THE APPLICATION OF GENETIC TECHNIQUES IN COMMUNITY HEALTH SURVEILLANCE.

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This original thesis has been submitted in order to meet the requirements for the degree Master of Medical Science in the Faculty of Medical Sciences at the University of the Orange Free State.

Bloemfontein, 1999

DECLARATION

I declare that the dissertation hereby handed in for the degree of M. Med. Sc. at the University of the Orange Free State, Bloemfontein, is my own independent work and that I have not previously submitted the same work for a degree at/in another university/faculty.

Kim Stanley

December 1999

ACKNOWLEDGEMENTS

I would like to extend my sincerest thanks to the following persons and institutions for their help:

Prof. Lynda Chalkley, my supervisor, for her encouragement, help, guidance and support during the past years.

Emmolina Venter, Philip Matthee, Anneke van der Spoel van Dijk, Marisa Pienaar and Jaco van Niekerk for preliminary/initial studies on *N. gonorrhoeae* and *M. tuberculosis.*

SAIMR, Kimberley for the rifampicin-resistant *M. tuberculosis* strains.

Public Health Laboratory, Bloemfontein for sputum samples.

The Free State Technicon for the collection of water samples.

The National Research Foundation for grant and running costs.

DEDICATION

To my family for their support and love.

ABSTRACT

The study was designed to investigate the application of a range of genetic methods for the detection and monitoring of bacterial pathogens responsible for key Free State community health issues. The rapid detection and differentiation of potentially pathogenic organisms from water sources is vital for the safety and the state of health of many. Conventional culture methods can be complex and time-consuming, whereas detection by the polymerase chain reaction (PCR) is rapid but could be impaired due to regional strain sequence variations and detection of dead cells. *Neisseria gonorrhoeae* is treated syndromically in South Africa. To ensure continued efficacy of antibiotics, resistance development and plasmid content (β-lactamase type or *tetM*-conjugative type) are important factors to be monitored. The study of pulmonary tuberculosis, which has re-emerged as a significant problem in the developed as well as the developing world, is greatly benefited by genetic techniques. DNA fingerprinting is a powerful method and may be used in the context of *Mycobacterium tuberculosis* surveillance for determining transmission versus reactivation rates and for following patient compliance. The first-line antibiotics employed against *M. tuberculosis* in the Free State are isoniazid (INH), rifampin (RIF), ethambutol and pyrazinamide. Resistance to rifampin is known to arise as a result of missense and other mutations occurring in a discrete 23 amino acid region (69 bp) of the *rpoB* gene. Detection of such mutations can be performed by PCR-based methods.

The objectives of the study were as follows: (1) surveillance of community and environmentally acquired infections including waterborne pathogens (conventional and PCR detection techniques), *N. gonorrhoeae* (randomly amplified polymorphic DNA [RAPD] and plasmid analysis) and *M. tuberculosis* (genomic fingerprinting); (2) to determine antibiotic susceptibilities of *N. gonorrhoeae* and *M. tuberculosis;* (3) to investigate the acquisition and dissemination of tetracycline resistance in *N. gonorrhoeae* and the development of rifampin antibiotic resistance in *M. tuberculosis* (*rpoB* gene sequencing).

One hundred and five water samples (shaken and brushed from containers, sewage effluent and river water) were collected during March – May 1999. The detection of waterborne pathogens *Escherichia coli, Shigella* sp., *Salmonella* sp. and *Listeria monocytogenes* was performed by the widely versatile PCR technique. The primer sets were designed to detect the verotoxin genes of Enterohaemorrhagic *E. coli* (EHEC), the invasive plasmid antigen gene of *Shigella* sp. and Enteroinvasive *E. coli* and the enterotoxin gene of *Salmonella* sp. A final primer set was used to amplify the listeriolysin O gene of *Listeria monocytogenes*. Where possible the suitability of primers against local clinical strains was shown to be successful. Selective and enrichment media was employed to provide presumptive confirmation of the detection of the pathogens by PCR. PCR detection revealed four cytotoxic *E. coli,* seven *ipaH Shigella* sp., ten enterotoxin *Salmonella* species, and thirteen listeriolysin *Listeria monocytogenes* strains in the waters examined. Culture confirmed only a single *Salmonella* sp. This indicated a higher potential for rapid detection (compared with conventional culture methods) of waterborne pathogens by PCR especially when the bacteria could have entered a non-culturable but viable state. The problem of residual DNA from non-viable bacteria being detected by PCR is still a setback to this particular genetic technique. The detection of four verotoxin containing EHEC, followed by the inability to confirm the *E. coli* serovar 0157:H7 (culture, immunomagnetic separation and latex agglutination) emphasises the dangers in concentrating efforts to detect only one specific serovar when screening water samples.

The *N. gonorrhoeae* investigated were isolated from the Bloemfontein community during 1993-1997. To overcome the problems and difficulties in speciating and strain typing *Neisseria* for epidemiological surveillance, RAPD surveillance analysis was performed. The primer used had been shown to exhibit excellent discriminatory power for the differentiation of *N. meningitidis* strains. The results (significantly enhanced by RAPD analysis beads) showed that this analysis can be used to augment auxotype/serovar typing of *N. gonorrhoeae* populations. With observed shifts in clinical isolation sites of *Neisseria* species, the RAPD technique has potential use for taxonomic studies of *Neisseria*. Investigations into tetracycline resistance development in *N. gonorrhoeae* were performed by

amplification of *tetM* genes by PCR. The PCR products were digested with *Hpa*II and the fragments separated on agarose gels. Plasmid analysis was performed using a plasmid Miniprep DNA purification system. *TetM*-conjugative and conjugative plasmids were restricted with enzymes *Bgl*I, *Sma*I and *Hinc*II and fragments separated on agarose gels. The conjugative (24.5 MDa) plasmid was present in 29/102 (28.4%) strains while the *tetM*-conjugative (25.2 MDa) plasmid was present in 48/102 (47%) strains. The Bloemfontein *N. gonorrhoeae* strains carried both African and Asian β–lactamase plasmids. Seventy percent of strains showed increased tetracycline resistance (≥ 2 µg/ml) while 42% of strains exhibited high-level (16-128 µg/ml) resistance. The restriction of *tetM*-conjugative and conjugative plasmids isolated in 1996 revealed different profiles to those previously described showing that these plasmid types are continuing to evolve. Amplification of a fragment of the *tetM* gene provided a simple and quick method for predicting high-level tetracycline resistance. On restricting the 43 high-level tetracycline-resistant strains (MICs ≥ 16 µg/ml) all were found to contain the American-type *tetM* gene and 25.2 MDa plasmids were demonstrated. The establishment of *tetM*-conjugative plasmids containing the American-type *tetM* gene is increasing, 2% in 1994 to 47% in 1997.

Three hundred and thirteen sputum samples were collected from the Rocklands community in Bloemfontein. Subsequent sputum samples were collected to monitor community response to reassessment and to ensure eradication. Detection of *M. tuberculosis* (MTB) was accomplished by Ziehl-Neelsen (ZN) staining and conventional culture on Löwenstein-Jensen (LJ) agar slopes. Thirty three sputum samples were ZN positive, with LJ detecting an additional 7 *M. tuberculosis* isolates. Discrepancies in ZN and LJ results were confirmed by amplifying a 123 bp fragment of the IS*6110*. PCR also indicated the need for additional diagnostic methods as 11% of isolates were not detected by ZN or LJ. The BACTEC system was used for confirmation as well as for susceptibility testing. Only 63% of persons receiving treatment returned after 1-3 months indicating possible non-compliance. A single patient (old case) had a maintained ZN positive result for 6 months with full susceptibility to all antibiotics tested. The standard method of fingerprinting involved *Pvu*II restriction endonuclease

digestion of genomic DNA followed by Southern blotting and probing for IS*6110* elements. The fingerprinting of 50 INH -and/or RIF-resistant strains from 1997 revealed 32 diverse profiles. Non-adherence and the emergence of resistant clonal groups were evident. Five clonally related clusters were evident that were either localised or had disseminated to different districts in the Free State. Of 26 person's initial samples (ZN+/LJ+) investigated in 1998, 25 diverse fingerprint profiles were found. Fingerprinting of 11 rifampicin-resistant strains (1998) showed the emergence of many diverse resistant strain types. The possible spread of TB in a hospital ward was revealed through shared fingerprint profiles of two samples. The monitoring of rifampin resistance through sequencing of a key region 157 bp of the *rpoB* gene was performed. Previously reported mutation sites were evident in the study; 516, 526, 531 and 533. The two local 1997 clonal groups (identified by fingerprint profile) did not share mutated *rpoB* alleles. This could possibly be explained by clonally related susceptible strains independently developing sub-clones bearing distinct *rpoB* alleles. Inaccuracies in susceptibility testing were evident as a Bloemfontein strain reported to be rifampin-susceptible presented with a variant *rpoB* allele. From 13 MTB (new cases, 1998) screened for the *rpoB* gene and subsequently sequenced it was found that two ZN/LJ positive samples had missense mutation at positions 516 and 526. A reduced outcome would result with these patients emphasising the need for accurate susceptibility testing to be conducted earlier than presently stipulated. Eleven rifampin-resistant strains (1998) revealed only one strain without *rpoB* gene mutations in the 157 bp region examined. The same mutated codon was evident with two strains (with shared fingerprint profile from same hospital ward) again strongly implying dissemination of a strain type between patients. A family community from a semi-rural area (Bainsvlei) situated 15 km from Bloemfontein was investigated. The Bainsvlei family member's samples from 1995, 1997 and 1998 revealed the same fingerprint profile (shared by other family members in 1995) and same mutated *rpoB* codon indicating the persistence of a rifampin/isoniazid-resistant strain. Subsequent information on the brother's past MTB infections and treatment showed that a possible reinfection of a multiply-resistant strain could have occurred. The situation has not been fully resolved due to lack of community involvement and funding.

Genetic techniques investigating infectious diseases in the community setting certainly provides required rapid results and epidemiological information essential for the future success of infection control programmes.

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ABBREVIATIONS

BHI	Brain heart infusion
bp	Base pairs
BPB	Bromophenol blue
CDC	Centers for Disease Control and Prevention
co	Contaminated
CTA	Cystine-tryptic digest agar
Da	Dalton
DOTS	Directly Observed Treatment Short-course
EHEC	Enterohaemorrhagic *E. coli*
EIEC	Enteroinvasive *E. coli*
EMB/ETHAM	Ethambutol
EPEC	Enteropathogenic *E. coli*
ETEC	Enterotoxigenic E. *coli*
FC	Final concentration
FIG.	Figure
GC	Gonococcal
HIV	Human Immunodeficiency Virus
HL	Heat labile
INH	Isoniazid
IS	Insertion sequence
kb	Kilobases
KDa	Kilodalton
LCR	Ligase Chain Reaction
LJ	Löwenstein-Jensen
MDa	Megadalton
MDR TB	Multi-drug Resistant TB
MOF	Modified oxidation-fermentation
MTB	*Mycobacterium tuberculosis*
MTSB	Modified tryptone soy broth

(List of abbreviations continued)

MWM	Molecular weight marker
NAP	ρ-Nitro-α-acetylamino-β-hydroxypropiophenane
NCCLS	National Committee for Clinical Laboratory Standards
PCR	Polymerase Chain Reaction
PZA	Pyrazinamide
RAPD	Randomly Amplified Polymorphic DNA
RFLP	Restriction fragment length polymorphism
RIF	Rifampicin
SLT	Shiga-like toxin
SMAC	Sorbitol MacConkey agar
SM/STREPT	Streptomycin
ST	Heat stable
TB	Tuberculosis
UK	United Kingdom
USA	United States of America
VT	Verotoxin
VTEC	Verotoxigenic *E. coli*
WHO	World Health Organisation
XLD	Xylose lysine decarboxylase
ZN	Ziehl-Neelsen

CHAPTER 1
INTRODUCTION

1.1 GENERAL

Techniques employing the polymerase chain reaction (PCR) are uniform, the only variables being primers (readily on hand once assessed) and cycling conditions (easily altered). PCR has several advantages over probe techniques. For probe detection to be successful many hybridisation methods require a large amount of cells per colony, whereas PCR requires only a small amount of target DNA (Hill, 1996). The PCR method provides a rapid alternative, as it does not require growth of cells prior to detection. This may be extremely helpful in screening water samples that contain viable but non-culturable cells (Hill, 1996). In addition, if the PCR primers are genus and/or species specific bacterial identification can be performed without the requirement for extensive biochemical reactions. A serious problem concerning detection by polymerase chain reaction is that dead cells can yield positive results if the segment of DNA involving the primer sites is intact. The effect of false positive results may be reduced by conducting a brief growth step (3-5 cell doublings) followed by dilution before undertaking PCR (Varnam & Evans, 1991). This method is useful only if the level of dead cells is relatively constant. Traditionally, identification of bacteria, meant isolation and propagation steps. Growth of the organisms being the prerequisite for various identification tests to be performed. The rapidly expanding use of genetic techniques involving PCR for not only phylogenetic, evolutionary and diagnostic studies offers an opportunity for alternative epidemiological approaches.

1.2 WATERBORNE PATHOGENS

1.2.1 Pathogenic Bacteria Investigated

The study focused on *E. coli*, *Shigella*, *Salmonella* and *Listeria* although further pathogenic bacteria that are considered major waterborne pathogens include *Yersinia*, *Vibrio*, *Campylobacter* and *Helicobacter* species.

Escherichia is the type genus of the family *Enterobacteriaceae,* while *Escherichia coli* is the type species (Howard, 1994). It is the most common aerobic organism in the gastrointestinal tract of humans and many other animals and therefore faecal pollution of water is rife. Symptoms vary according to the type of *E. coli* infection. Five groups of *E. coli* strains exist: the enteropathogenic *E. coli* (EPEC), enteroadherent-aggregative, enteroinvasive (EIEC), enterohaemorrhagic (EHEC) or verotoxigenic (VTEC) and enterotoxigenic (ETEC) (Varnam & Evans, 1991). The latter two groups are most frequently the cause of diarrhoeal disease throughout the world (Lang *et al*, 1994). The *E. coli* strains of EHEC, ETEC, EIEC and EPEC groups all contain toxigenic genes (Varnam & Evans, 1991). ETEC produce heat labile (LT) and heat stable (ST) enterotoxins, EHEC produce Shiga-like toxins (SLT), EIEC produce toxins similar to SLTs and EPEC has been reported to have forms of all toxin types (Olsvik *et al*, 1993; Read *et al*, 1992; Varnam & Evans, 1991). The highest death rates reported are linked to the serovar O157:H7 of the EHEC group, which causes haemorrhagic colitis with bloody diarrhoea (Read *et al*, 1992). Outbreaks of *E. coli* O157:H7 have been traced not only to contaminated food, but to drinking water and lake water (Dev *et al*, 1991; Keene *et al,* 1994; Yu & Bruno, 1996). After an outbreak of EHEC in the USA in 1993, the US Department of Agriculture Food Safety and Inspective Service developed a series of presumptive and confirmatory tests for the detection of *E. coli* O157:H7 (Yu & Bruno, 1996). Other common *E. coli* serotypes besides 0157 belonging to the haemorrhagic category are 026 and 011 (Varnam & Evans, 1991). The *E. coli* strains designated EIEC possess invasive properties and are antigenically related to

Shigella species (Olsen *et al*, 1995).

The genus *Shigella* consists of four species; *Sh. dysenteriae* (subgroup A), *Sh. flexneri* (subgroup B), *Sh. boydii* (subgroup C) and *Sh. sonnei* (subgroup D) (Cowan & Steel, 1993). *Shigella* can cause either classic bacillary dysentery (subgroup A), mild diarrhoea with additional symptoms (subgroup B, C) or only diarrhoea (subgroup D) (Varnam & Evans, 1991). The most severe form of shigellosis is caused by *Sh. dysenteriae* type I and the Shiga toxin it produces may contribute to the necrotic lesions of the colon and probably to the diarrhoea associated with this disease (Howard, 1994). *Shigella* species can contain large plasmids carrying invasive genes (Olsen *et al*, 1995).

The genus *Salmonella* is another typical member of the family *Enterobacteriaceae* with serovars adapted to man and animals (Varnam & Evans, 1991). Symptoms include typhoid or enteric fever (*S. typhi*), gastroenteritis or salmonellosis (*S. typhimurium*) and extraintestinal infections (Howard, 1994). Invasive ability is essential for the development of diarrhoea with *Salmonella* producing at least three enterotoxins, and at least one endotoxin and cytotoxin (Varnam & Evans, 1991).

Of the seven *Listeria* species currently recognised, *L. monocytogenes* is associated with disease in man (Varnam & Evans, 1991). Ingestion of *L. monocytogenes* results in listeriosis characterised by meningitic listeriosis (affecting newborns and children), foetal, septicaemic and oculoglandular listeriosis (Varnam & Evans, 1991). Virulence is associated with listeriolysin (a 58 KDa protein of the streptolysin O family) the gene of which resides in the chromosome (Varnam & Evans, 1991).

1.2.2 Conventional Methods Employed to Detect Waterborne Pathogens

Isolation of specific bacteria from the environment can be a complex and time-consuming procedure, often involving pre-enrichment, enrichment and selective plating. Sensitivity (the ability to recover the bacteria for which the medium was

designed) and selectivity (the ability to prevent the growth of bacteria other than those for which the medium was designed) are two important points that have to be taken into account to improve isolation of the required bacterial species.

In order to resuscitate *E. coli* isolates nutrient broth or nutrient agar can be used (Varnam & Evans, 1991). In addition to particular selective enrichment media e.g. MacConkey broth, the incubation temperature should be elevated to increase sensitivity and reduce incubation time. Some strains of *E. coli* O157:H7 do not, however, grow at such temperatures and may lose virulence plasmids (Varnam & Evans, 1991). Selective plating involves differentiation of lactose fermenters but an increasingly large percentage of pathogenic strains of *E. coli* fail to ferment this sugar. Both non-lactose and lactose fermenters should be selected from conventional media for further testing, necessitating the then time-consuming task of screening a large number of colonies (Varnam & Evans, 1991). EIEC strains often have atypical biochemical characteristics, being lactose negative or with slow lactose reaction and are often regarded as non-motile (Olsen *et al*, 1995). Failure to ferment sorbitol and failure to produce beta-glucuronidase are characteristics of serovar O157:H7, two differential features exploited in selective isolation media (Howard, 1994). Competing flora may obscure *E. coli* 0157:H7 colonies thereby giving rise to false negative results.

On general purpose selective media colonies of *Shigella* may be difficult to differentiate from *Proteus* species. Specifically designed media e.g. Salmonella/Shigella agar results in colonies that are readily confused with those of H_2S negative strains of *Citrobacter* and *Proteus*. Selenite containing media is most commonly used for the selective enrichment of *Salmonella* as the incorporation of cystine enhances growth. Selective media, Brilliant Green, XLD, Hektoen contain in addition to inhibitors, "diagnostic" differentiation systems for preliminary divisions of lactose/sucrose fermenters and non-fermenters (Varnam & Evans, 1991).

Isolation of *Salmonella* follows a similar process as *Shigella.* Likewise on a selective agar such as XLD it is also hard to differentiate between colonies of *Salmonella* and *Proteus*. Rapid culture methods for *Salmonella* species are

tending to replace conventional methods as the latter are too time consuming (up to 5 days). These improved methods include commercial kits e.g. Salmonella Rapid Kit and immobilisation of *Salmonella* by flagellar antibodies.

No single enrichment technique can recover all strains under all circumstances. This is particularly true for *Listeria monocytogenes*. A choice has to be made if low temperatures and non-selective media or higher temperatures and selective media are to be used for the enrichment procedure (Varnam & Evans, 1991). Broths such as Hayes enrichment, University of Vermont and Fraser are commonly used (Varnam & Evans, 1991). Fraser broth involves a two stage enrichment procedure which although minimizing inhibition of *Listeria monocytogenes* during enrichment, the extra manipulations involved have restricted application (Varnam & Evans, 1991).

To detect and confirm the presence of pathogens in water supplies therefore requires numerous types of media, identification kits and antisera. There is still no consensus as to which is the best media to use for each of the above mentioned pathogens. This together with the fact that each of these bacteria requires several time-consuming steps to culture and identify, means that the infective agent may well be long gone before reported. Besides traditional cultural techniques, serological techniques and enzyme linked immunoabsorbent assays and genetic techniques such as DNA hybridisation and polymerase chain reaction (PCR) can be used (Howard, 1994). These are highly specific and sensitive means of detection, however, the problem of false positives can not be easily overcome (Varnam & Evans, 1991).

1.2.3 PCR Techniques

E. coli serves as an object of intense basic genetic study and so considerable work has been conducted on the application of PCR-based detection and identification methods on strains of this pathogenic species (Hill, 1996). PCR has been used to demonstrate the presence of genes encoding for the production of both enterotoxins and cytotoxins or verotoxins and recently, adhesion factors

from strains of *E. coli* (Olsen *et al*, 1995). The use of the primers responsible for the amplification of a 322 bp fragment from the LT gene have been extensively reported, with different hybridisation techniques being developed to confirm detection and identification through PCR (Olive, 1989). Tamanai-Shacoori *et al* (1992) succeeded with this, by performing a PCR reaction to amplify the LT gene, followed by hybridization with a LTp probe, prepared from plasmid DNA and restricting with *Hind*III. This method proved to be extremely specific as visible PCR bands were obtained with only a single LT positive bacterium. Primers STIa and STIb are commonly used to achieve amplification fragments from heat stable toxins of ETEC (Olsen *et al*, 1995; Lang *et al*, 1994). The primer set ES-151 and ES-149 were used to target conserved sequences in verotoxins (VT) 1, 2 and variants of the VTEC (Read *et al*, 1992).

