigators meeting for Enhanced Surveillance on 5-6 November 2008.

### POST-GRADUATE STUDENTS:

Ananta Nanoo (MSc Epidemiology, University of the Witwatersrand)

Masters Dissertation: The impact of cotrimoxazole usage in HIV on cotrimoxazole resistance in non-typhoidal *Salmonella*

Rugola Mtandu (MSc Epidemiology, University of the Witwatersrand)

Masters Dissertation: The impact of HIV on clinical-microbiologic features and mortality among patients with invasive non typhoidal *Salmonella* infection in South Africa. (Graduated in December 2008)

Nevashan Govender (MSc)

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

Sarika Dwarika (MSc)

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

Brett Archer (Masters in Public Health (Specialization in Epidemiology and Biostatistics), University of Pretoria.

Research Report: Epidemiology of typhoid fever in South Africa, 2003-2007 (submitted).