Due to the genetic similarities between *Shigella* and EIEC, many PCR methods have been developed that detect both genera (Schoolnik, 1993; Ye *et al,* 1993). These detection methods use primers associated with invasive loci, insertion sequences, toxin genes and outer membrane proteins. A common PCR target is a region of the invasive gene (*ial*) associated locus. This technique has been reported as being simple, sensitive and specific enough for routine diagnosis (Ye *et al*, 1993). In a similar vein, simple PCR procedures have been performed to detect DNA sequences from the invasion plasmid antigen *ipaH* (Gaudio *et al*, 1997). The outer membrane protein (PhoE) of members of the family *Enterobactericeae*, consists of conserved and hypervariable regions. Two oligonucleotide primers based on DNA sequences encoding two different cell surface exposed regions of the *E. coli* K12 PhoE protein, have been designed for specific detection *of E. coli, Shigella* and even *Salmonella* (Spierings *et al*, 1993). The specificity was good, whereby only an *Escherichia fergusonnii* (closely related to *E. coli*) strain out of a group of other *Enterobactericeae*, reacted with the probes.

A favourite target for PCR based amplification of *Listeria monocytogenes* is the *hly*A (listeriolysin O) gene (Hill, 1996). A novel multiplex PCR using three specific primers namely LI1, LM1/LM2, has been developed (Siggens, 1995). Primer LI1 amplifies a DNA region of the 16S rRNA that is specific to the genus *Listeria* and

LM1 and LM2 primers derived from the listeriolysin O gene are specific *for L. monocytogenes* (Siggens, 1995).

1.3 *NEISSERIA GONORRHOEAE*

1.3.1 Background

N. gonorrhoeae infection has been treated syndromically in South Africa since 1993. Different first line antibiotics are administrated in different provinces. Since its introduction as a first line treatment in 1992, a quinolone such as ciprofloxacin has been widely used in South Africa and Bloemfontein. Already the loss of two previously effective and cost efficient antibiotics, penicillin and tetracycline has occurred.

1.3.2 Identification and Speciation for Surveillance Studies

Neisseria are becoming progressively more difficult to speciate and to strain type for epidemiological surveillance. *N. gonorrhoeae* and *N. meningitidis* strains are establishing themselves in each others previously defined clinical sites (Winterscheid *et al,* 1994; Gregory & Abramson, 1971; Galaid *et al*, 1980). It can no longer be predicted with any certainty that a genitourinary/anal isolate will be *N. gonorrhoeae*, as *N. meningitidis* has been associated with generalised defined clinical sites (Morello *et al*, 1991). Likewise oropharyngeal gonorrhoea may produce acute pharyngitis or tonsillitis (Morello *et al*, 1991). Physiologically similar *Moraxella catarrhalis* has also been found in cases of neonatal conjunctivitis (Navarro *et al*, 1993).

Differentiation between *N. gonorrhoeae*, *N. meningitidis* and closely related bacteria (such as *Moraxella catarrhalis*) can be achieved by several methods: identification systems such as API NH (bioMérieux, sa, France), where 12 identification tests plus the detection of penicillinase is performed, modified growth media, serological based tests and finally molecular techniques such as randomly amplified polymorphic DNA (RAPD) analysis. The biochemical differentiation between *N. meningitidis* and *N. gonorrhoeae* is limited as it is

based primarily on glucose and maltose reaction.

As a means of confirming species of *N. meningitidis* and *N. gonorrhoeae*, serological tests have been recommended (Sarafian & Knapp, 1989). Serological classification is achieved by using polyvalent or monovalent antibodies (Poh *et al*, 1996). This requires the keeping of expensive antisera and identification may still remain elusive if the strain is untypable or cross- / non-specific reactions occur (Poh & Lau, 1993; Knapp *et al*, 1984).

Employing the RAPD method, provides an alternative means of discriminating between closely related strains (Welsh & McClelland, 1993). Other genetic methods such as pulsed-field gel electrophoresis and ribotyping can be time-consuming, expensive and labour intensive. Primers for the standard PCR assays are chosen arbitrarily with annealing to the genome performed at low stringency (Welsh & McClelland, 1993). Primers of 10 bases tend to produce fewer PCR products than primers of 18 or more bases (Welsh & McClelland, 1993). Subsequent PCR results in the amplification of sequences bounded by these low stringency-annealing events (Welsh & McClelland, 1993). If a RAPD profile is not reproducible it could be due to differences in the primer-to-template ratio, annealing temperature and magnesium concentration (Hill, 1996). The RAPD technique not only provides a sensitive means of discrimination and identification, but also can be developed into a method for fast data collection for use in population genetics. This technique therefore has the potential to assist in the definitive identification of *N. gonorrhoeae*, *N. meningitidis* and *M. catarrhalis* and possibly be applied in the differentiation of *N. gonorrhoeae* strains.

1.3.3 Plasmid Analysis

N. gonorrhoeae is one of several bacterial species that has undergone sub-typing studies through plasmid analysis i.e. the comparisons of plasmid profiles. The development of rapid and inexpensive techniques for extracting plasmid DNA and separating plasmids by electrophoresis has led to the widespread use of plasmids in epidemiological investigations (Smaminathan & Matar, 1993).

Knowledge of a strain's plasmid content and associations is essential not only for epidemiological purposes, but for the surveillance of antimicrobial resistance as plasmids may carry drug resistant genes or code for virulence factors.

β-Lactamase plasmids are endemic in isolates from North America, the Caribbean, Europe, Asia and Africa (Roberts, 1989). *N. gonorrhoeae* carries a TEM type - lactamase which is active against benzylpenicillin, ampicillin and cephaloridine substrates (Heffron *et al*, 1977; Roberts, 1989). This β-lactamase is present in a TnA region. β-Lactamase plasmids that have been described in *N. gonorrhoeae* strains include the 2.9, 3.05, 3.2 and the 4.4 MDa plasmid (Roberts, 1989).

The 24.5 MDa conjugative plasmid was first observed in 1974 in penicillinase and non-penicillinase producing strains of *N. gonorrhoeae* (Roberts, 1989). The 24.5 MDa plasmid may be present alone or co-exist with 2.6 MDa and gonococcal β-lactamase plasmids (Roberts, 1989). The 24.5 MDa conjugative plasmid does not carry detectable markers for antibiotic resistance, while the 2.6 MDa (cryptic) plasmid has an unknown function (Roberts, 1989).

N. gonorrhoeae strains containing the 25.2 MDa *tetM*-conjugative plasmid have been isolated in North America, Europe, Great Britain and the Netherlands (Roberts, 1989). Strains in the Netherlands and North America in addition to the 2.5 MDa plasmid have also been shown to carry the 3.2 MDa β-lactamase plasmid and 2.6 MDa cryptic plasmid (Roberts, 1989). The 25.2 MDa plasmid was formed by the transposition of the *tetM* determinant, encoding tetracycline resistance onto the 24.5 MDa plasmid. The 25.2 MDa plasmid was initially associated with the 3.2 MDa β-lactamase plasmid, therefore it was thought that the 25.2 MDa plasmid was created in a *N. gonorrhoeae* population which carried the 3.2 MDa plasmid (Roberts, 1989). The first isolates of *N. gonorrhoeae* shown to carry the 25.2 MDa tetracycline resistance conjugative plasmids in southern Africa were isolated in 1993 (Chalkley *et al*, 1997).

Both the 25.2 and the 24.5 MDa conjugative plasmids can mobilise the

gonococcal 4.4 and 3.2 MDa β-lactamase plasmids to a variety of bacterial species (Roberts, 1989). However, when the 24.5 MDa plasmid is used to mobilise the β-lactamase plasmids, only *N. gonorrhoeae* and some of the *N. cinerea* transconjugates receive or maintain the 24.5 MDa plasmid (Genco *et al*, 1984).

1.3.4 Tetracycline Resistance

Chromosomal-encoded tetracycline resistance is mediated through three different mechanisms; energy dependent efflux of tetracycline, chemical alteration of the tetracycline molecule rendering it inactive and ribosomal protection by reduced binding of tetracycline to the bacterial ribosome (Chopra *et al*, 1992). Resistance conferred by removal of tetracycline is mediated by *tet* genes A-F, K and L encoding for efflux proteins (Chopra *et al*, 1992). The Class X resistant determinant is responsible for chemical alteration of the tetracycline molecule and classes M, O and Q for ribosomal protection (Chopra *et al*, 1992). Classes M and O are the only tetracycline-resistant genes to be located on the plasmid and chromosome, while Class Q is found solely on the chromosome, associated with a conjugative transposon.

Ribosomal protection is the predominant mechanism of resistance in *Neisseria* species conferred by the Class M tetracycline-resistant determinant (*tetM*). The TetM determinant encodes for a protein, which appears to protect the translation apparatus (Bäckman *et al,* 1995). It has been reported that TetM products may have ribosome-dependent guanosine triphosphatase activity or that they are capable of acting in a catalytic manner by modifying ribosomal proteins or rRNA (Chopra *et al,* 1992). The effect of the *tet* locus in *N. gonorrhoeae* is to confer low-level resistance resulting in MICs of ≥ 2 μg/ml but seldom exceeding 8 μg/ml (Ison *et al,* 1993).

Plasmid-mediated high-level resistance to tetracycline was first reported in 1985 (Bäckman *et al*, 1995). The 25.2 MDa conjugative plasmid carrying the *tetM*

gene is responsible for mediating high-level resistance (MICs 16 –128 µg/ml) in *N. gonorrhoeae* (Gascoyne *et al*, 1991). The prototype for *tetM* is the 5 kb fragment cloned from a *Streptococcus agalactiae* strain (de Barbeyrac *et al*, 1996; Roberts *et al*, 1986). Rapid detection of plasmid-mediated high-level TetM resistance to tetracycline in *N. gonorrhoeae* has been developed by means of PCR amplification (Ison *et al*, 1993). The PCR screening method proposed by Ison *et al* (1993) produces a *tetM* gene fragment of 765 bp. No false positives were found with strains exhibiting chromosomal resistance to tetracycline (Ison *et al*, 1993). Restriction endonuclease analysis of the *tetM* gene and the *tetM*-conjugative plasmid has revealed different patterns, the Dutch and the American type (Gascoyne-Binzi *et al*, 1993). Two new 25.2 MDa TetM-encoding plasmids have been demonstrated in South Africa on the basis of restriction site analysis (Chalkley *et al*, 1997). One was similar to the American type plasmid and a second resembled an American/Dutch hybrid (Chalkley *et al*, 1997).

1.4. *MYCOBACTERIUM TUBERCULOSIS*

1.4.1. Background

Mycobacterium is the only genus of the family *Mycobacteriaceae.* The distinguishing characteristics of this genus include acid fastness and the presence of mycolic acid (Howard, 1994). Of the thirty-one described species, *Mycobacterium tuberculosis* (MTB) that causes the disease tuberculosis (TB) is the most recognised (Howard, 1994). A third of the world's population has been infected with *M. tuberculosis* (Blumberg, 1995). In Africa 7.4 million new cases and South Africa 158 689 new cases were reported in 1995 (Medical Research Council, 1996). In addition to these startling statistics, resistance development in MTB is threatening efforts to control the disease. In a global surveillance, the World Health Organisation found antituberculosis drug resistance in 35 countries and regions, indicating the problem is wide spread and of international concern (Pablos-Mendez *et al*, 1998).

Tuberculosis is considered the most important opportunistic infection in patients infected with the human immunodeficiency virus (HIV) (Blumberg, 1995). In the western Cape in 1996, 4% of all TB clinic attenders were HIV positive and in the absence of active HIV screening, this percentage was considered an underestimate (London, 1996). In 1996 in South Africa, 27% of persons infected with HIV were diagnosed with tuberculosis (Medical Research Council, 1996). In countries such as in Africa, where high rates of both exist, HIV and TB are regarded as dual epidemics threatening to overwhelm already strained health care systems (Blumberg, 1995).

1.4.2 Laboratory Diagnosis

Sputum microscopy for acid fast bacilli is the foundation of laboratory diagnosis and of any TB control programme, as a positive smear identifies individuals who

are most infectious, and therefore those that carry the greatest burden of disease (Blumberg, 1995). MTB microscopy is not only performed for diagnosis of TB, pre-treatment microscopy is also used to assess response to treatment and to diagnose cure, by repeating sputum examination two months after treatment and at the end of treatment (Blumberg *et al,* 1997). A standardised recording and reporting system is used in laboratories to provide information to better the tuberculosis programmes at all levels, national, provincial, regional and district.

M. tuberculosis, despite being slow growers, can be cultured on simple synthetic media. The non-selective culture media include those that are egg-based such as Löwenstein-Jensen (LJ), Petragnani and American Thoracic Society or the agar-based media such as Middlebrook 7H10 and 7H11 (Shinnick & Good, 1995). Selective culture media involves the modification and addition of antimicrobial agents such as nalidixic acid and cycloheximide to the LJ and Middlebrook media (Howard, 1994). Inoculated solid media should be checked after 1-2 weeks and thereafter 4-6 weeks for growth before being discarded as negative (Howard, 1994). A commercially available radiometric system (BACTEC, Becton Dickinson, Johnston Laboratories, Md, USA) detects growth of mycobacterium in selective liquid media (Blumberg, 1995). The BACTEC system was first introduced in 1971 with initial systems being BACTEC 226, BACTEC 301 followed by BACTEC 406 (Scrivanos, 1995). The system detects growth by measuring released radioactively labelled CO_2 from ^{14}C palmitic acid (Shinnick & Good, 1995). Results are obtained on average after 4 weeks, but readings can continue for a further 3 weeks. Newer systems have been introduced which are more automated and the substrates are not labelled with radioisotopes. Such techniques use acridium-ester labelled DNA probes and chemiluminescence (Evans *et al*, 1992) or fluorescence such as with the Becton Dickinson MGIT system (Shinnick & Good, 1995).

Rapid direct tests that detect MTB nucleic acid are an appealing alternative for the laboratory diagnosis of tuberculosis (Eisenach *et al*, 1993). The serious limitations of conventional techniques (e.g. time to obtain results) can be overcome by tests such as DNA hybridisation, ligase chain reaction (LCR) and PCR. DNA probe systems are now commercially available which can identify

MTB within a few hours, while target amplification can be completed within five hours (Blumberg, 1995). Descriptions of numerous PCR-based assays for the detection and identification of *Mycobacterium* species have been published (Shinnick & Good, 1995). One such method involves a segment of DNA repeated in the chromosome of MTB as a target for amplification (Thierry *et al*, 1993). The segment is a 123 bp sequence in IS*6110*, which makes an ideal target for amplification by PCR because of specificity (Eisenach *et al*, 1993).

1.4.3 Strain Typing

Restriction fragment length polymorphisms (RFLPs) were used conventionally, in the context of MTB, to confirm suspected cases of TB transmission (Yang *et al*, 1994) and for the assessment of laboratory contamination, ensuring the relevance of epidemiological results (Torrea *et al*, 1995). Presently RFLP (in combination with Southern blotting and hybridisation with specific probes) is regarded as a powerful tool, which can be used in TB surveillance to establish a number of important facts. These include identifying persistent clones in communities and the monitoring of clonal dissemination of antibiotic resistant strains (Dellagi *et al*, 1993). Information on the pressing issue of antibiotic non-compliance i.e. whether the community acted responsibly can be deduced from DNA fingerprinting. The monitoring of the movement of resistant strains across geographical borders and countries through DNA fingerprinting is necessary in epidemiological studies (Hermans *et al*, 1990; Yang *et al*, 1994). A key factor in the surveillance and control of TB, contact tracing, is aided by fingerprinting, as index cases can be identified (Warren *et al*, 1996). The emergence of the AIDS pandemic has caused profound changes in the epidemiological aspects of tuberculosis (Yang *et al*, 1995, Daley *et al*, 1992). RFLP typing can monitor the risk of infection with a defined MTB clone for HIV–seropositive and seronegative individuals (Yang *et al*, 1995).

1.4.4 Treatment Regimens

The goals of antituberculosis chemotherapy are to convert the sputum cultures to negative, prevent the emergence of drug resistance and to assure a complete cure without relapse (Davidson & Le, 1992). To achieve this in the shortest time possible, the original 2 year period required for treatment has been replaced by more effective treatment over 6 months (Davidson & Le, 1992).

Isoniazid (INH) is the most widely used antituberculosis agent. In many respects it is an ideal agent – bactericidal (depending on concentrations), relatively nontoxic, easily administered and inexpensive (Howard, 1994). Rifampin is also bactericidal with most strains of MTB inhibited *in vitro* by concentrations of 0.5 μg/ml. (Centres for Disease Control, 1992). Pyrazinamide (PZA) is active against organisms in macrophages, as it favours the acid environment. PZA is hydrolysed to pyrozinoic acid (the active bactericidal agent by the enzyme PZA aminohydrolase (Centres for Disease Control, 1992). Ethambutol (EMB) is a synthetic agent active only against mycobacteria, by interfering with the synthesis and stabilisation of RNA (Davidson & Le, 1992). Streptomycin was one of the earliest drugs used against MTB (Howard, 1994). It is not only actively bactericidal (interfering with bacterial protein synthesis) but also prevents the emergence of resistant organisms (Davidson & Le, 1992). The recommended treatment of pulmonary TB in South Africa is currently the first line antituberculosis drugs, INH, rifampicin, PZA and EMB in combination for 2 months followed by rifampin and INH for 4 months (Blumberg *et al,* 1997). In cases of multi-drug-resistant MTB (MDR TB), where *in vitro* resistance of TB bacilli to INH and rifampin occurs, a therapeutic regimen of at least 3-5 alternative drugs to which the organism is shown to be susceptible should be administrated (Blumberg, 1995). The alternative drugs available are the 4-fluorinated quinolones – ciprofloxacin, ofloxacin and sparfloxacin; cycloserine and its derivatives including terizidone, kanamycin or amikacin and thiacetazone (Young, 1993). Individualised treatments are characterised by a 4 month intensive phase with a continuation phase of 12-18 months (Davidson & Le, 1992). Patients who are treated unsuccessfully are a major health problem and

their isolation from the community is necessary to prevent spread of untreatable resistant strains (Goble *et al*, 1993). In management of HIV-positive and negative patients with MDR TB a standardised or an individual approach exists when selecting a drug regimen (Chintu & Mwinga, 1999). The use of thiacetazone and the ethical issues involved are two relevant and debatable points (Nunn *et al*, 1991).

The Directly Observed Treatment Short-Course (DOTS) programme, a tuberculosis control strategy designed and implemented to improve treatment completion and outcome (World Health Organisation (WHO), 1997). This control programme involves the persons infected with confirmed MTB being observed taking the combination of short-course anti-TB drugs, by health workers or volunteers. The DOTS strategy is reported to consistently produce 85% cure rates (WHO, 1998). Many countries, such as Korea, have experienced increased cure rates and declines in overall drug resistance since introducing the DOTS strategy, however, no setting in the world has seen rates of MDR TB decrease (Farmer & Kim, 1998). In 1998, WHO adopted the DOTS-plus strategy as first described by Farmer & Kim (1998) for MDR TB. Community based programmes depend on various inputs for their success, these include financial and political commitment from government and province, particularly in developing countries. Infected persons' compliance and co-operation in conjunction with the community is essential for the immediate and long term success of such control programmes.

1.4.5 Antibiotic Resistance

The first-line antibiotics employed against *M. tuberculosis* in the Free State are isoniazid (INH), rifampin (RIF) and pyrazinamide (PZA) and ethambutol (EMB) (Blumberg *et al*, 1997). They are administered together to lower the risk of resistance mutations developing to the individual agents (Howard, 1994). A global antituberculosis drug resistance surveillance performed by the WHO (1994-1997) revealed an overall prevalence of 12.6% for single drug resistance. Resistance to isoniazid (7.3%) or streptomycin (6.5%) was more common than

resistance to rifampin (1.8%) or ethambutol (1.0%) (Pablos-Mendez *et al*, 1998). INH resistance in 1996, in Bloemfontein, was about 20% and when RIF resistance occurred, it was in the vast majority of strains in conjunction with INH resistance (van der Spoel van Dijk *et al*, 1996).

Multi-drug-resistant TB is defined as the *in vitro* resistance of TB bacilli to INH and RIF (Blumberg, 1995). MDR TB is mainly a man-made problem and is a consequence of any of the following factors: use of monotherapy, poor patient or poor doctor compliance, omission of some of the prescribed agents, suboptimal dosage or insufficient number of drugs in a regimen due to cost or access and poor absorption of drugs. (Goble *et al*, 1993; Villarino *et al*, 1992). Several "hot zones" of ongoing transmission of MDR TB have been identified, including Russia, Estonia, Latvia and Côte d'Ivoire (Farmer & Kim, 1998). The prevalence of MDR TB in South Africa and globally is 1-2% (Blumberg, 1995). MDR TB's nosocomial nature poses an epidemiological hazard, with transmission of infection not only to patients but also to health care workers (Young, 1993).

1.5 OBJECTIVES

The objectives of this study were as follows:

1) Surveillance of community and environmentally acquired infections.

a) Waterborne pathogens: conventional and PCR detection techniques.

b) *Neisseria gonorrhoeae*: RAPD and plasmid profiles.

c) *Mycobacterium tuberculosis*: genomic fingerprinting.

2) Determine antibiotic susceptibilities of:

a) Neisseria gonorrhoeae

b) Mycobacterium tuberculosis

3) Investigate the development of antibiotic resistance in:

a) *Neisseria gonorrhoeae:* acquisition and dissemination of tetracycline resistance.

b) *Mycobacterium tuberculosis*: Rifampin resistance

CHAPTER 2

MATERIALS AND METHODS

2.1. WATERBORNE PATHOGENS

2.1.1 Strains

One hundred and five water samples were collected from the Bloemfontein area during March – May, 1999. These included water that was shaken and brushed from containers ("clean"), as well as sewage effluent and river water. The water samples (50 ml) were transferred to sterile centrifuge tubes (50 ml) and centrifuged for 30 min at 3000 **xg**. The supernatant was discarded and 200 µl of the resuspended pellet was divided: A) 100 µl portion was stored directly at – 70°C and B) to 100 µl, 1 ml of Brain Heart Infusion Broth (BHI) (Difco, Detroit, Michigan, USA) was added and the inoculated broth incubated at 27°C for 2 h. Following incubation sterile glycerol (final 10% v/v) was added and tubes stored at – 70°C.

Ten serologically identified clinical strains of *Salmonella* were collected between December 1998 and July 1999. The *Salmonella* were sub-cultured on blood agar plates (Difco) and incubated at 37°C for 18 h. The confluent growth was then inoculated into bacterial preservers (Protect, Technical Service Ltd, Lancashire, UK) which were stored at – 70°C.

2.1.2 Gene Detection by PCR

2.1.2.1 DNA preparation

DNA was extracted from the water sample pellets retrieved and stored directly at – 70°C (see section 2.1.1) by means of the High Pure PCR Template Preparation Kit according to the protocol provided (Boehringer Mannheim, Mannheim, Germany). The pellets were resuspended in 200 µl phosphate buffered saline. Lysozyme at a final concentration of 20 mg/ml was added to the suspension and the tubes incubated for 18 h at 37°C. Subsequently 200 µl of binding buffer (6 M guanidine-HCl, 10 mM urea, 10 mM Tris-HCl, 20% (v/v) Triton X-100, pH 4.4) and proteinase K (final concentration 20 mg/ml) was added

and the mixture incubated for a further 18 h at 55°C. After incubation the samples were mixed with 100 µl isopropanol (99%) and transferred into the filter tubes provided. These in turn were placed in the collection tubes and centrifuged for 1 min at 6000 x**g**. The flowthrough was discarded and 1 ml of Wash buffer (20 mM NaCl, 2 mM Tris-HCl, pH 7.5) was added to the filter tube. Centrifugation followed for 1 min at 6000 x**g**. The wash and centrifugation steps were repeated. To remove residual wash buffer the samples were then centrifuged for 10 secs at 14 000 x**g**. The nucleic acid retained on the filter was then eluted by adding 200 µl of elution buffer (10 mM Tris, pH 8.5) and collected after centrifugation for 1 min at 6000 x**g**. DNA preparation was stored at 4°C until required.

A bench lysis procedure (see section 2.2.1.2) was performed on selected pure isolated *Listeria* and *E. coli* colonies. The resulting DNA lysates were stored at 4°C until needed.

2.1.2.2 Primers

The primer set EVC-1 and EVC-2 (TaKaRa Biomedicals, Otsu, Japan) was used to detect the VT genes of Enterohaemorrhagic *E. coli,* PCR amplification product 171 bp. The primer set IPA-1 and IPA-2 (TaKaRa Biomedicals) was used to amplify the detectable *ipaH* gene of *Shigella* sp. and enteroinvasive *E. coli* (EIEC) with an amplification product of 242 bp. The third primer set STN-1 and STN-2 (TaKaRa Biomedicals) was used for the detection of the enterotoxin gene of *Salmonella* sp., PCR amplification product 264 bp. Finally the primer set LM1 and LM2 (Siggens, 1995) was used to amplify the listeriolysin O gene of *L. monocytogenes* with an amplification product of 702 bp.

2.1.2.3 Amplification

Amplification was performed in a total volume of 25 µl. The reaction mixture for all three TaKaRa primer sets comprised: 1.5 µl of DNA preparation, 2.5 µl 10 x buffer, 2.5 mM of each dNTP (dATP, dCTP, dGTP and dTTP), and 19 pmols of each primer. Positive controls (1 pg/µl) were included in each amplication assay. Positive control DNA EC3 (TaKaRa Biomedicals) that was designed to amplify 685 bp fragment by PCR primer EVC-1 and EVC-2, was used as the template. Positive control DNA SS (TaKaRa Biomedicals) that was designed to amplify

689 bp fragment by PCR primer IPA-1 and IPA-2, was used as the template. Positive control DNA SN (TaKaRa Biomedicals) that was designed to amplify 690 bp fragment by PCR primer STN-1 and STN-2, was used as the template.

The reaction mixture for the *L. monocytogenes* primer set comprised: 10 mM Tris-HCl (pH 8.3), 50 mM KCl and 1.5 mM $MgCl_2$, 2.5 mM of each dNTP (dATP, dCTP, dGTP and dTTP) and 25 pmols primer. Heat lysis was performed on two API (API Listeria, bioMérieux, sa, France) confirmed *L. monocytogenes* cultures and the lysates were used as positive controls.

Following detection of *Listeria* and *E.coli* colonies by culture methods – lysates of the pure cultures were used in a confirmation PCR with primers LM1/LM2 and EVC-1/EVC-2 respectively.

Amplification was performed in a thermocycler (Perkin-Elmer, Norwalk, USA). A single cycle of 94°C for 5 min was performed before 0.6 units/µl of *Taq* DNA polymerase. (TaKaRa Taq™, TaKaRa Biomedicals) was added with the temperature at 55°C. Amplification proceeded as follows: 1 cycle of 72°C for 10 min, 35 cycles of 94°C for 1 min, 55°C for 1 min and 72°C for 1 min, with a final extension time of 10 min at 72°C.

For the *Listeria* PCR a single cycle of 96°C for 5 min was performed before 0.5 units/µl SuperTherm Taq DNA polymerase (Southern Cross Biotechnology, Cape Town, SA) was added with the temperature at 55°C. Amplification proceeded as follows: 1 cycle of 72°C for 5 min, 30 cycles of 95°C for 1 min, 50°C for 1 min and 72°C for 1 min, with a final extension time of 72°C for 1 min.

2.1.2.4 Gel electrophoresis

The amplification products were separated on 3% (w/v) agarose gels (NuSieve 3:1, FMC BioProducts, Maine, USA). PCR products (20 µl) were mixed with 3 µl 10 x TAE (40 mM Tris-HCl, 20 mM acetic acid, 1 mM EDTA, pH 8) and 1.5 µl saturated bromophenol blue (BPB). Separation was performed in a mini-submarine electrophoresis apparatus at 84 V for 1 h 45 min in 1 x TAE running

buffer. The PCR products were visualised by staining with ethidium bromide and photographed under UV illumination. A 100 bp DNA ladder (Advanced Biotechnologies, Surrey, UK) was used as size marker in each gel run.

2.1.3 Conventional Isolation

The 105 water samples inoculated in BHI broth (Difco) were incubated for an additional 2 h at 22°C. The bacterial suspensions were centrifuged (3000 x**g**/15 min), the supernatant discarded and the pellet stored at –70°C with sterile glycerol (final 10% v/v).

2.1.3.1 Screening for *E. coli* 0157:H7

E. coli 0157:H7 strains were isolated by means of a one stage as well as two stage enrichment process. The selected PCR positive water pellets were inoculated into 0157 MTSB or Modified Tryptone Soy Broth (Lab M, International Diagnostics Group, Bury, England) as well as directly onto SMAC or Sorbitol MacConkey agar (Lab M). The enrichment broth was incubated at 42°C for 18 h before inoculation onto the selective agar. The SMAC plates were incubated at 37°C for 18 h. Typical sorbitol negative colonies, indicative of *E. coli* 0157:H7 were translucent and glossy, entire and convex in shape

Twenty two (including PCR positive) water samples (see 3.2.1) were inoculated into MTSB and incubated for 6 h at 42°C. Captivate™ particles (Captivate™ 0157, Lab M) were added and the suspension was mixed at room temperature for 30 min. The tubes were then inserted into a magnetic separator (DYNAL MPC® – E, Dynal, Oslo, Norway) for 3 min to separate the particles. The supernatant was removed and washed in phosphate buffered saline (0.15 M sodium chloride, sodium phosphate, 0.05% (v/v) Tween 20, pH 7.4). The wash step was repeated, each time resuspending the particles in the wash before separation. After washing, the particles were resuspended in a final 100 µl of wash solution and 50 µl of the suspension was inoculated onto SMAC and SMAC agar plates supplemented with cefixime and tellurite (Lab M). The agar

plates were incubated for 18 h at 37°C and examined for typical sorbitol negative (translucent) colonies. Latex test reagent kit containing latex particles coated with antiserum against *E. coli* 0157 antigen (E. coli 0157 Latex Test Reagent Kit, Pro-lab Diagnostics, Ontario, Canada) was used as presumptive identification of the *E. coli* serovar by agglutination.

2.1.3.2 Screening for *Shigella*

An initial screening for *Shigella* species was performed with the "clean" water pellets that were resuscitated in BHI broth (Difco), by culturing onto Hektoen Enteric agar (Lab M). The agar plates were incubated at 37°C for 18 h. Sewage effluent and river water pellets were inoculated into Rappaport Vassiliadis broth (Lab M) and incubated at 37°C for 18 h. The broth cultures were then subcultured onto Hektoen Enteric agar (Lab M). Plates were examined after 18 h at 37°C for potential green *Shigella* colonies. The presumptive *Shigella* colonies were then subcultured onto Xylose Lysine Decarboxylase agar (XLD, Lab M). The XLD plates were incubated at 37°C for 18 h. Pink positive colonies were confirmed to be *Shigella* species with API 20E (bioMérieux, sa, France).

Selected *Shigella* PCR positive water pellets were also screened employing the Rappaport Vassiliadis (Lab M) and XLD (Lab M) media in the method described above.

2.1.3.3 Screening for *Salmonella*

An initial screening for *Salmonella* species was performed with the "clean" water pellets that were resuscitated in BHI broth (Difco), by culturing onto Hektoen Enteric agar (Lab M). The agar plates were incubated at 37°C for 18 h. Sewage effluent and river water pellets were inoculated into Rappaport Vassiliadis broth (Lab M) and incubated at 37°C for 18 h. The broth cultures were then subcultured onto Hektoen Enteric agar (Lab M). Plates were examined after 18 h at 37°C for potential green and black H_2S positive *Salmonella* colonies and/or green H_2S negative *Salmonella* colonies. The presumptive *Salmonella* colonies were then subcultured onto Xylose Lysine Decarboxylase agar (XLD, Lab M). The XLD plates were incubated at 37°C for 18 h. Transparent positive colonies

with black centres were confirmed to be *Salmonella* species with API 20E (bioMérieux).

Selected *Salmonella* PCR positive water pellets were also screened employing the Rappaport Vassiliadis (Lab M) and XLD (Lab M) media in the method described above.

2.1.3.4 Screening for *Listeria monocytogenes*

A two stage isolation process was used involving primary and secondary enrichment broths. Selected *Listeria monocytogenes* PCR positive water pellets were inoculated into Fraser Broth (Lab M) containing half Fraser supplement (Lab M). After incubation at 30°C, the primary enrichment broth suspension was inoculated into Fraser Broth containing full strength supplement. Blackening of the Fraser broth, either after 24 h or 48 h incubation at 35°C indicated the presence of potential *Listeria*. Fraser broth (regardless of the colour change) was subcultured onto the selective media (Palcam agar, Lab M) and plates incubated for 48 h at 30°C. The presence of grey/green draughtsman colonies with black halos indicated potential *Listeria* sp. API Listeria (bioMérieux) was used to confirm the presence of *Listeria monocytogenes* strains.

2.2. *NEISSERIA GONORRHOEAE*

2.2.1 Randomly Amplified Polymorphic DNA (RAPD) Analysis

2.2.1.1 Strains

A series of *N. gonorrhoeae* isolates from southern Africa (1993-1995) and Bloemfontein strains isolated in 1996-1997 were investigated. *N. meningitidis* strains isolated in South Africa during 1991-1997 and a group of *Moraxella catarrhalis* strains and *Neisseria* species isolated in Bloemfontein in 1995 were also investigated. Conventional biochemical identification was performed as described by Knapp & Holmes (1983) with the additional identification of some problematic strains employing API NH system (bioMérieux, sa, France) and Biolog GN Microplate system (Biolog, Hayward, California, USA).

2.2.1.2 DNA preparation

N. gonorrhoeae strains were grown overnight at 37°C in a 5% CO_2 atmosphere on Gonococcal (GC) agar plates (Oxoid, Basingstoke, Hampshire, England) supplemented with 5% horse blood plus heating to "chocolate" and on cooling with 1% IsoVitalex (BBL Microbiology Systems, Cockeysville, MD, USA). The *N. meningitidis* and *M. catarrhalis* strains were cultured on chocolate agar plates (Blood agar & Bacto agar base, Oxoid) to which on cooling to 80°C, 2% whole sheep blood was added. Confluent growth from a quarter plate was suspended in 250 µl of TE buffer (50 mM Tris hydrochloride, 20 mM EDTA, pH 7.5). To the cell suspension 1.25 µl of lysozyme at a concentration of 10 mg/ml, was added and the suspension was incubated at room temperature for 10 min. Lysis was enhanced by the addition of 250 µl of 2% Triton X-100 in 50 mM Tris (pH 8.5) and 1.25 µl of proteinase K (5 mg/ml). After a further incubation period of 10 min at 37°C, the lysates were stored at 4°C.

2.2.1.3 RAPD amplification

A 10 bp oligonucleotide primer AB9-07: 5' TCGCTGCGGA from a RAPD primer kit (Advanced Biotechnologies) was used to produce specific RAPD profiles. Ready-To-Go RAPD® analysis beads (Pharmacia Biotech, Brussels, Belgium) were used to perform PCR reactions. The beads were resuspended in a final volume of 25 µl which contained 25 pmols of primer and 1.25 µl of lysate. Amplification was performed in a thermocycler (Perkin Elmer) under the following cycling conditions: 1 cycle of 5 min at 94°C, 5 min at 36°C and 5 min at 72°C. Forty cycles of 1 min at 94°C, 1 min at 36°C, 2 min at 72°C were performed, with an additional final extension of 5 min at 72°C.

PCR products were separated by electrophoresis on a 2% (w/v) agarose gel (NuSieve 3:1, FMC BioProducts) for 2 h at 80 V in a mini-submarine electrophoresis apparatus. After ethidium bromide staining the products were visualised and photographed under UV illumination. A DNA molecular weight marker X (Boehringer Mannheim) was used as a size marker in each gel run.

2.2.2 Plasmid Profile Analysis

2.2.2.1 Plasmid purification

N. gonorrhoeae plasmid DNA was purified using Wizard Plus Minipreps DNA Purification System (Promega, Madison, WI, USA) according to the manufacturers' protocol. Growth from half a GC agar plate was resuspended in 200 µl of Cell Resuspension Solution. Cell Lysis Solution and Neutralisation Solution (200 µl each) were added and the tubes inverted until the suspension had cleared. The lysate was then centrifuged for 5 min at 4000 x**g**. Resin and lysate was combined into a Minicolumn/Syringe assembly and a vacuum applied. Diluted Column Wash was added, followed by reapplication of the vacuum. DNA was eluted by the addition of 50 µl of sterile water to the minicolumn and collected by centrifugation for 20 sec at 4000 x**g**.

The purified plasmid DNA (15 µl) was electrophoresed (3 h at 80 V) on 0.7%

agarose gels (SeaKem, FMC Bioproducts). After ethidium bromide staining the plasmid profiles were examined and photographed under UV light. Supercoiled DNA ladder (Promega) was used as a size marker in each gel run.

2.2.2.2 Plasmid restriction analysis

Thirty *tetM*-conjugative plasmids from cultures grown with 8 mg/l tetracycline selection and 8 conjugative plasmids from strains isolated in 1996 were restricted with 1 µl *Bgl*I, *Sma*I and *Hinc*II (10 units, Promega). Fragments were separated on 0.9% and 1.5% agarose gels. Molecular weight marker X (Boehringer Mannheim) was included in each gel run as a reference.

2.2.3 Susceptibility Tests

Minimum inhibitory concentrations (MICs) to tetracycline were determined by the NCCLS agar dilution method (NCCLS, 1997) using GC agar base (Oxoid) supplemented with 1% IsoVitalex (BBL) and 5% lysed horse blood. Susceptibility and resistance breakpoints were implemented according to NCCLS approved standards (NCCLS, 1998)

2.2.4 *TetM* Gene Amplification

2.2.4.1 DNA preparation

N. gonorrhoeae strains were grown on supplemented GC agar plates. After incubation at 37°C for 18 – 24 h in a 5% CO_2 atmosphere, strains exhibiting growth were subjected to cell lysis (Section 2.2.1.2).

2.2.4.2 Amplification of the *tetM* gene

Amplification of the *tetM* gene was performed in a total volume of 100 µl, using primer A) 5' GGCGTACAAGCACAAACTCG corresponding to bases 1264 through 1283 and primer B) 5' TCTCTGTTCAGGTTTACTCG corresponding to sites 2020 through 2001 (Ison *et al*, 1993). The reaction mixture comprised: 10

mM Tris-HCl (pH 8.3), 50 mM KCl, 2.5 mM $MgCl_2$, 200 µM of each dNTP (dATP, dCTP, dGTP, dTTP respectively) and primers at a concentration of 100 pmols. PCR was performed in a thermocycler (Perkin Elmer). After a single cycle of heating to 94°C for 5 min and cooling to 50°C, 2 Units of *Taq* DNA polymerase (Thermoprime Plus, Advanced Biotechnologies) was added. Following an extension period of 72°C for 5 min, thirty cycles were performed; 94°C for 30 sec 50°C for 20 sec, 72°C for 20 sec with a final extension time of 5 min.

The PCR product (8 µl) was mixed with 2.5 µl 10 x TAE, 8.5 µl sterile water and 1 µl saturated BPB. Products were separated on 2% (w/v) agarose gels (NuSieve 3:1, FMC Bioproducts), visualised by staining with ethidium bromide and photographed under UV illumination. A 100 bp ladder (Boehringer Mannheim) was used as a size marker in each gel run.

2.2.4.3 *TetM* gene analysis

The *tetM* genes obtained by PCR were purified using a Wizard ™ PCR Preps DNA Purification System (Promega) [see appendix].

The purified PCR product was digested with restriction enzyme *Hap*II (Amersham Life Science, Amersham, Buckinghamshire, UK). The reaction mixture (final volume = 20 µl) consisted of PCR product (8-15 µl), 100 mM Tris-HCl (pH 7.5), 100 mM $MgCl_2$, 10 mM dithiothreitol and 1 µl *Hap*II (10 units/µl; Promega) enzyme. After incubation at 37°C for 18 h, the restriction fragments were separated by gel electrophoresis on a 2% (w/v) agarose gel (NuSieve 3:1, FMC Bioproducts) for 3 h at 80 V in a mini-submarine apparatus. A 100 bp ladder (Boehringer Mannheim) was included in each gel run as a size marker.

2.3. *MYCOBACTERIUM TUBERCULOSIS*

2.3.1. Samples

Screening was performed on sputa collected from 313 patients from the Kagisanong and Thusong Clinics, Bloemfontein, from March through to May 1998. The Ziehl-Neelsen results performed by the Public Health Laboratory on the samples were noted. The samples underwent digestion by means of the N-acetyl-L-cysteine sodium hydroxide method in order to decontaminate the sample for culture (Nolte & Metchock, 1995). Equal volumes of specimen and decontamination reagent (1N NaOH, 0.1N sodium citrate, N-acetyl-L-cysteine) were added to a 50 ml plastic centrifuge tube. The reagent and specimen were placed on a shaker 200 x**g** for 20 minutes before the tube was filled with phosphate buffer (pH 6.8). The mixture was then centrifuged (3000 x**g**, 15 min), the supernatant discarded and the pellet resuspended with a further 10 ml of phosphate buffer. Centrifugation and addition of buffer was repeated until the pH of the mixture was neutral.

Sputum samples from family members living and working in the Bainsvlei area of Bloemfontein in 1995/96 as well as a single sample from 1997 and 1998 were examined. The South African Institute Medical Research Laboratories, Kimberley kindly supplied rifampin-resistant *M. tuberculosis* cultures isolated from August 1998 to January 1999.

Fifty isoniazid and/or rifampicin antibiotic resistant strains were collected between March and July 1997 from Bloemfontein and districts in the Free State province: Bloemfontein (9 samples), Bainsvlei (1 sample), Thaba`Nchu (9 samples), Welkom (7 samples), Kroonstad (3 samples), Hoopstad (3 samples), Qwa-Qwa (3 samples), Allanridge (1 sample), Marseilles (3 samples), Parys (1 sample), Ficksburg (2 samples), Virginia (2 samples), Odendaalsrus (3 samples), Trompsburg (1 sample), Christiana (1 sample) and Bloemhof (1 sample).

2.3.2 Staining Procedure

A Ziehl-Neelsen (ZN) stain was performed directly on the decontaminated sputum specimen or from numerous organisms from a solid media slant. Carbol Fuchsin stained the *Mycobacterium tuberculosis* red, while 5% acid alcohol was used to decolourise with Löffler's methylene blue as counterstain. The stains were examined with the 100x oil immersion objective of a microscope, with good growth (3+) being reported when 1-9 tuberculosis bacilli per field were observed.

2.3.3 Culture of *M. tuberculosis*

2.3.3.1 Conventional agar method

The digested specimens (1 ml) were inoculated onto a nonselective egg based culture media, Löwenstein-Jensen (LJ). Components include Bacto Löwenstein Medium Base (Difco) containing 1.2% (v/v) glycerol, one homogenised whole egg per litre and malachite green (0.025 g/100ml). Heating at 85°C for 45 min coagulated the medium. The LJ slopes were incubated in a slanted position for at least 1 week at 37°C. All cultures were examined after 5-7 days of incubation and weekly thereafter for 4-6 weeks. Once a week the screw top caps were loosened for a short interval to air the cultures. Before reporting a culture as positive, after the suitable time period, a Ziehl-Neelsen stain was performed (section 2.3.2) to confirm the presence of the characteristic red stained bacilli.

2.3.3.2 BACTEC system

The BACTEC 12B liquid media and system (BACTEC 460, Becton Dickinson) was used for growth and identification of certain samples where the culture and stain results did not correlate. Also return patient samples were inoculated into this Middlebrook broth (Becton Dickinson). The digested specimens (0.5 ml) were inoculated directly into the broth along with 0.1 ml of reconstituted polymixin B, amphotericin B, nalidixic acid, trimethoprim, and azlocillin (BACTEC® PANTA™ Plus Kit, Becton Dickinson) to reduce bacterial and fungal contamination. The reconstituting fluid contains polyoxyethylene stearate, which

promotes growth. The 12B vials were incubated at 37°C with readings taken once a week for a maximum of 6-8 weeks or until the growth index (G.I.) was greater than 400 (maximum G.I. = 999). The samples producing positive growth indices were 1) confirmed to be acid-fast bacilli with a ZN stain and 2) inoculated onto a blood agar plate incubated aerobically at 37°C to exclude actinomyces or other extraneous microorganisms. BACTEC 12B vials containing G.I. positive cultures (4 ml) were transferred into fresh vials with a disc impregnated with 5 μg of ρ-nitro-α-acetylamino-β-hydroxypropiophenane (NAP). The remainder of the positive culture vial was used as a growth or positive control. After incubation and daily readings of both control and NAP, the bacterial suspension was reported as *M. tuberculosis* if the G.I. reading of the vial containing the NAP was ≤30 and the control was ≥200. If the G.I. of the NAP was reported as being >30, ZN staining and further testing was done to establish the presence of *Mycobacteria* other than tuberculosis or a contaminant.

2.3.4 Susceptibility Testing

The BACTEC® S.I.R.E Drug Kit (Becton Dickinson) was used for rapid drug susceptibility testing. Bacterial suspension (0.1 ml) from the 12B bottles (with G.I. reading of greater than 400 and less than 800) was added to the 12B bottles containing the test drugs: streptomycin (final concentration (FC) in 12B bottle = 2 μg/ml), isoniazid (FC = 0.1 μg/ml), rifampin (FC = 2 μg/ml) and ethambutol (FC = 2.5 μg/ml). Bottles were incubated at 37°C. Control 12B bottles were diluted 1:100, one for each samples tested. Bottles were incubated at 37°C. Growth index readings were taken using the BACTEC 460 system (Becton Dickinson) until the controls reached a value of ≥30 (minimum 4 days, maximum 12 days). If the rate of increase or amount of change over that of the previous day (ΔG.I.), was less in the drug vial than the control, the population was susceptible, if more the strain was resistant.

2.3.5 *M. tuberculosis* Detection by Polymerase Chain Reaction

The digested sample (0.2 ml, section 2.3.1) was transferred into a microcentrifuge tube and 1 ml of washing buffer (1% Triton 1N, 20 mM Tris-HCl [pH 8.3]) was added. The sample and buffer was mixed and centrifuged for 5 min at 14000 x**g**. The supernatant was discarded and the washing process was repeated until the pellet was clean. Alkaline lysis was performed using 0.1 N NaOH (0.1 ml) added to the clean pellet / 0.1 ml of supernatant. This mixture was placed on a vortex for 5 min and then centrifuged briefly at top speed in a microcentrifuge. The tubes were placed in a dry bath at 95°C for 15 min and were finally neutralised with 1 M Tris-HCl (pH 7.8) and centrifuged for 20 min at 14000 x**g**.

PCR of a 123 bp sequence in IS*6110* was performed essentially as described by Eisenach *et al* (1990). The sequences of the primers were (5' to 3') CCT GCG AGC GTA GGC GTC GG and (3' to 5') CTC GTC CAG CGC CGC TTC GG (Eisenach *et al*, 1990; Eisenach *et al*, 1993).
Amplification was performed in a total volume of 25 µl. The reaction mix comprised 5 µl of the DNA lystate, 0.01% (w/v) gelatin, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2.5 mM $MgCl_2$, 200 µM of each dNTP, 0.8 µM of primer, 0.5 units of *Taq* DNA polymerase (Thermoprime Plus, Advanced Biotechnologies) and 0.1 units uracil DNA glycosylase (Boehringer Mannheim). A lysate of a confirmed *Mycobacterium tuberculosis* culture was included in the amplification process as a positive control.

Amplification: The PCR was performed in a thermocycler (Perkin Elmer) with a three step initial phase of 94°C for 5 min, 37°C for 20 min and 95°C for 5 min. This was followed by a denaturation step at 94°C (1 min), an annealing step at 70.5°C (1 min) and an extension step at 72°C (1 min) for a total of 30 cycles. A further extension step at 72°C for 30 min then completed the reaction.

The PCR products were separated by running on a 4% NuSieve 3:1 (FMC BioProducts) agarose gel for 3 h at 80 V using TBE buffer (108 g/L Tris, 55 g/L

boric acid, 9.3 g/L EDTA, pH 8) in a mini-submarine apparatus. After ethidium bromide staining the PCR products were examined and photographed under UV illumination. A molecular weight marker V (Boehringer Mannheim) was used as a size marker in each gel run.

2.3.6 IS*6110* Fingerprinting of *M. tuberculosis*

2.3.6.1 *Pvu*II restriction and Southern blot

The confluent growth on the LJ slope was collected (approximately equivalent to 200 µl) and suspended in TE buffer (10 mM Tris-HCl, 1mM EDTA, pH 8). Heating for 1 h at 80°C was sufficient to kill the cells (Eisenach *et al*, 1990).
Genomic DNA was isolated from the heat killed cells by means of a High Pure PCR Template Preparation Kit (Boehringer Mannheim) [section 2.2.2.1]. The DNA preparations were stored at 4°C. Agarose gel (0.5%) electrophoresis was performed to determine approximate visually determined DNA concentrations.

Genomic DNA was restricted with *Pvu*II in a reaction mix (final volume 20 µl) which comprised: approximately 2 µg of genomic DNA, 10 units *Pvu*II in a potassium and Tris acetate buffer (Amersham Life Science). The restriction mixture was incubated for 20 h at 37°C.

A 0.8% agarose gel (SeaKem HGT, FMC Bioproducts) was loaded with the 20 µl *Pvu*II digested genomic DNA mixture, 2 µl saturated BPB and 2 µl 10 x TAE buffer. Separation was performed in 1x TAE buffer at 40 V for 18 h in a maxi horizontal electrophoresis apparatus. Pre-treatment of the gel involved exposure to UV light for 5 min and rinsing the gel in 0.25 M HCl for 10 min, followed by two rinses of 20 min duration in 0.4 M NaOH.
The genomic DNA was transferred onto Hybond-N^+ membrane (Amersham Life Science) using a vacuum blotter (BioRad Laboratories, Hercules, CA, USA). A single layer of 3MMChr filter paper (Whatman Int., Maidstone, England) as well the Hybond-N^+ membrane (Amersham) were soaked in 10 x SSC (1.5 M sodium chloride, 0.15 M sodium citrate, pH 7) prior to the vacuum being drawn and the

gel being flooded with 10 x SSC for 2.5 h.

2.3.6.2 Detection of IS*6110* fragments

The *Mycobacterial* insertion sequence probe was prepared by labelling a 245 bp fragment obtained by amplification. The primers INS-1 and INS-2 used in the PCR were described by van Embden *et al* (1993). The amplified product was purified (Promega, Wizard™ PCR Preps DNA Purification System, Madison, USA) and agarose gel (4%) electrophoresis was performed to estimate DNA concentrations. Labelling was performed by a Gene Images Labelling Detection Kit (Amersham) [see appendix].

The blots were placed in 0.25 ml/cm^2 AlkPhos Direct hybridization buffer (Amersham) and prehybridised for 30 min at 60°C. The labeled probe was then added before hybridisation was performed (18 h, 60°C) in a Hybridiser HB1 oven (Techne, Cambridge, England). The blot was transferred to a primary stringency buffer and washed at 60°C for 10 min. Two further washes were performed under similar conditions. An excess of wash buffer was used for the secondary wash (5 min, 25°C) performed, as with the primary wash, in triplicate [see appendix for composition of primary and secondary washes]. Chemiluminescent signal generation and detection was achieved using the CDP-*Star* kit (Amersham) [see appendix]. An exposure time of 1 h was sufficient to produce a good signal.

2.3.7 *rpoB* Gene Detection

Chromosomal DNA was isolated from LJ slope cultures as well as directly from washed sputum digests (section 2.3.5) with the High Template Pure PCR Preparation Kit (Boehringer Mannheim) [section 2.2.2.1].

The primer set used for PCR amplification of a 157 bp fragment of the *rpoB* gene was TR8/TR9 (Pretorius *et al*, 1996).

TR8 5' TGCACGTCGCGGACCTCCA

TR9 5' TCGCCGCGATCAAGGAGT

The DNA amplification reaction mixtures (for a final volume of 100 µl per reaction) consisted of 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2.5 mM $MgCl_2$, 200 µM each dNTP (dATP, dCTP, dGTP, dTTP, respectively), 25 pmol of each primer and 0.5 units of *Taq* DNA polymerase (SuperTherm Taq, Southern Cross Biotechnology). The PCR was performed in a thermocycler (Perkin Elmer). Cycling was performed as follows: 96°C for 5 min, 72°C for 5 min, followed by 35 cycles, 93°C for 1 min, 58°C for 2 min, 72°C for 2 min with a final extension period of 5 min at 72°C.

The PCR product was analyzed by electrophoresis on 1.8% agarose gels (NuSieve 3:1, FMC Bioproducts), this was followed by staining with ethidium bromide and the product was visualised under UV light. A 100 bp ladder (Southern Cross Biotechnology) was used as a size marker in each gel run.

The 157 bp PCR product was recovered from a TAE agarose gel using a Nucleon GX kit (Amersham) [see appendix]. This involved the use of a supplied resin to bind and separate DNA from the agarose gel matrix. The purified gene product underwent preparation for sequencing with the Thermo Sequenase kit (Amersham) and the DYEnamic™ ET terminator cycle sequencing premix kit (Amersham) [see appendix]. Forward (TR9) primer was used in the amplification step with all samples. The reverse primer (TR8) was subsequently used to confirm any mutations in the gene sequence. The prepared samples (1.5-2 µl) were loaded on a gel and the Department of Microbiology and Biochemistry (UOFS, Bloemfontein) sequenced the samples by using the ABI Prism™ 377 system (Perkin Elmer).

CHAPTER 3
WATERBORNE PATHOGENS

3.1 INTRODUCTION

Detection of the principal enteric pathogens in waters is of the utmost importance concerning community health. Unfortunately surveillance of microorganisms other than faecal coliform and faecal streptococci are performed in very few countries (Polo *et al*, 1998). Studies have shown that fresh and seawater samples negative for indicators have been positive for the presence of salmonellae and shigellae (Polo *et al*, 1998). Therefore, the reliability of indicator-based standards to predict the presence of pathogenic microorganisms remains a matter of debate (Polo *et al,* 1998).

The detection of waterborne pathogenic microorganisms by conventional methods involves the use of a variety of media and is very labour intensive. The basic method for most pathogens isolated from water or food incorporates the procedures of resuscitation, enrichment, selection and finally identification by serological and biochemical methods (Varnam & Evans, 1991). These steps after isolation are time-consuming with each phase often requiring 24 hour incubation periods.

The advent of gene probe techniques have allowed the development of powerful tests by which particular bacterial strains can be rapidly identified without the need for isolating pure cultures (Hill, 1996). The use of gene probes is closely associated with the development of PCR-based tests (Hill, 1996). There are now an ever-increasing number of waterborne pathogens that can be detected by using PCR. The survival and injury of the waterborne enteropathogenic bacteria is influenced by such factors as water temperature (Terzieva & McPeters, 1991). This makes PCR extremely useful in screening samples that contain not only viable but also non-culturable cells (Hill, 1996). PCR can thus detect the presence of a potential health hazard far better than methods that resuscitate and coerce these cells to grow on selective media in the laboratory (Hill, 1996). The variety of primers available for demonstrating the presence of waterborne

bacteria is immense. From the large range of reported primers used to detect various bacterial species in water, commercially available or published selected primers can be chosen to perform PCR amplification screening. Oligonucleotide primers used in the present study were directed at toxins (cytotoxins and enterotoxins) and invasive plasmid antigen genes for the *Enterobacteriaceae* and a haemolysin gene for *L. monocytogenes*.

The VTEC or EHEC produces a wide variety of closely related verocytotoxins (verotoxins/VTs) or Shiga-like toxins (SLTs) (Read *et al*, 1992; O' Brian *et al,* 1993). The specific *E. coli* serotype O157:H7 produces either SLTI and SLTII or SLTII only (Varnam & Evans, 1991). They are a family of protein cytotoxins that are active on Vero cells and may play a role in the pathogenesis of VTEC-related disease. (Read *et al*, 1992; O'Brian *et al,* 1993). The toxin structural gene or genes probably reside on the genome (Varnam & Evans, 1991). The primers EVC-1 and EVC-2 (TaKaRa Biomedicals) are directed at the different antigenic types of verotoxins i.e. VT1 (SLT1), VT2 (SLT2) and the verotoxin 2 variants (VT2 vha, VT2 vhb and VT2 vpl) of the EHEC.

The *ipaH* loci comprises a multicopy antigen gene family unique to shigellae and EIEC (Buysse *et al*, 1995). Sequencing and molecular characterisation of the *ipaH* gene in *Sh. flexneri* has been investigated (Hartman *et al*, 1990), although amplification using primers from the revealed sequence has not been extensively reported. The primer set IPA-1/IPA-2 (TaKaRa Biomedicals) was used for the detection of the plasmid antigen genes of *Shigella* and EIEC strains.

Salmonella possess heat labile, heat stable toxins, cytotoxins and enterotoxins. Unlike the cholera toxin-like enterotoxin and heat labile enterotoxins localised in the periplasm, the *Salmonella* enterotoxin is cytoplasmic in nature (Prasad *et al*, 1992). Based on nucleotide sequence analysis, Chopra *et al* (1994) found the *stn* gene to contain 749 bp that would encode a protein having a molecular size of 29 073 Da. Through Southern blot analysis, the enterotoxin gene of *S. typhimurium* was found to be located on the plasmid but not on the chromosome (Yang & Tan, 1989). It is immunogenic but appears to be antigenically distinct from cholera and *E. coli* enterotoxins (Kaura & Sharma, 1998). Primer pair STN-

1 and STN-2 (TaKaRa Biomedicals) was used to amplify a 264 bp region of the enterotoxin genes of *Salmonella* species (*stn* genes).

L. monocytogenes produces a species specific haemolysin or listeriolysin. Primers LM1 and LM2 employed in the present study are derived from the listeriolysin gene O (Siggens, 1995). This gene (*hlyA*) for listeriolysin production resides in the chromosome at a region crucial for virulence (Varnam & Evans, 1991).

The primers and their design are referenced from international sources, with the possibility that specificity is mainly applicable to strains relevant to a particular location. Detection by PCR of a number of bacterial species and strains from South Africa may not therefore always be as effective as a study indicates. Such impaired PCR detection could be due to regional strain target sequence variation in that designated primers and cycling conditions especially annealing temperatures may not be ideal for local strains. Using the primers, where possible, against clinical samples will help confirm whether they are relevant to South African locations. Investigating genetic (PCR) technique versus conventional methods will aid in developing quick and accurate means of surveillance for environmental pathogens.

3.2 RESULTS

3.2.1 PCR Detection

Nine of the ten serotype confirmed *Salmonella* species all produced a 264 bp fragment of the *stn* gene (Table 3.1). They represented four different *Salmonella* serotype groupings. The primer application appeared suitable for investigations on South African strains. The DNA template preparation kit used (for all control and water samples) was also suitable for analysis with the added advantage of removing any possible PCR inhibitory substances.

E. coli cytotoxin weak PCR products of 171 bp were demonstrated from four strains (Table 3.2, Figure 3.1). Seven weak PCR positives (amplification product 242 bp) were obtained from the assay whereby primers targeted at the *ipaH* gene of *Shigella* sp. and EIEC were used (Table 3.2). The weak bands were not resolved in photo reproduction. The TaKaRa control SS and non-specific bands were, however, clearly evident (Figure 3.2). The PCR screening for the *Salmonella* enterotoxin gene revealed ten samples which produced the predicted size amplification product, 264 bp (Table 3.2, Figure 3.3). Of the ten positives, three had weak bands (sample numbers 85, 86 and 92). The two API confirmed *L. monocytogenes* controls produced positive PCR results. The listeriolysin O gene was detected in 13 of the water samples by amplification of the predicted 702 bp product (Table 3.2, Figure 3.4).

TABLE 3.1 PCR detection of the *stn* gene in clinical *Salmonella* serotypes.

SALMONELLA **SEROTYPE GROUPING**	**NUMBER OF SAMPLES** (n = 10)	**PCR DETECTION OF *STN* GENE**
Group B	3	+
Group C	1	-
Group C1	1	+
Group C4	1	+
Group D1	4	+

TABLE 3.2 PCR detection of pathogens from different water sources.

WATER SOURCE (total)	DATE	NUMBER OF SAMPLES	SAMPLE NUMBERS	PCR DETECTION *			
				E. coli (weak)	*Shigella* (weak)	*Salmonella*	*L. monocytogenes*
Containers (24)	15/03/99	12	1 – 12				
	12/04/99	12	78 – 89	1	4	3	1
Tap (4)	15/03/99	2	13,14				1
	12/04/99	2	16,17		1		
River (35)	17/03/99	5	26 – 30				
	29/03/99	8	42 – 49	1	1		2
	30/03/99	8	60 – 67		1	1	2
	07/04/99	8	68 – 75			2	1
	14/04/99	6	94,95,103-106				
Sewage	17/03/99	11	15 – 25				
effluent (42)	29/03/99	10	31,33 – 41	1			3
	30/03/99	10	50 – 59				2
	14/04/99	11	90 – 93,96-102	1		4	1
		n = 105		n = 4	n = 7	n = 10	n = 13

* With the exceptions of sample 66 *Salmonella/L.. monocytogenes*; 81 EHEC/*Shigella*; 83 *Salmonella/L. monocytogenes*; 90 EHEC/*L. monocytogenes* only one pathogen per sample was demonstrated.

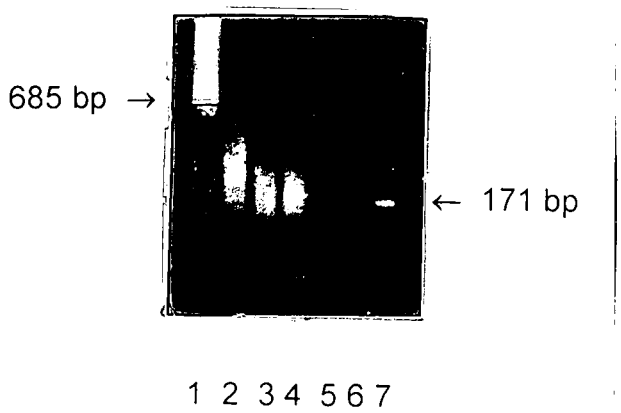


FIGURE 3.1 PCR detection of Enterohaemorrhagic *E. coli* cytotoxins using primers EVC-1 & EVC-2. Only lanes with a positive PCR fragment 171 bp are given.
Lanes: **1**, TaKaRa control EC3 (685 bp); **6**, strain 90; **7**, strain 35.

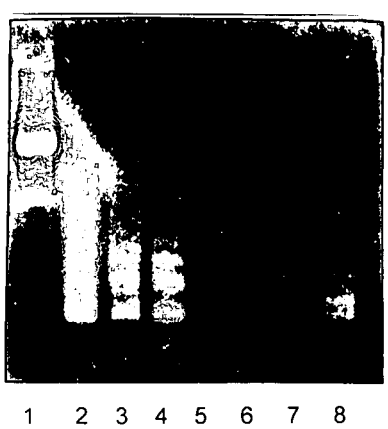


FIGURE 3.2 PCR screening for *ipaH* genes of *Shigella* sp. with primers IPA-1 and IPA-2.
Lanes: **1**, TaKaRa control SS (689 bp); **3-5, 7, 8**, non specific binding

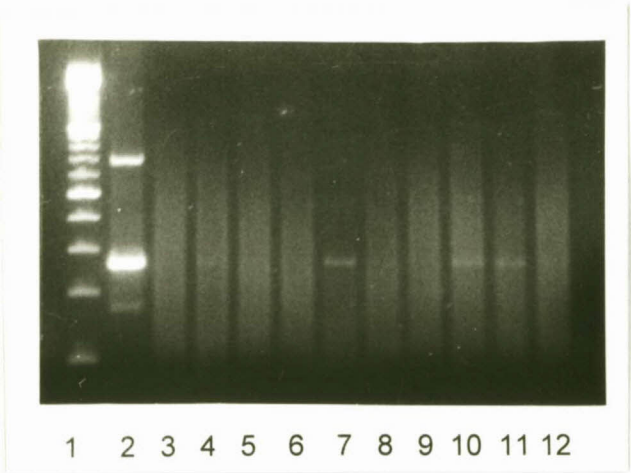


FIGURE 3.3 PCR detection of *Salmonella* enterotoxins using primers STN-1 and STN-2. Only lanes with a positive PCR fragment 264 bp are given.

Lanes: **1**, 100 bp marker; **2**, TaKaRa control SN control (690 bp) and positive control (264 bp); **4**, strain 86; **5**, strain 85; **7**, strain 83; **10**, strain 96; **11**, strain 93; **12**, strain 92. Lanes 4,5 & 12 = weak PCR positive fragment.

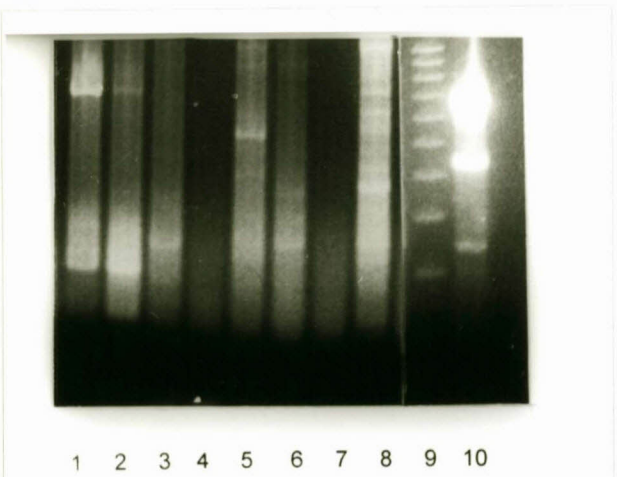


FIGURE 3.4 PCR detection of *Listeria monocytogenes* using primers LM1 and LM2. Only lanes with a positive PCR fragment 702 bp are given.

Lanes: **1**, strain 39; **2**, strain 40; **8**, strain 33; **9**, 100 bp marker; **10**, positive control (702 bp).

3.2.2 Standard Culture Methods

3.2.2.1 Screening for *E. coli* 0157: H7

The four PCR positive EHEC water samples (35, 42, 81, 90) were selected for confirmation by culture methods. The lysates of the pure suspected *E. coli* 0157:H7 cultures, when used as DNA template in the PCR assay did not reproduce the amplification product 171 bp. Two of the four strains (strains 35 & 90) produced growth (seen as turbidity) in the MTSB selective for 0157:H7. The same two strains, 35 and 90, demonstrated translucent sorbitol negative colonies on the SMAC agar.

Twenty two (including the four PCR positive) EHEC water samples were directly inoculated into MTSB for 6 h at 42°C and subjected to antigen immunomagnetic separation (Captivate™ 0157 kit, Lab M). Four samples (35, 90, 36 & 99) produced sorbitol negative colonies on CT-SMAC agar plates. No agglutination was seen when the latex reagent containing the 0157 antibody was added to a suspension of the sorbitol negative colonies.

3.2.2.2 Screening for *Shigella*

The initial screening of water samples revealed no detection of the characteristic green *Shigella* colonies on the Hektoen Enteric agar. On re-screening of the seven PCR positive waters, one presumptive pink *Shigella* colony, sewage effluent number 97, was identified. API 20E testing demonstrated this presumptive colony to be a *Pseudomonas* species.

3.2.2.3 Screening for *Salmonella*

The initial screening demonstrated the presence of two presumptive green and black *Salmonella* colonies on the Hektoen Enteric agar; from waters 63 and 35. On subculturing these two positive colonies onto XLD agar, the characteristic translucent colonies with black centres where detected. API 20E testing demonstrated strain number 35 to be *a Citrobacter frendii*, while strain number 63 was confirmed to be a *Salmonella* species. Re-screening of the ten PCR positive *Salmonella* waters saw four presumptive strains of *Salmonella* species

being isolated by means of enrichment and selective media (water numbers 93, 96A, 96B and 97). API 20E testing confirmed strain number 96A to be a *Salmonella* species. The remaining presumptive colonies were *Proteus* species (numbers 96B and 97) and a *Citrobacter* species (number 93).

3.2.2.4 Screening for *Listeria monocytogenes*

Thirteen PCR positive *Listeria monocytogenes* waters were selected for screening using Fraser broth. The metabolism of aesculin, as seen by blackening of half Fraser broth is indicative of potential *Listeria*. None of the suspect *L. monocytogenes* demonstrated blackening of the broth after 24 h. The second stage of enrichment in Fraser broth revealed that four water samples contained microorganisms capable of producing blackening after 48 h. Regardless of colour change to the broth all thirteen strains were subcultured onto Palcam agar. One sewage effluent sample (number 39) showed grey/green draughtsman colonies with black halos indicative of *Listeria* species on the Palcam agar. API Listeria indicated this culture not to be a *L. monocytogenes* strain.

The confirmation *L. monocytogenes* PCR performed with the lysate of the isolated pure culture (number 39) revealed no 702 bp product. This correlated with the API Listeria result.

3.3 DISCUSSION

The PCR assays performed on the water samples provided a simpler and rapid means of screening environment samples for pathogenic bacteria. This genetic technique omits the steps of culture by means of enrichment and selective steps, which can be very time consuming (5-7 days). Serological or biochemical testing is always required to confirm presumptive bacterial colonies, which further extends this time period by 1-2 days.

PCR detection revealed four cytotoxin containing *E. coli,* seven *ipa*H containing *Shigella* species, ten enterotoxin containing *Salmonella* species, and thirteen listeriolysin containing *L. monocytogenes* strains. Organisms growing at different rates or over-growing others could have resulted in the greater number of *Listeria, Salmonella* and *Shigella* and *E. coli* 0157:H7 being detected by PCR and not by the conventional culture methods. Culture did however, detect one *Salmonella* strain not detected by PCR. The amplified fragments were easily distinguished in the TaKaRa product as the size of the fragment of the control template differed from that of the specific gene.

The disadvantage of PCR assays is that the DNA (chromosomal and plasmid) extracted from water samples can remain detectable for long periods after the bacteria are no longer viable in the environmental setting (Hill, 1996). *Shigella* species are known to be particularly fragile in their ability to survive long periods outside the host (Varnam & Evans, 1991), which could explain the detection of the invasive plasmid antigen locus DNA in seven samples while *Shigella* was not isolated by conventional methods. No clinical *Shigella* samples were available to be tested with the IPA primer set. These primers definitely aid in increasing the detection of *Shigella* species and EIEC from dysentery stool samples (Gaudio *et al*, 1997) but simultaneously this plasmid probe cannot be used to differentiate among *Shigella* serotypes and EIEC strains (Houng *et al*, 1997). PCR assays have the capability to detect any damaged cells, which can not be recovered by

conventional methods. Virulence plasmid loci like the invasion plasmid antigen genes of *Shigella* and EIEC are extremely unstable and loss of the plasmid will result in non-invasive bacteria (Hill, 1996). PCR screening based on plasmid invasion genes is therefore specifically aimed at important pathogenic strains that may by culture selection / differentiation remain elusive.

The immunomagnetic separation method was investigated to further find a rapid means of directly isolating *E. coli* 0157:H7 from a high background of other flora, which could obscure colonies giving rise to false negative results. This particular *E. coli* serovar warrants rapid identification techniques to assist the 4-5 day conventional methods, as it is responsible for the serious condition of haemorrhagic colitis. Confirmation by biochemical tests, however, is essential as *E. hermanii* will not only agglutinate with the 0157:H7 antiserum but also fail to ferment sorbitol. Four presumptive *E. coli* 0157:H7 strains were isolated by immunomagnetic separation. However, no agglutination was seen when the latex reagent containing the 0157 antibody was added to a suspension of the sorbitol negative colonies isolated.

The culture methods of detection were lengthy and expensive as compared to the PCR technique. Attempts have been made to shorten various isolation stages, but this has resulted in an unacceptably high risk of obtaining false negatives (Varnam & Evans, 1991). The selective and enrichment media commercially available from which to chose was extensive, with no single media being able to isolate all species equally successfully. The isolation process has in some cases been made simpler by the inclusion of various "diagnostic" systems in the selective media chosen for example metabolism of aesculin by *Listeria* sp. in Palcam agar producing distinctive black zones around colonies. Despite this, differentiation between colonies appearing similar to the pathogen under investigation, on selective agar was difficult. It should also be noted that species specific PCR methods can assist in the rapid identification of presumptive pathogenic isolates.

The culture methods of enrichment and selective steps revealed no *Salmonella, Shigella, L. monocytogenes* and *E. coli* 0157:H7 species in the four clean water

samples collected from taps. Taking water samples brushed and shaken from containers (n = 24) was important as these containers which are often not hygienically cleaned/stored can provide a means of transmitting pathogenic bacteria to the water carried therein.

Two *Salmonella* were detected by culture from the 77 sewage effluent samples and river waters. Neither *Shigella* species nor *L. monocytogenes* were detected. The manure of cattle and faeces of chicken and possibly also pigs and sheep are often the cause of contamination of the environment including watercourses. Sewage effluent running off into river waters could cause serious infection by the various bacteria discussed. The rapid detection of harmful bacteria in various water sources is important, particularly in rural and informal housing settings where hygiene standards are difficult to maintain.

CHAPTER 4

NEISSERIA GONORRHOEAE

4.1 INTRODUCTION

N. gonorrhoeae, a member of the family *Neisseriaceae*, remains one of the most common sexually transmitted diseases (Howard, 1994). In the United States, 700 000 cases were reported during 1988 (Morello *et al,* 1991). The human body is the only natural host to *N. gonorrhoeae.* Two important factors responsible for the high prevalence of gonorrhoea is firstly the large reservoir of asymptomatic females and males who unknowingly transmit the disease to their sexual partners and secondly the increasing resistance of *N. gonorrhoeae* to available antimicrobial agents (Morello *et al*, 1991).

N. gonorrhoeae can be cultured on Thayer Martin agar or a GC agar base supplemented with IsoVitalex, with growth stimulated by carbon dioxide. Colonies are about 0.5 mm to 1 mm in diameter and appear grey to white, opaque, raised and glistening (Morello *et al*, 1991). *N. gonorrhoeae* are typically gram-negative, diplococci, intracellularly located, depending on the specimen type (Morello *et al*, 1991).

The sugar (glucose, fructose, maltose, saccharose) utilisation patterns are the key biochemical tests for a basic identification and thus differentiation between closely related *Neisseria* species. Both *N. gonorrhoeae* and *N. meningitidis* utilise (with acid production) glucose, while neither utilise fructose and saccharose (Cowan & Steel, 1993). *N. gonorrhoeae* does not use maltose, while the majority of *N. meningitidis* strains do. False positive results for *N. gonorrhoeae* arise because a small percentage (10%) of *N. meningitidis* can present with negative results for maltose utilisation. Similarly 3% of *N. gonorrhoeae* and *N. meningitidis* strains do not produce acid from glucose (API NH, bioMérieux). One of the standard basal media for detecting acid production from carbohydrates by *Neisseria* spp, is a cystine-tryptic digest agar (CTA) base (Morello *et al*, 1991). Problems that occur with CTA carbohydrate media include i) carbohydrate degradation is not observed due to the lack of sufficient inoculum

or that production of ammonia from peptone partially or completely neutralises any acid produced from carbohydrates, ii) degradation of both maltose and glucose due to a mixed meningococci and gonococci inoculum and iii) all carbohydrates appear degraded due to contamination of inoculum (Morello *et al*, 1991). An oxidation-fermentation medium was developed to try and alleviate some of these problems (Knapp & Holmes, 1983). A modified oxidation-fermentation (MOF) medium then followed as a more sensitive means of detecting carbohydrate utilisation by *Neisseria* spp. and *M. catarrhalis* (Knapp & Holmes, 1983). *M. catarrhalis* displays the same morphological characteristics as *Neisseria* i.e. gram-negative cocci/diplococci and does not utilise glucose or maltose, the main differentiation tests (Morello *et al,* 1991).

Plasmid analysis of *N. gonorrhoeae* strains can reveal information on the origin, evolution as well as dissemination of plasmids and resistance genes (Roberts, 1989). The treatment of isolated plasmid DNA with restriction enzymes before electrophoretic separation allows one to identify and group profiles that vary from those previously described (Swaminathan & Matar, 1993). Other detection methods of *tetM*-mediated resistance besides plasmid analysis screening is by growth on agar containing 10 µg/ml tetracycline, disc testing and MIC determination. Confirmatory methods encompass for example dot blotting and Southern blotting.

Studies of the different tetracycline resistant plasmid types from tetracycline-resistant *N. gonorrhoeae* isolates acquired from different countries around the world, has shown the Dutch-type plasmid to be predominant in isolates from the Netherlands and Asia, and a predominance of the American-type tetracycline-resistant plasmid in isolates from the United Kingdom and eastern and central Africa (Gascoyne-Binzi *et al*, 1994). These plasmid types have independently disseminated amongst *N. gonorrhoeae* strains throughout the world and suggests that the *tetM* gene within these plasmids are stable (Gascoyne-Binzi *et al*, 1994). However, the existence of American-type *tetM* gene on tetracycline resistant plasmids that have restriction endonuclease patterns similar to the Dutch-type tetracycline-resistant plasmid suggests, that further evolution of the tetracycline resistant plasmids has occurred (Gascoyne-Binzi *et al*, 1994).

The *tetM* determinant is often associated with conjugative chromosomal elements, which code for their own transfer, such as the Tn*1545* and Tn*916* transposon families (Gascoyne-Binzi *et al*, 1993). These transferable elements allow the determinants to be introduced into new species on plasmids capable of being maintained in the host or by integration into indigenous hosts, followed by mobilisation or conjugation (Roberts, 1989). Therefore plasmid/transposon-mediated resistance has enormous dissemination capabilities as compared with chromosomal resistance. Swartley *et al* (1993) revealed, however, that the TetM-encoding conjugative plasmids of *N. gonorrhoeae* and *N. meningitidis* contain a class of a TN*916*-like transposon, which suffered deletions on insertion and so transposition is not maintained, therefore limiting dissemination capabilities.

In order to predict the number of different plasmids and associated antibiotic resistant genes in addition to ongoing gene and plasmid exchange, plasmid studies have been shown to be extremely helpful (Roberts, 1989). The characterisation of *N. gonorrhoeae* strains isolated in South Africa by genetic techniques such as RAPDs, plasmid profiles and plasmid restriction analysis has been shown to be useful in epidemiological investigations (Chalkley *et al*, 1997).

4.2 RESULTS

4.2.1 RAPD Analysis

The profiles obtained with the RAPD analysis beads were quite extensive. The thermostable polymerases AmpliTAQ and Stoffel fragment incorporated into this system provide for excellent reproducible RAPD profiles in the range 0.3-1.6 kb. The RAPD analysis of the *N. gonorrhoeae* and the *N. meningitidis* strains allowed for the comparison of profiles between the two species of *Neisseria* and grouping within the species.

The non-pathogenic *Neisseria* species: *perflava*, *flava, mucosa*, *cinerea* and *polysaccharea* were identified using conventional biochemical tests. Of seven strains described as having identification discrepancies, three strains were identified as *N. flava* but by the API system as *N. perflava* (Figure 4.1, lanes 14-16); the remaining four strains as *N. perflava* by conventional methods but as *N. mucosa* by the Biolog identification system (Chalkley, personal communication) (Figure 4.1, lanes 17-19 and 21). Except for two *N. mucosa* strains RAPD analysis was unable to resolve any of the *Neisseria* strains into species subsets (Figure 4.1).

The RAPD profiles of the *N. meningitidis, N. gonorrhoeae* and *M. catarrhalis* strains isolated in 1991-1995, showed individual strain variability whilst maintaining species specific banding patterns (Figure 4.1). A single or doublet band(s) of approximately 290 bp was found in all *N. meningitidis* and *N. gonorrhoeae* strains but not in the other *Neisseria* species.

The RAPD profiling of 26 *N. gonorrhoeae* strains isolated in 1997 revealed six groups (A-F). Group A (13 strains), B (6 strains), C (3 strains), and D (2 strains) all exhibited three characteristic bands at 1.5, 1.2 and 0.98 kb (Figure 4.2). Thirty-one *N. gonorrhoeae* isolates from 1996 had also shown this constant banding pattern (Stanley & Chalkley, 1998). Only two isolates; strains 56 and 68

(Figure 4.2, lanes 4 and 2) did not conform. The *N. meningitidis* strain's RAPD profiles (1996 & 1997) were too variable to reveal recognisable grouping (Figure 4.2, lanes 13-20).

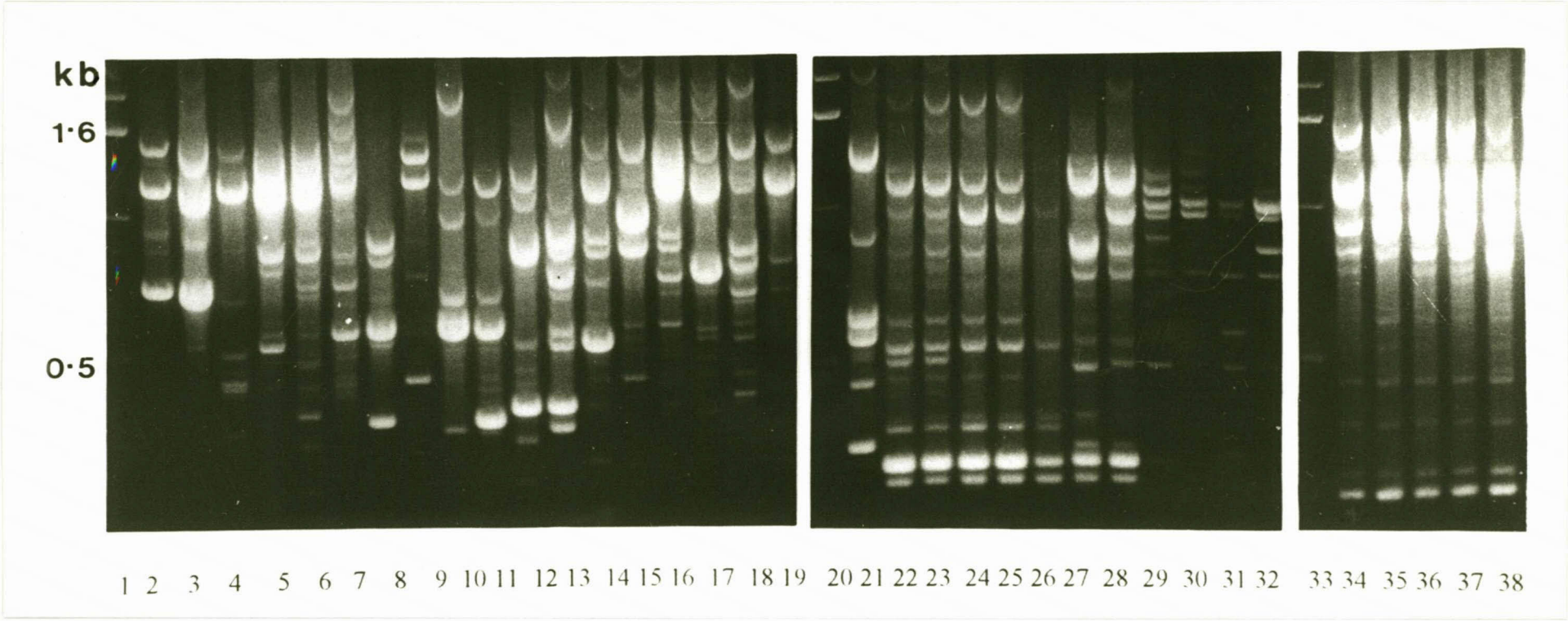


FIGURE 4.1 RAPD profiles of *Neisseria* species and *Moraxella* strains isolated in 1995, *N. meningitidis* strains 1991-1994 and *N. gonorrhoeae* 1994-1995.
Lanes: **1, 20, 33,** Molecular weight marker; **2-5**, *N. perflava;* **6** and **7**, *N. flava;* **8-11**, *N. mucosa;* **12**, *N. cinerea;* **13**, *N. polysaccharaeae;* **14-16**, *N. flava/perflava* identification discrepancies; **17-19 & 21**, *N. perflava/mucosa* identification discrepancies; **22-28**, *N. meningitidis*; **29-32**, *M. catarrhalis*; **34-38**, *N. gonorrhoeae.*

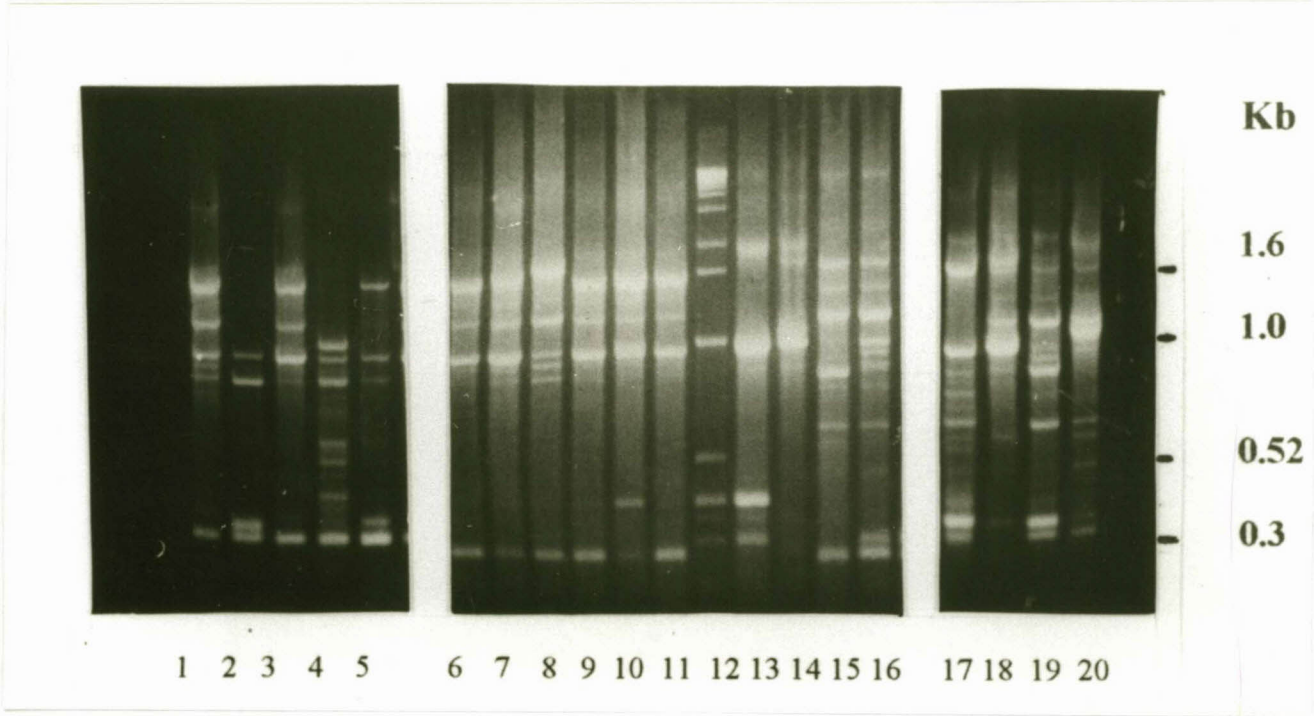


FIGURE 4.2 Comparative RAPD profiles of *N. gonorrhoeae* strains isolated in 1997 and *N. meningitidis* strains isolated during 1996-1997. Lanes 1-11, *N. gonorrhoeae*; Lanes 13-20, *N. meningitidis.* RAPD profile groups of *N. gonorrhoeae* strains are given in brackets.
Lanes: **1**, strain 92 (C); **2**, strain 68 (F); **3**, strain 9 (B); **4**, strain 56 (E); **5**, strain 61 (B); **6**, strain 83 (A); **7**, strain 77 (C); **8**, strain 80 (C); **9**, strain 99 (A); **10**, strain 37 (D); **11**, strain 20 (A); **12**, molecular weight marker X; **13**, strain 11; **14**, strain 10; **15**, strain 9; **16**, strain 8; **17**, strain 7; **18**, strain 5; **19**, strain 4; **20**, strain 3.

4.2.2 Plasmid Analysis

Plasmid profiles of the 102 *N. gonorrhoeae* strains isolated in 1997 revealed the presence of both conjugative and *tetM*-conjugative plasmids responsible for high levels of resistance (Figure 4.3). The conjugative (24.5 MDa) plasmid was present in 29/102 (28.4%) strains while the *tetM*-conjugative (25.2 MDa) plasmid was present in 48/102 (47%) strains. Four of five African β–Lactamase plasmids were found in conjunction with a 24.5 MDa conjugative plasmid. Four Asian β–lactamase plasmids were found each in association with a 25.2 MDa *tetM*-conjugative plasmid.

The cryptic plasmid (2.6 MDa) was present in 99/102 (97.1%) strains, with 3 strains showing a reduction in this plasmid. Four strains were seen to carry 8 MDa plasmids, three had an additional plasmid of 11 MDa, again with a loss of the cryptic plasmid copy number (Figure 4.3). In three 1997 isolates, *tetM*-conjugative plasmids were found in association with the 8 MDa plasmid.

The restriction endonuclease fingerprinting performed on 30 *tetM*-conjugative plasmids from strains isolated in 1996 revealed 7 different restriction profiles to those of previously described American and Dutch plasmid types (Figure 4.4 and Table 4.1). Of the 8 conjugative plasmids investigated, 3 plasmids possessed different restriction profiles. (Figure 4.5 and Table 4.2).

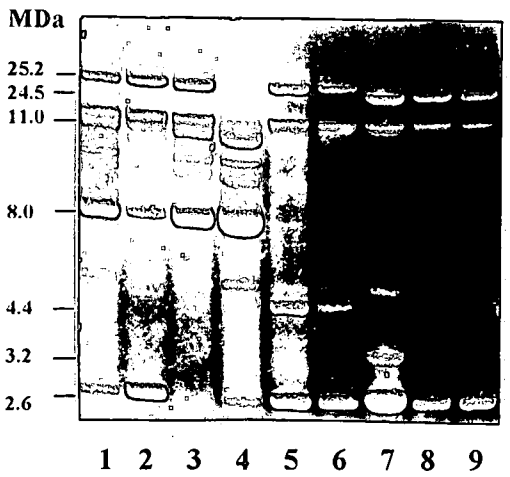


FIGURE 4.3 Representative plasmid profiles from *N. gonorrhoeae* strains isolated in 1997.
Lanes: (**1 & 3**) strains 57 and 83, 25.2 MDa *tetM* conjugative + 11 MDa + 8 MDa + 2.6 MDa weak cryptic plasmid; (**2**) strain 61, 25.2 MDa *tetM* conjugative + 8 Mda + 2.6 MDa cryptic plasmid; (**4**) strain 25, 11 MDa + 8 MDa + 2.6 MDa weak cryptic plasmid; (**5 & 6**) strains 23 and 51, 25.2 MDa *tetM* conjugative + 4.4 MDa Asian β-lactamase + 2.6 MDa cryptic plasmid; (**7-9**) strains 27and 5 and 22, 24.5 MDa conjugative + 3.2 MDa African β-lactamase + 2.6 MDa cryptic plasmid.

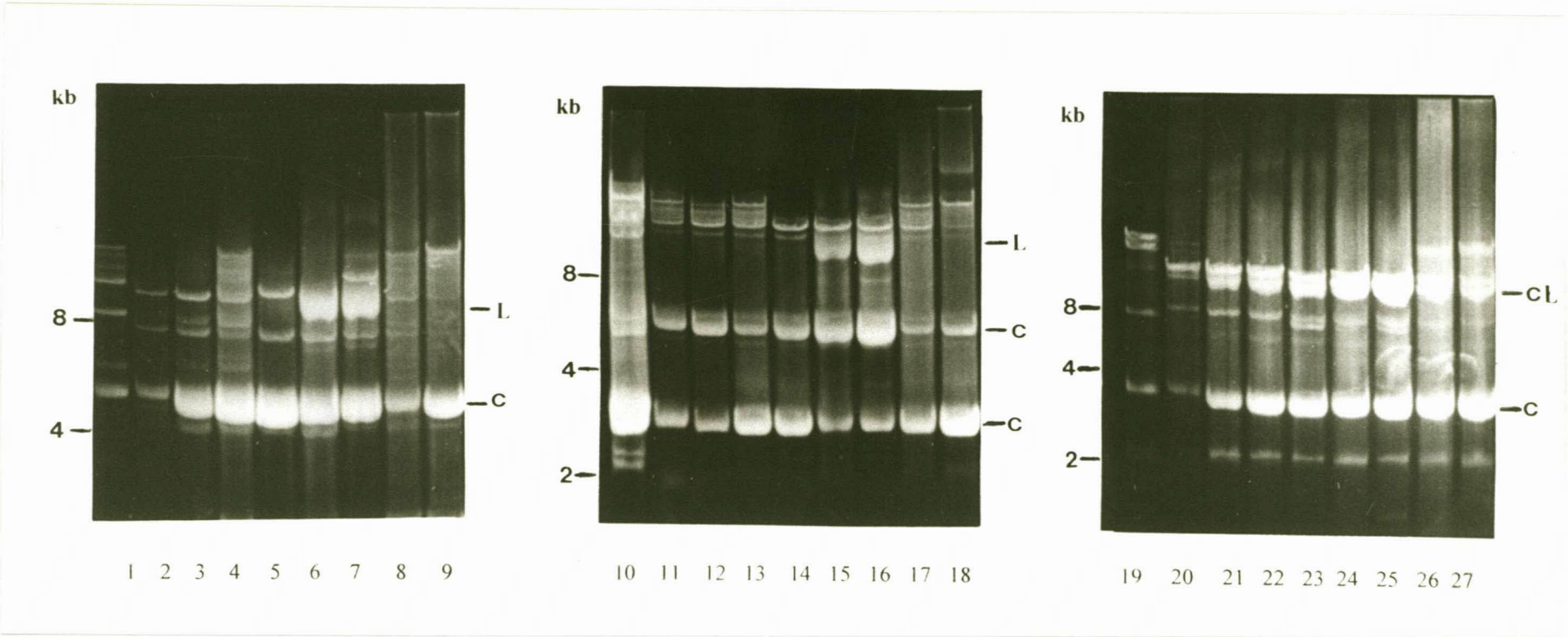


FIGURE 4.4. Representative restriction profiles of *tetM*-conjugative plasmids from strains isolated in 1996.
Lanes: 1-9 restriction enzyme *Bgl*I 0.9% agarose gel, 10-18 restriction enzyme *Sma*I 1.5 % agarose gel, 19-27 restriction enzyme *Hinc*II 1.5%agarose gel. Lanes: (**1, 10, 19**) control strain carrying the Dutch *tetM* conjugative plasmid; (**2,11,20**) control strain carrying the American *tetM* conjugative plasmid; (**3, 12, 21**) strain 30; (**4, 13, 22**) strain 20; (**5, 14, 23**) strain 18; (**6, 15, 24**) strain 15; (**7, 16, 25**) strain 12; (**8, 17, 26**) strain 10; (**9, 18, 27**) strain 2. High intensity bands as indicated are due to the following whole or partially restricted plasmid types [C] and 4.4 MDa Asian β–lactamase plasmids (strains 12 and 15) [L].

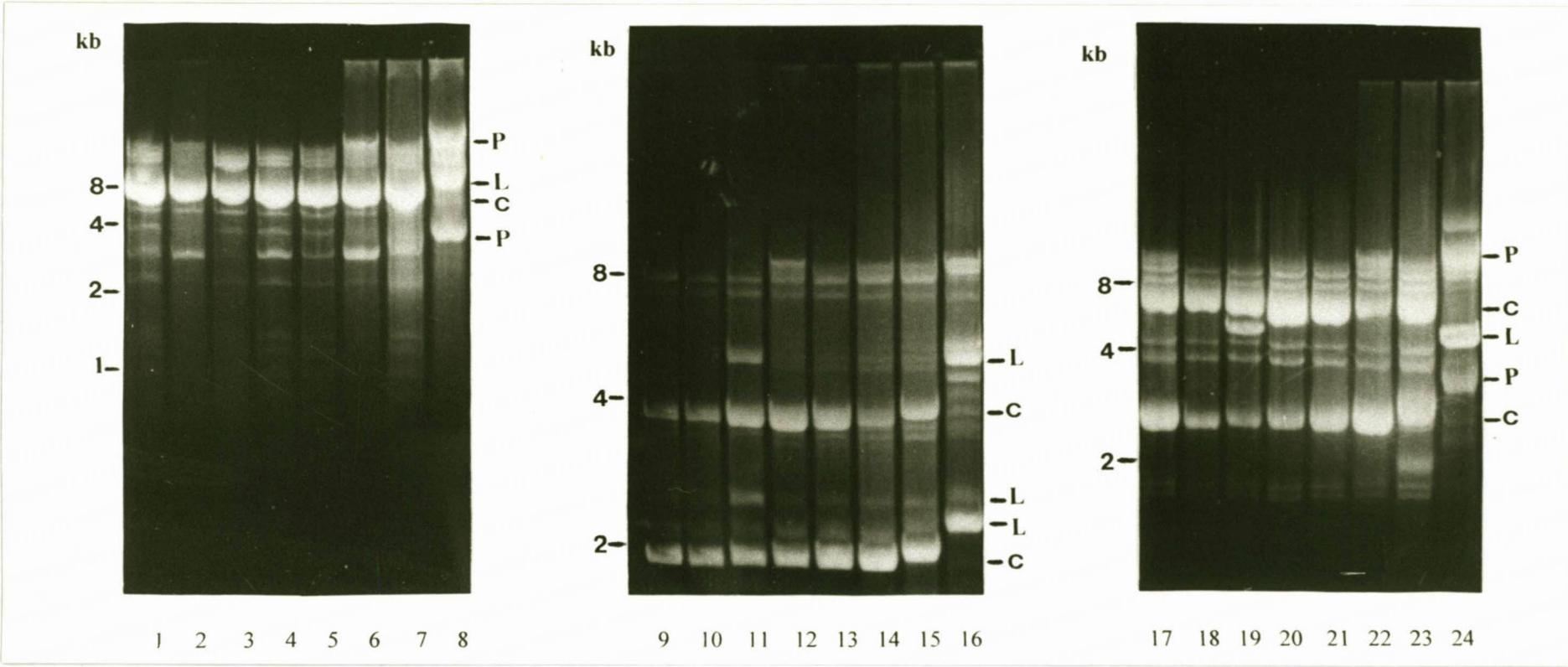


FIGURE 4.5 Representative restriction profiles of conjugative plasmids from strains isolated in 1996. Lanes: 1-8 restriction enzyme *Bgl*II 1.5% agarose gel, 9-16 restriction enzyme *Sma*I 0.9% agarose gel, 17-24 restriction enzyme *Hinc*II 0.9% agarose gel. Lanes: (**1, 9, 17**) strain 89; (**2, 10, 18**) strain 62; (**3, 11, 19**) strain 51; (**4, 12, 20**) strain 39; (**5, 13, 21**) strain 36; (**6, 14, 22**) strain 25; (**7, 15, 23**) strain 13; (**8, 16, 24**) strain 7. High intensity bands as indicated are due to the following whole or partially restricted plasmid types; cryptic plasmids [C], African β–lactamase plasmids of 3.2 MDa (strain 7) and 3.4 MDa (strain 51) [L] and an 8 MDa plasmid [P] in strain 7 that carried no cryptic plasmid.

TABLE 4.1 Restriction profiles of *tetM*-conjugative plasmids.

STRAIN	TETRACYCLINE MIC (µg/ml)	RESTRICTION ENZYMES *BglI*	*SmaI*	*HincII*
American	16	A	1	a
2	64	B	1	b
10	64	C	1	b
12	128	D	2	c
15	16	E	3	a
18	64	F	3	c
20	128	G	1	a
30	64	H	1	a

TABLE 4.2 Restriction profiles of conjugative plasmids.

STRAINS	RESTRICTION ENZYMES *BglI*	*SmaI*	*HincII*
13, 25, 36, 62, 89	N	4	D
7	M	5	E
39	N	6	D
51	N	7	F

4.2.3 Minimum Inhibitory Concentrations

The MICs of tetracycline for the isolates were determined and it was found that 72/102 strains (70.5%) showed increasing resistance (≥ 2 µg/ml). High-level resistance (16-128 µg/ml) was observed in 43/102 (42%) of the strains. Twenty three strains showed intermediate tetracycline resistance (0.5-1 µg/ml) and only 7 strains were susceptible (MICs ≤ 0.25 µg/ml). Ten of seventy-two (13.9%) strains exhibited resistance that was not due to the presence of the *tetM* gene.

4.2.4 *TetM* Gene Analysis

The amplification of the *tetM* gene from 48 strains exhibiting tetracycline MICs ≥ 8 µg/ml, produced a fragment of predicted size, 756 bp. The *Hpa*II digestion of the amplified *tetM* genes was performed to reveal any genetic diversity within the gene. All of the restricted *tetM* genes were of the American-type (Figure 4.6). The 43 high-level tetracycline-resistant strains (MICs ≥ 16 µg/ml) all contained the American-type *tetM* gene and 25.2 MDa plasmids were demonstrated. The *tetM* gene was also found in an additional five resistant strains (MICs 8 µg/ml) and the 25.2 MDa plasmid demonstrated. In these strains containing a *tetM* gene, 25.2 MDa plasmids were readily demonstrated, however, a single strain for which no ethidium bromide / UV visible 25.2 MDa plasmid was detected, visualisation was only achieved by means of a Southern blot employing a *tetM* gene probe.

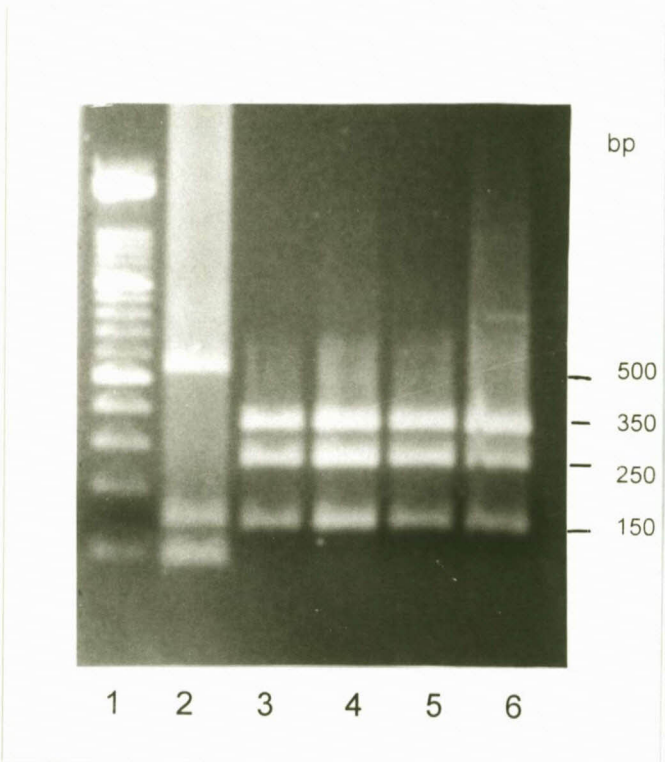


FIGURE 4.6 Representative *Hpa*II restricted *tetM* genes of *N. gonorrhoeae.* Lanes: **1**, 100 bp ladder; **2**, Dutch-type *tetM*; **3**, American-type *tetM*; **4**, strain 41; **5,** strain 100; **6**, strain 34.

4.3 DISCUSSION

Genetic techniques were employed in the study of *Neisseria* species and seen to enhance knowledge on various aspects, from differentiation between closely related species to screening for tetracycline resistance.

The RAPD analysis was performed with pre-mixed, pre-dispensed beads (Ready-To-Go® RAPD Analysis Beads, Pharmacia Biotech). These analysis beads are reported to result in genomic polymorphisms being detected at multiple loci using only nanogram quantities of DNA. The RAPD analysis did indeed result in multiple and reproducible profiles containing extensive information. The reproducibility of profiles is important when determining the reliability of any fingerprint based method (Welsh & McClelland, 1993). The occurrence of non-clonality of *Neisseria* was clearly reflected in the complex genetic profiling obtained. RAPD analysis has been successfully applied to study *Neisseria* populations within a relatively narrow time frame and physical environment (Stanley & Chalkley, 1998). The comparative RAPD analysis between the non-pathogenic *Neisseria* species and *N. meningitidis*, *N. gonorrhoeae* and *Moraxella* strains revealed that the species specific banding patterns were maintained and this information can be used as an accurate identification method. The non-pathogenic *Neisseria* were found to be more genetically variable. The conventional biochemical tests and the more recent commercial carbohydrate identification systems, API and Biolog, are not always definitive in their performance and strains vary in their ability to produce acid from glucose and maltose (bioMérieux; Cowan & Steel, 1993). Therefore, RAPD profiling provides a less time consuming alternative to these tests.

Large intra-species genetic diversity was evident with the RAPD profiles of the *N. gonorrhoeae* strains isolated in 1997. The strains were divided into six genomic groups. Despite their diversity the majority of strains still produced bands characteristic to *N. gonorrhoeae*.

Plasmid analysis of the 102 *N. gonorrhoeae* strains isolated in 1997 revealed that 47% of strains contained the *tetM*-conjugative plasmid. This shows an increase of almost 20% as compared to 1996, where only 30/100 strains from Bloemfontein contained the 25.2 MDa plasmid (Chalkley, personal communication). Changing plasmid profiles of *N. gonorrhoeae* was evident as seen with the loss of cryptic plasmids and the presence of additional 8 and/or 11 MDa plasmids. Their functions are presently unknown but further investigation of strains carrying these plasmids will provide information as to their continued maintenance in Bloemfontein *N. gonorrhoeae* populations.

The restriction endonuclease fingerprinting of the *tetM*-conjugative plasmids isolated in 1996 revealed the characteristic pattern of the American (42.8 kb) plasmid type in the majority of *N. gonorrhoeae*. The three restriction enzymes used especially *Bgl*I produced a large number of restriction fragments with a variety of sizes, thus maximising the difference between the strains (Gascoyne *et al*, 1991). The fingerprinting resulted in the identification of seven different profiles to those previously described. Similarly three of the conjugative plasmids also possessed varied restriction profiles. Both plasmid types appear to be evolving which could result in the future acquisition of further resistance genes.

Both *tetM* and non-*tetM*-mediated tetracycline resistance has increased dramatically over the recent years (Chalkley, personal communication). Of the *N. gonorrhoeae* strains investigated, 70% were fully resistant to tetracycline, 23% showed intermediate resistance with only 7% considered susceptible.

Inherent errors/limitations in experimental procedures were also evident in that 1) strains with MICs 8 µg/ml tetracycline and not ≥ 16 µg/ml were seen to possess *tetM* genes and 2) visualisation of a *tetM*-conjugative plasmid was only achieved on performing a Southern blotting technique.

The PCR reaction to amplify a fragment of *tetM* gene offered a simple method of detection and/or conformation of *tetM* presence and provided the ability to predict high-level tetracycline resistance, since all such strains contained the

gene. The *Hpa*II restriction of the *tetM* gene revealed all strains to be of the American-type. *TetM*-conjugative plasmids containing the American-type *tetM* gene have continued to establish themselves; from 2% in 1994 to 47% in 1997 strains.

It is therefore imperative that antibiotic resistance and plasmid content is monitored and investigated in order to try and prevent agents still in use against *N. gonorrhoeae* such as ciprofloxacin, ceftriaxone and azithromycin joining the ranks of redundant agents. Utilising genetic techniques to achieve this provides a thorough and simple means of surveillance.

CHAPTER 5

MYCOBACTERIUM TUBERCULOSIS

5.1 INTRODUCTION

Tuberculosis kills three million persons a year, making it the leading infectious cause of death. (Blumberg, 1995). The World Health Organisation (WHO) and the Centres for Disease Control and Prevention (CDC) calculate that tuberculosis is responsible for about 27% of the preventable deaths worldwide (Blumberg, 1995). In 1993 the WHO declared TB a global emergency.

There are several staining methods of detecting the acid-fast nature of *M. tuberculosis*, Ziehl-Neelsen, Kinyoun and flourochrome staining techniques (Shinnick & Good, 1995). In all methods the detection limit is about 5×10^3 bacilli per millilitre of specimen. It is reported that the acid-fast stain cannot be used to distinguish between the various members of the *Mycobacteriaceae* (Scrivanos, 1995). Organisms other than mycobacteria may also demonstrate various degrees of acid fastness for example *Rhodococcus* sp. and *Nocardia* sp. leading to false smear-positive results (Blumberg *et al*, 1997).

Detection by growth can be performed on solid media; LJ agar slopes and by the radiometric method of BACTEC employing liquid media. Solid media still plays an irreplaceable role in mycobacterial culture due to its high sensitivity and cost-effectiveness (Tortoli *et al*, 1998). The problem of false-positive signals has not as yet been reported with the BACTEC system, but this is counterbalanced by BACTECs` well-known difficulty of microscopic confirmation of the presence of acid-fast organisms when testing is performed on early positive vials. Therefore, delays of BACTEC positive confirmation until vials reach a growth index (G.I.) near a value of 400 lengthens the turn-around time (Tortoli *et al*, 1998). Rules regulating the use of radiolabelled materials (12B BACTEC bottles) may represent an insuperable hindrance (Tortoli *et al*, 1998). Tests such as the NAP test (inhibition of p-nitro-α-acetylamino-β-hydroxypropiophenone, a precursor to chloramphenicol) is used to identify presumptive 12B BACTEC positive vials as MTB (Howard, 1994). This test is expensive, takes 4 days to obtain a result, and

may be difficult to interpret in a small percentage of cases, particularly where *M. kansasii* is isolated (Blumberg, 1995).

Traditional bacteriological methods are slow and expensive and so nucleic acid amplification assays provide a more timely diagnosis (Eisenach *et al*, 1993). The insertion sequence, IS*6110* of *M. tuberculosis* makes an ideal target for amplification by PCR (Eisenach *et al*, 1993). The insertion sequence's repetitive nature increases the sensitivity of detection by a factor of 10-16 compared with any other proposed target that occurs only once in the chromosome (Eisenach *et al*, 1990). Two to twenty eight IS*6110* copies are present in MTB strains, while strains of *M. bovis* contain only one to three copies (Cave *et al*, 1991). The MTB complex comprising *M. tuberculosis*, *M. avium* and *M. bovis* will give an IS*6110* PCR positive fragment of 123 bp.

For strain identification of *M. tuberculosis*, DNA fingerprinting has been internationally standardised and is based on IS*6110* insertions (van Embden *et al*, 1993). Restriction analysis has indicated that the sequence of IS*6110* is conserved across strain and species lines (Cave *et al*, 1991). This method allows strains from different geographic areas to be compared and the movement of individual strains to be tracked (van Embden *et al*, 1993). Such data provides important insights into local and global transmission of tuberculosis. This DNA screening method can be used to characterise strains with important properties such as high infectivity and/or multi-drug resistance (van Embden *et al*, 1993). The standard method involves restriction endonuclease digestion of genomic DNA, followed by Southern blotting and probing for fragments containing IS*6110* elements. The restriction enzyme *Pvu*II is recommended as it cleaves the IS*6110* sequence only once (van Embden *et al*, 1993). In order to compare fingerprints between *M. tuberculosis* isolates separated on different gels and in different laboratories the size of each IS*6110* – hybridising fragment must be determined (van Embden *et al*, 1993). Standardised fingerprinting data can be collected and analysed employing computer programs (van Embden *et al*, 1993).

For strains isolated in Bloemfontein during 1996, antibiotic resistance was reported as 7% isoniazid and 1.4% isoniazid/rifampin resistance (van der Spoel

van Dijk, personal communication). The genetic basis of rifampin resistance was first described in *E. coli* (Whelen *et al*, 1995). The molecular mechanism of RIF activity involves the inhibition of DNA-dependent RNA polymerase (Musser, 1995). In *E. coli,* this enzyme is a complex oligomere composed of four different subunits (α, β, β` and σ encoded by *rpoA*, *rpoB*, *rpoC*, and *rpoD* respectively) in a core or holoenzyme form (Miller *et al*, 1994). Rifampin binds to the β subunit of *E. coli* RNA polymerase inhibiting transcription (Musser, 1995).

Resistance to rifampin is known to arise as a result of missense and other mutations occurring in a discrete 23 amino acid region (69 bp) of the *rpoB* gene (Musser, 1995). Detection of these mutations can be performed by a combination of PCR amplification of the *rpo* gene, single-strand conformation polymorphism (SSCP) and DNA sequence analysis (Telenti *et al*, 1993; Miller *et al*, 1994). A newer technique, which combines elements of both SSCP and DNA sequencing, is dideoxy fingerprinting (ddf) (Sarkar *et al*, 1992). Results from the different methods are, however, difficult to interpret in a clinical setting. Using automated DNA sequencing, Kapur *et al* (1994) confirmed that greater than 90% of rifampin-resistant strains have sequence alterations in a 69 bp region of the *rpoB* gene - also identified were several mutant *rpoB* alleles not previously described. Subsequent studies on mutation analysis have revealed two additional polymorphisms outside of the 69 bp core region, therefore extending the size of the core region to 81 bp (Kapur *et al,* 1994; Musser, 1995). Mutation studies have shown that the level of rifampin resistance is dependent on the type of mutation involved (Bodmer *et al*, 1995). The rapid genetic detection of such mutations can play an important role in the diagnostic laboratory and management of patients with tuberculosis. It is not only possible to use mutations in the *rpoB* gene to predict rifampin resistance, but since there is a 95% probability that such strains will also be isoniazid-resistant, this locus may also be used in the prediction of multi-drug resistant strains.

5.2 RESULTS

5.2.1 Screening

5.2.1.1 Conventional methods

Ziehl-Neelsen screening showed that 33/313 (10.5%) of sputum samples collected from the community (1998) were positive, corresponding to 30 persons. Seventy-eight percent of the samples were ZN negative. Figure 5.1 shows the numbers of samples tested and grouped according to ZN screening and culture results, including contaminated cultures, while Figure 5.2 shows results on the number of persons. LJ culture over ZN staining identified seven additional persons, however, 10% contamination occurred with the LJ agar culture method.

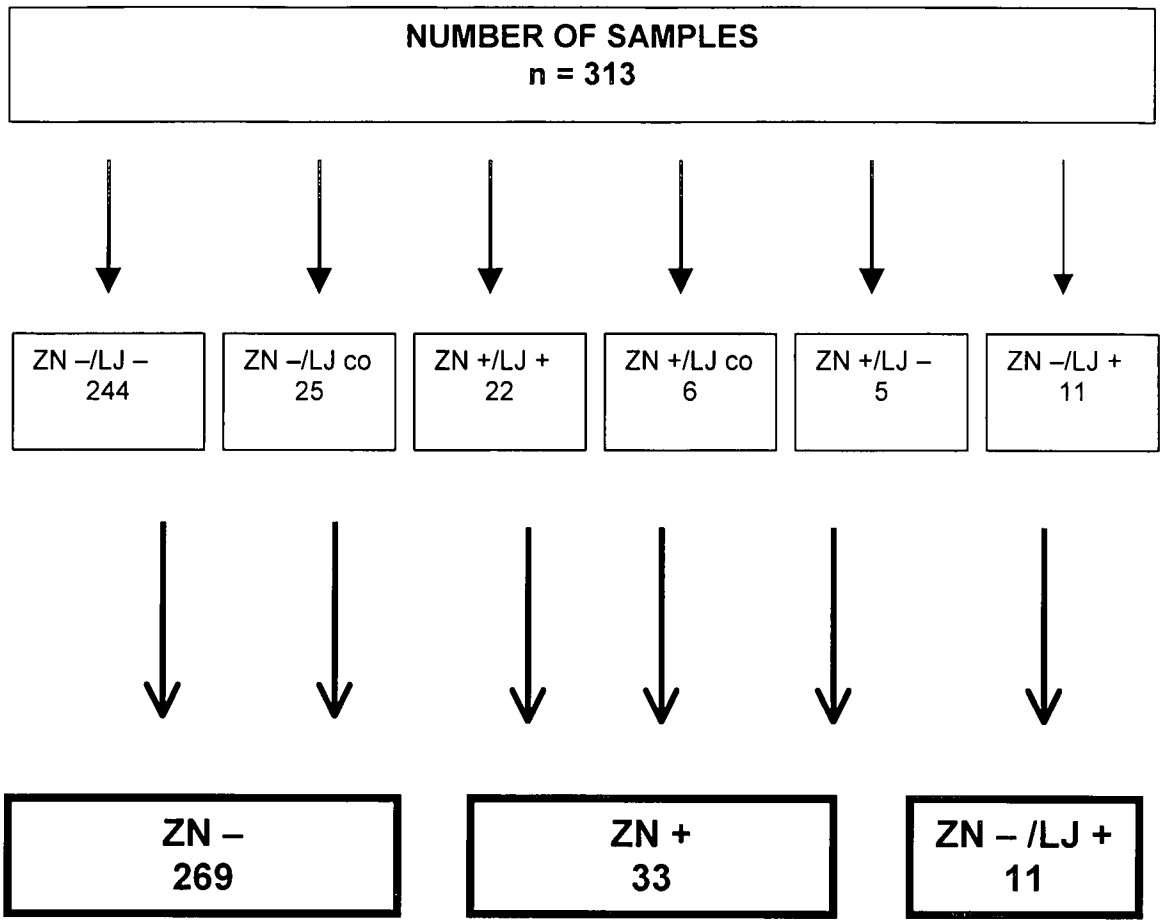
5.2.1.2 BACTEC and PCR confirmation

When results of ZN and culture did not correlate PCR amplification of a 123 bp sequence in IS*6110* was performed on the sputum digests (Figure 5.3). Where possible, selected digests were also inoculated into 12B bottles and the growth of MTB confirmed.

PCR and BACTEC confirmed the ZN positive result from 5 persons (6 samples) whose LJ cultures were contaminated (Figure 5.3). Thirteen of the 25 ZN negative digests with corresponding contaminated LJ cultures were randomly chosen for PCR. Nine samples gave positive results, 2 of which were weak (Figure 5.3). One sample from the group of 13 was cultured by the BACTEC system producing a positive growth index. This person's sputum digest sample in fact producing 1 of the 2 weak PCR results. Direct inoculation from MTB cultured in the 12B bottles onto blood agar plates revealed no contamination.

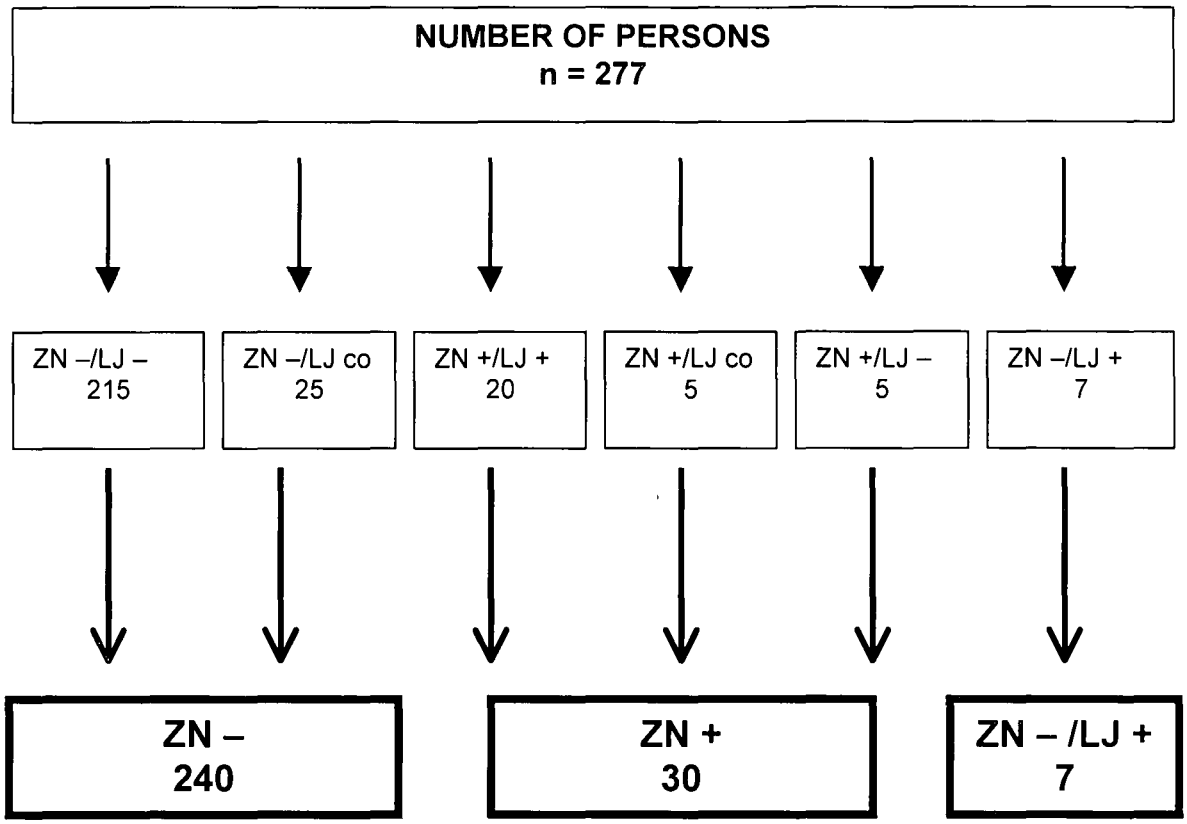
Confirmation methods also revealed that 9/11 ZN negative / LJ positive samples had positive PCR results (Figure 5.4). Again, a single digest was

FIGURE 5.1 ZN screening and LJ culture of sputum samples.



co = contaminated

FIGURE 5.2 Microscopy and culture results on persons screened for *M. tuberculosis*.



co = contaminated

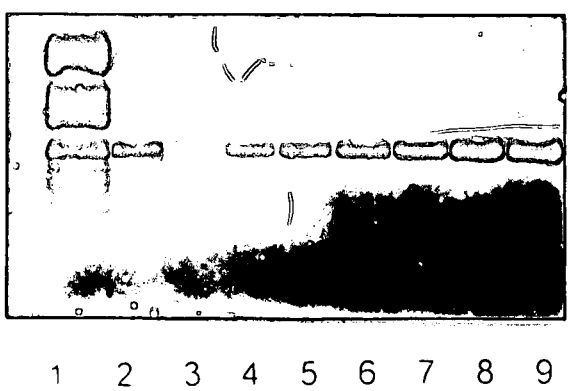
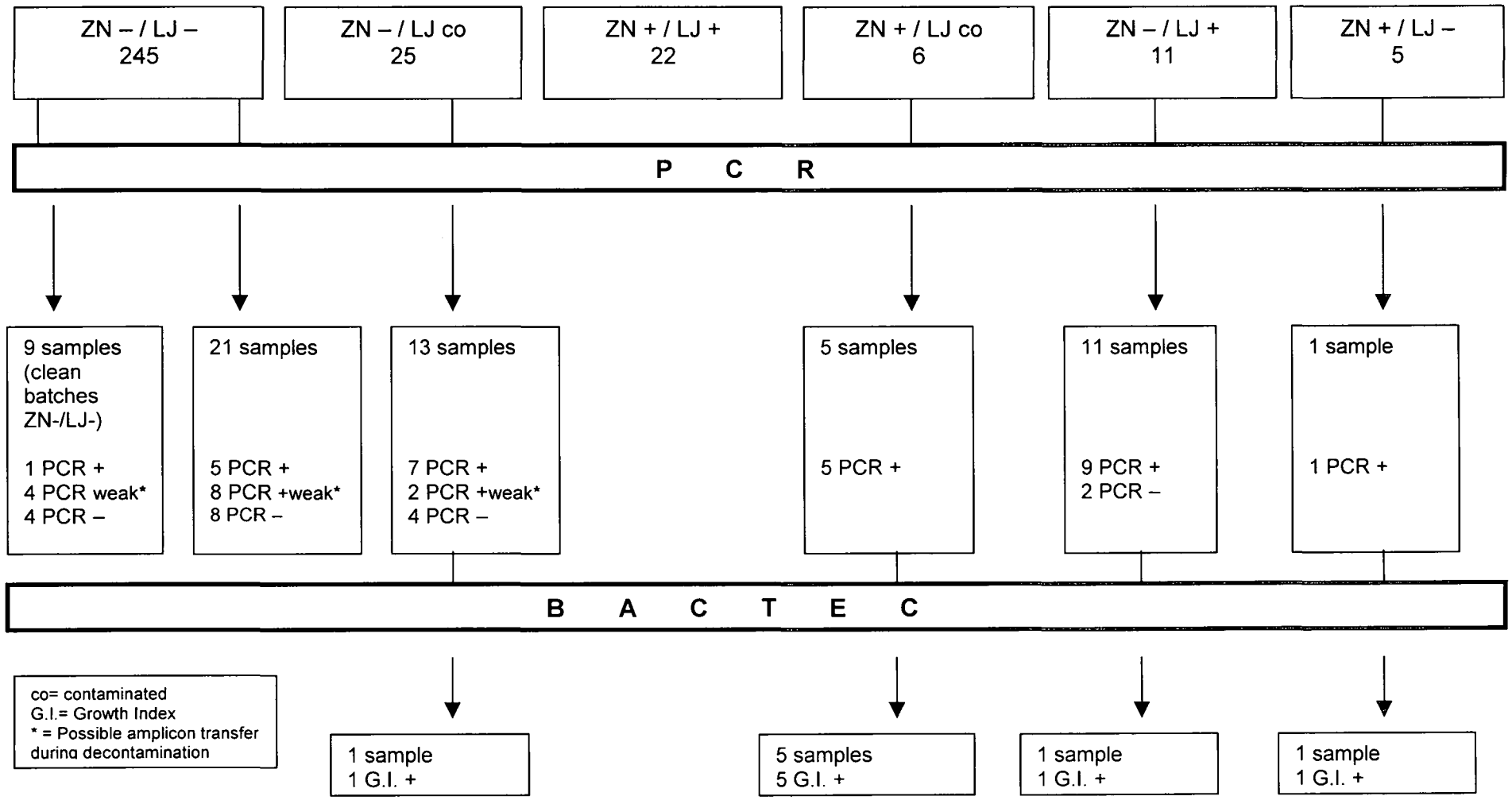


FIGURE 5.3 Representative IS*6110 M. tuberculosis* PCR results.
Lanes: **1**, MWM V; **2**, positive control; **3**, negative control; **4** strain 215 (ZN–/LJ co.); **5**, strain 204 (ZN–/LJ –); **6**, strain 103 (ZN+/LJ co.); **7**, strain 177 (ZN+/LJ co.); **8**, strain 226 (ZN+/LJ –); **9**, strain 82 (ZN –/LJ +).

FIGURE 5.4 PCR and BACTEC confirmation of sputum samples.



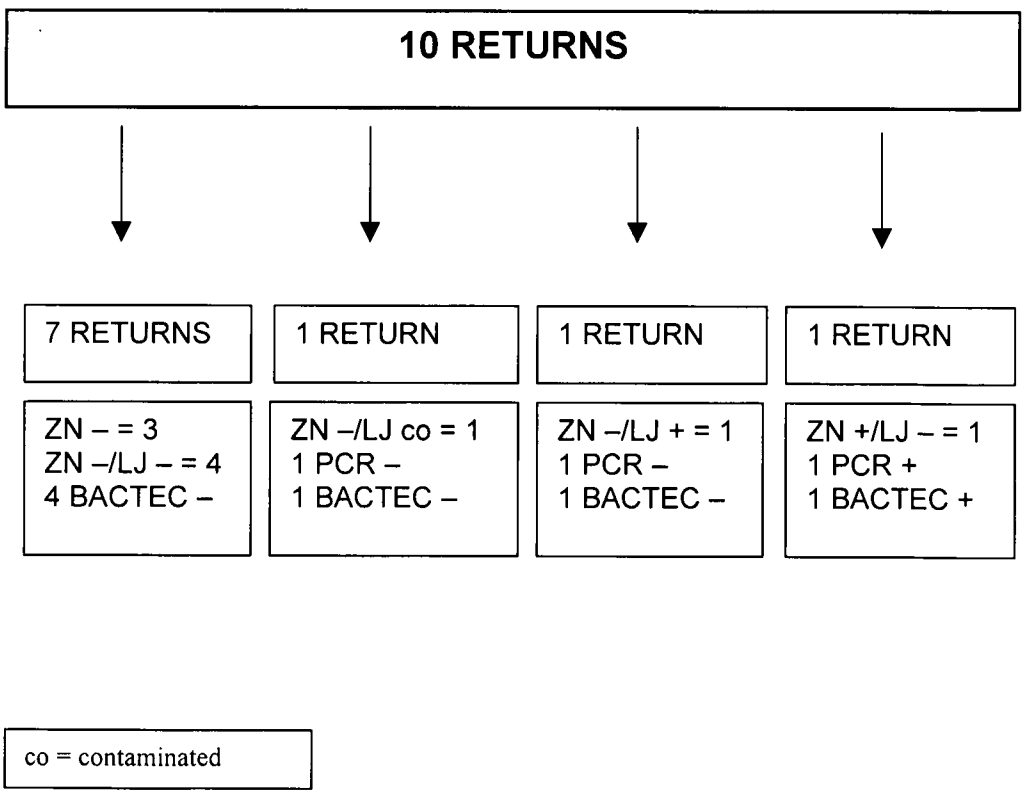
cultured by the BACTEC system and produced a positive growth index reading. Five ZN positive confirmed sputum samples failed to grow on LJ agar slopes. Insufficient amounts of digest halted confirmation by PCR and BACTEC on four of these samples.

Finally 30 ZN negative / LJ negative samples were chosen for PCR assessment, nine of which were taken from clean batches (all digests decontaminated on a certain day found to be ZN-/LJ-). PCR revealed 1) 1/9 clean batch samples produced a strong positive result and 2) 4/9 produced weak bands, 3) 5/21 produced strong PCR positives (Figure 5.3) and 4) 8/21 samples produced weak bands that could possibly be as a result of amplicon carry over.

5.2.2 Screening of Return Cases

Only ten persons out of the 27 new cases that were ZN positive and that received therapy returned to the clinics. Results of the techniques pertaining to return cases varied considerably (Figure 5.5). Patients non-adhering to treatment or reassessment comprised 63%. Where possible susceptibility testing was performed on the return cases. One patient returning with positive ZN/PCR was seen to have an isoniazid susceptible strain initially and isoniazid-resistant strain on return. Four persons returned to provide a sputum sample (based on criteria other than the ZN negative result, for example chest X-ray or symptoms) three were ZN negative one was ZN positive.

FIGURE 5.5 Screening of new cases that received treatment.



Old cases refers to patients whose first return samples fell within the main screening collection. These persons then returned a second time after 3-6 months. Where possible ZN, culture and BACTEC testing was performed on these samples (Table 5.1). Patient 2 has a 'no cure' outcome as the 3-6 month return results remained positive despite the patient's *M. tuberculosis* strain being fully susceptible to all antibiotics tested (Table 5.1).

TABLE 5.1 Screening of old cases.

PATIENT	FIRST RETURN (2-3 months)	SECOND RETURN (3-6 months)	OUTCOME
1	ZN + / LJ –	ZN – / LJ – BACTEC –	CURE
2	ZN + / LJ –	ZN + / LJ + BACTEC + Fully Susceptible	NO CURE
3	ZN – / LJ + BACTEC - / PCR +	ZN –	POSSIBLE CURE
4	ZN + / LJ + BACTEC + Fully Susceptible	ZN –	POSSIBLE CURE

5.2.3 Fingerprinting

Fingerprinting of 50 INH-resistant *M. tuberculosis* strains isolated in 1997 revealed 32 strains with different profiles. Five clonally related groups (I - V) were evident (Figure 5.6, Table 5.2). Samples collected in Bloemfontein possessed fingerprint profiles which placed them within the groups I and II (Figure 5.6, lanes 1 and 2). The other clonal groups were disseminated throughout districts in the Free State.

Fingerprinting of 31 culture confirmed *M. tuberculosis* strains isolated in 1998 revealed diverse strain types (Figure 5.7). Four persons had repeat samples with like-fingerprinting profiles observed between the pairs (Figure 5.7 strains 9A and 9B, lanes 6 and 7; strains 20A and 20B, lanes 23 and 24). Twenty six distinct fingerprint profile patterns were evident from the 27 *M. tuberculosis* strains isolated from different persons. The fingerprinting of two cultures isolated from LJ slopes on the same day indicated that cross contamination had occurred (Figure 5.7 strains 4 and 5, lanes 3 and 4). Strain 8 (Figure 5.7, lane 5) showed a 1-band shift difference compared with strains 9A and 9B (Figure 5.7, lanes 6 and 7). The number of IS*6110* copies present in the *M. tuberculosis* isolates was difficult to determine when incomplete restriction had taken place. Despite detection of typical MTB colonies on LJ agar and the presence of acid fast bacilli, one strain only contained two IS*6110* copies which could be indicative of *a M. bovis* isolate (Figure 5.7 strain 18, lane 13).

Fingerprinting of 14 rifampicin-resistant strains (13 persons from 1998) revealed 12 different profiles (Figure 5.8). Two samples (17/9/1998 and 6/10/1998), however, collected from the same hospital ward shared profiles (Figure 5.8 strains R1 and R4, lanes 1 and 4). Samples with 1-3 insertion copies were noted as possible *M. bovis* strains (Figure 5.8 strains 5A and 5B, lanes 6 and 7). The remaining strains had IS*6110* profiles with between 3 – 24 bands. All but one (strain R2) of the 12 samples was reported as multi-drug resistant strains; resistant to not only rifampicin but isoniazid as well.

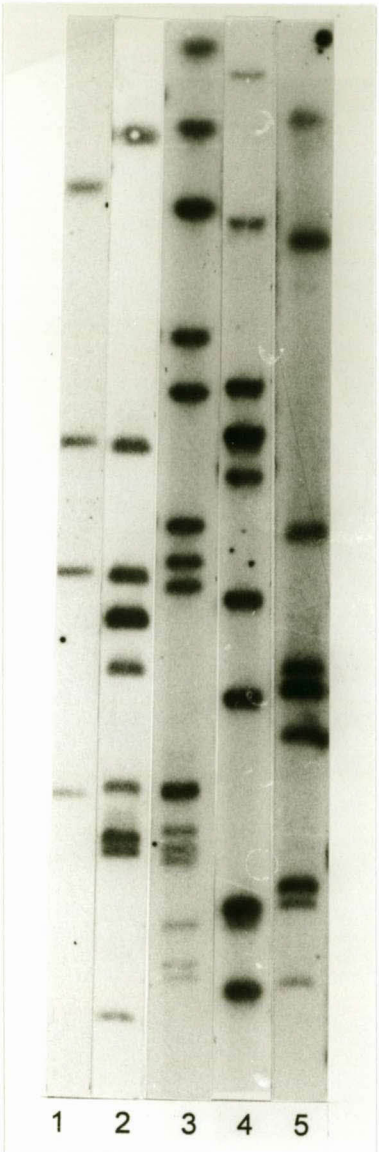


FIGURE 5.6 Representative IS*6110* fingerprint profiles of clonal groups from *M. tuberculosis* strains isolated during 1997 from districts in the Free State.

Lanes: **1**, clonal group I; **2**, clonal group II; **3**, clonal group III; **4**, clonal group IV; **5**, clonal group V.

TABLE 5.2 Clonally related strains isolated in the Free State.

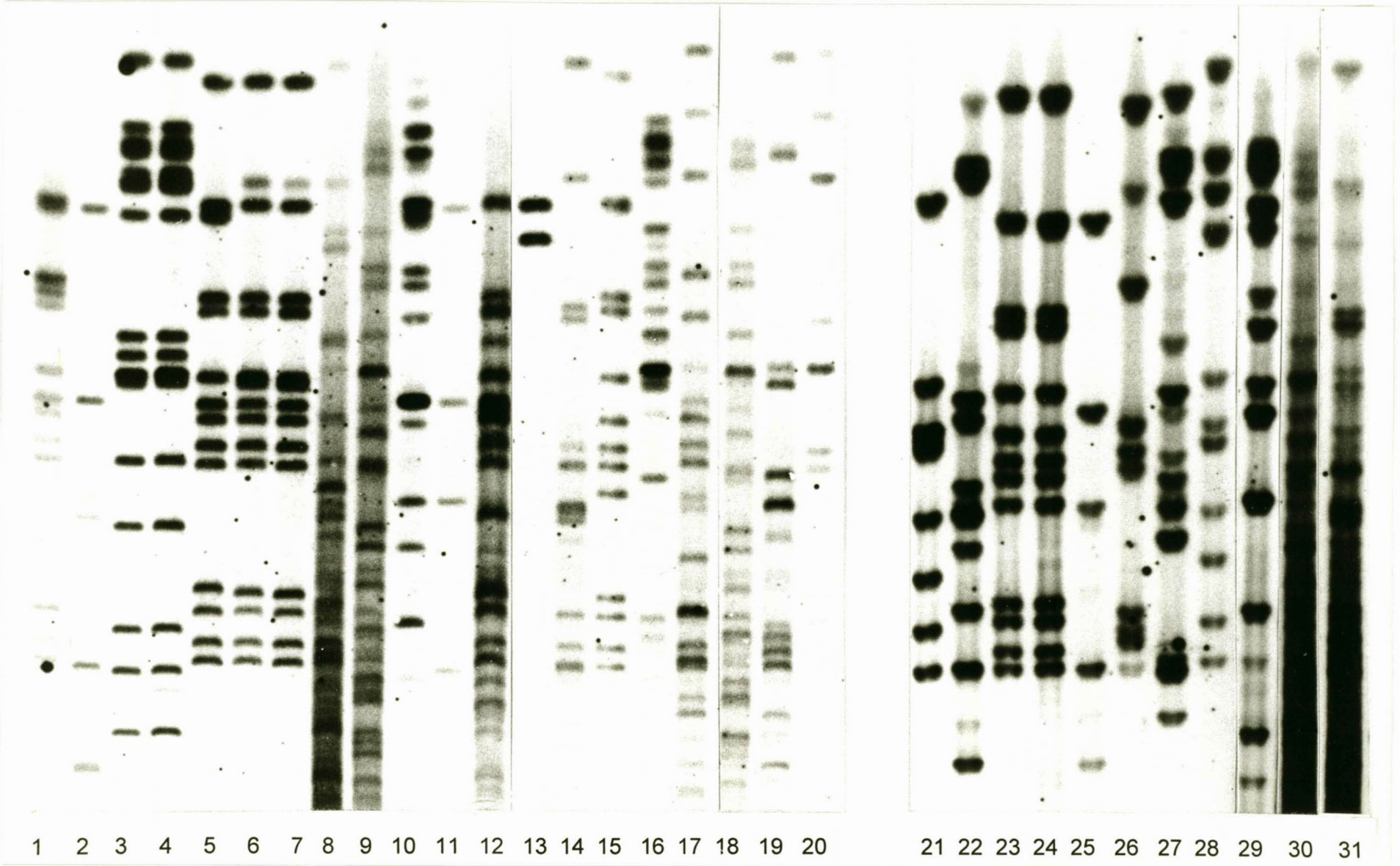
LOCATION	ANTIBIOTIC PROFILES				FINGERPRINT
	RIF	INH	STREPT	ETHAM	PROFILES
Welkom	R	R	R	S	I
Bloemfontein	S	R	S	S	I
Thaba'Nchu	R	R	S	S	I
Bloemfontein	S	R	S	S	II
Bloemfontein	S	R	S	S	II
Parys	S	R	S	S	II
Allanridge	R	R	S	R	II
Qwa-Qwa	S	R	S	S	II
Hoopstad	S	R	S	S	II
Hoopstad	R	R	S	S	II
Trompsburg	S	R	S	S	III
Bloemhof	S	R	S	S	III
Christiana	S	R	S	S	III
Qwa-Qwa	S	R	S	S	IV
Qwa-Qwa	S	R	S	S	IV
Marseilles	S	R	S	S	V
Marseilles	S	R	S	S	V
Marseilles	S	R	S	S	V

FIGURE 5.7

FIGURE 5.7 IS*6110* Fingerprint profiles of 1998 strains.

Lanes: **1**, strain 1A; **2**, strain 2; **3**, strain 4; **4**, strain 5; **5**, strain 8; **6**, strain 9A; **7,** strain 9B; **8**, strain 10; **9**, strain 12; **10**, strain 13; **11**, strain 14; **12**, strain 15A; **13**, strain 18; **14**, strain 19; **15**, strain 20B; **16**, strain 21; **17**, strain 22; **18**, strain 26; **19**, strain 33; **20**, strain 35; **21**, strain 6; **22**, strain 17; **23**, strain 20A; **24**, strain 20B; **25**, strain 27; **26**, strain 28; **27**, strain 29; **28**, strain 30; **29**, strain 16; **30**, strain 37; **31**, strain 25.

Lanes 1-20 - same Southern blot. Lanes 21-31 – same Southern blot


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
21 22 23 24 25 26 27 28 29 30 31

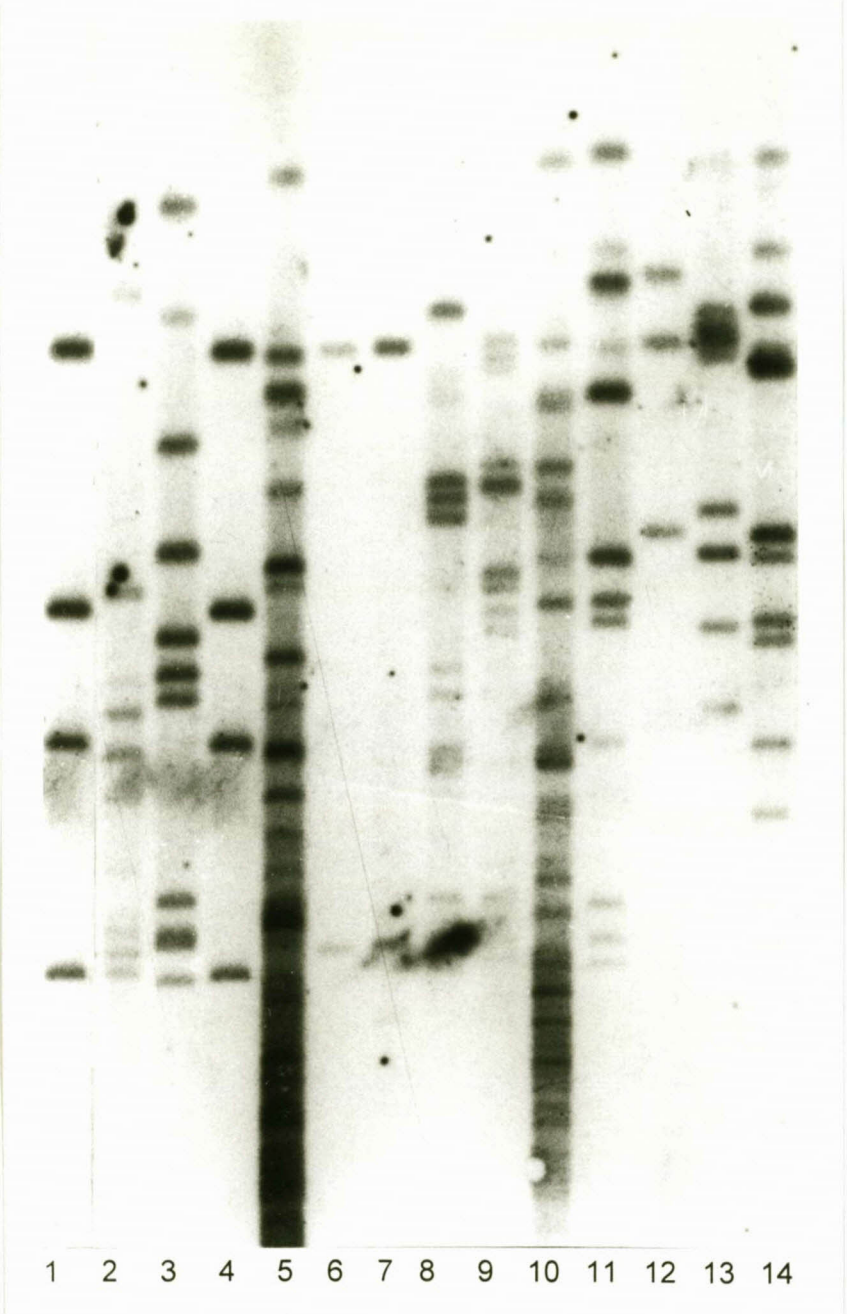


FIGURE 5.8 IS*6110* Fingerprint profiles of rifampicin-resistant *M. tuberculosis* strains (1998).

Lanes: **1**, strain R1; **2**, strain R2; **3**, strain R3; **4**, strain R4; **5**, strain R6; **6**, strain R5A; **7**, strain R5B; **8**, strain R8; **9**, strain R9; **10**, strain R10A; **11**, strain R11, **12**, strain R12; **13**, strain R13; **14**, strain R7

M. tuberculosis fingerprinting studies were initiated in Bloemfontein in 1995 and of epidemiological interest was a family group comprising two brothers, a sister and a wife from Bainsvlei, Bloemfontein (van der Spoel van Dijk *et al*, 1996). Brother 1 received treatment prior to January 1995, but had failed to comply with treatment. Brother 2 was treated in January while the sister in February. The shared fingerprinting profiles of all the Bainsvlei family members showed them to represent a clonal contact group, however, their resistance profiles differed (Table 5.3, Figure 5.9). The finding of a fully susceptible strain from the sister in May 1995, whilst still on treatment could be explained by incorrect susceptibility testing, although this was not the case (section 5.2.4). One brother (2) was found to continue to exhibit a positive ZN stain and *M. tuberculosis* culture result on returning in 1997 and 1998. The strain isolated in 1997 demonstrated the same IS*6110* fingerprint profile in addition to an isoniazid / rifampicin resistance profile. On returning to the clinic in 1998 with yet again a strain with the same resistance profile, the same fingerprint profile was found. A single non-family member isolated from the 50 strains in 1997 shared the same fingerprint profile as was found with the family members in 1996 and 1997. Together this single sample and the family members created a sixth Free State clonal group.

TABLE 5.3 Details of Bainsvlei family members.

	DATE	RIF	INH	FINGERPRINT
BROTHER 1	10-1-95	R	S	
	5-5-95	R	R	+
BROTHER 2	8-2-95	R	R	+
	26-5-95	R	R	+
SISTER	10-2-95	S	R	
	5-5-95	S	S	+
WIFE (Brother 2)	11-5-95	S	R	+

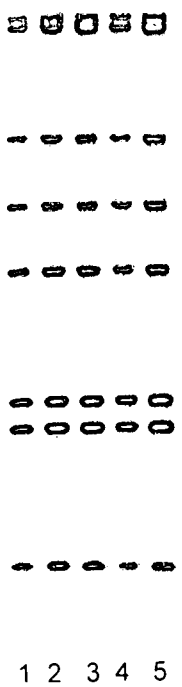


FIGURE 5.9 IS*6110* Fingerprint profiles from Bainsvlei family members.
Lanes: 1-4 – 1995 strains, 5 – 1997 strain.
Lanes: **1**, brother 1; **2**, brother 2; **3**, sister; **4**, wife; **5**, brother 2

5.2.4 *rpoB* Gene Sequencing

A group of eight *M. tuberculosis* strains from Bloemfontein and regions in the Free State isolated in 1997 were chosen for *rpoB* gene sequencing. These strains represent two of the 5 clonal groups evident from the 50 strains examined that contained rifampicin-resistant isolates (Table 5.2). All eight gave a 157 bp amplification product. Three strains reported to have susceptible phenotypes to rifampicin (resistant only to isoniazid) showed no mutations within the 157 bp region of the *rpoB* gene sequence confirming susceptibility data (Table 5.4). One rifampicin-susceptible strain by BACTEC, however, showed a mutated codon at position 516 (Table 5.4). The shared fingerprinting profiles of these two cluster groups had no apparent correlation to their rifampin mutations, perhaps indicating rifampin resistance had developed independently after the rifampin-susceptible strains had been contracted and treated (Table 5.4).

Seventeen *M. tuberculosis* strains isolated in 1998 from new cases (susceptibility testing not routinely performed) underwent amplification for the *rpoB* gene. Thirteen of the seventeen samples produced the predicted 157 bp amplification fragment. Sequencing of the *rpoB* gene revealed 1) no mutations were evident with 10 strains, 2) 1 strain carried the mutated codon GTC in position 516 and 3) 1 strain carried the mutated codon CCG in position 533.

Thirteen rifampin-resistant strains (1998) also underwent sequencing of the *rpoB* gene. All mycobacterial DNA produced a 157 bp fragment by amplification to act as template for sequencing. Two samples were excluded, as insufficient DNA was extracted to enable precise sequence data collection. Mutated codons were found in 10/11 resistant strains (Table 5.5). For nine strains amplified *rpoB* gene regions each had a single–base substitution leading to an amino acid change. Three *rpoB* genes had mutations in the previously reported position 526, three in position 531 and three had mutations at position 516. One strain had deletions involving codons 511 – 514 (Table 5.5). Altering the up stream primer so that a larger fragment of the gene could be amplified would allow for confirmation of this deletion in the reverse direction. Only one strain reported to have the

TABLE 5.4 Mutations occurring in the 157 bp sequence of the *rpoB* gene from *M. tuberculosis* isolated during 1997 from districts in the Free State.

Geographic location (Fingerprint profile group)	**Drug resistance profile #**	**Codon mutation**	**Amino acids affected (position)**
Welkom (I)	RIF,INH,SM	GAC→TAC	Asp→Tyr (516)
Bloemfontein (I)	INH,SM	No mutation	none
Thaba'Nchu (I)	RIF,INH	TCG→TTG	Ser→Leu (531)
Allanridge (II)	RIF,INH,EMB	TCG→TTG	Ser→Leu (531)
Hoopstad (II)	RIF,INH	GAC→GTC	Asp→Val (516)
Hoopstad (II)	INH	No mutation	none
Bloemfontein (II)	INH	No mutation	none
Bloemfontein (II)	INH	GAC→TAC	Asp→Tyr (516)

RIF = Rifampicin INH = Isoniazid SM = Streptomycin EMB = Ethambutol

The codon numbers are designated on the basis of alignment of the translated *E. coli rpoB* sequence with a portion of the translated *M. tuberculosis* sequence and are not the positions of the actual *M. tuberculosis rpoB* codons (Musser, 1995).

TABLE 5.5 Mutations occurring in the 157 bp sequence of the *rpoB* gene from rifampicin-resistant *M. tuberculosis*.

Strain number	**Geographic location**	**Drug resistance pattern ***	**Codon mutation**	**Amino acid (s) affected (position) #**
R1	±H1,Bloemfontein	RIF,INH,EMB	TCG→TTG	Ser→Leu (531)
R2	H2,Bloemfontein	RIF	no mutation	none
R3	H3,Bloemfontein	RIF,INH,EMB,SM	CAC→GAC	His→Asp (526)
R4	H1,Bloemfontein	RIF,INH,EMB	TCG→TTG	Ser→Leu (531)
R5A	Bloemfontein	RIF,INH,EMB	GAC→GTC	Asp→Val (516)
R6	Bainsvlei	RIF,INH	CAC→TAC	His→Tyr (526)
R7	Excelsior	RIF,INH,SM	CTG AGC CAA TTC (deletion)	Leu-Ser-Gln-Phe (511-514)
R8	Thaba'Nchu	RIF,INH,EMB	CAC→CAA	His→Glu (526)
R9	Henneman	RIF,INH,	GAC→GTC	Asp→Val (516)
R11	Odendaalsrus	RIF,INH,EMB	TCG→TTG	Ser→Leu (531)
R12	Botshabelo	RIF,INH	GAC→GTC	Asp→Val (516)

± H = hospital * RIF = Rifampicin INH = Isoniazid EMB = Ethambutol SM = Streptomycin

Ser = Serine; Leu = Leucine; Asp = Aspartic acid; Val = Valine; His = Histidine; Tyr = Tyrosine; Gln = Glutamine; Glu = Glutamic acid; Phe -= Phenylalanine

TABLE 5.6. Mutations occurring in the 157 bp sequence of the *rpoB* gene from *M. tuberculosis* isolated from a family community.

Family Member (year)	Antibiotic Profile RIF	INH	Codon mutation	Amino acid affected (position)
Brother 2 (1995)	R	R	CAC→TAC	His→Tyr (526)
Sister (1995)	S	S	No mutations	none
Wife of Brother 2 (1995)	S	R	No mutations	none
Brother 2 (1997)	R	R	CAC→TAC	His→Tyr (526)
Brother 2 (1998)	R	R	CAC→TAC	His→Tyr (526)

rifampin-resistant phenotype contained no mutations within the 157 bp region of the *rpoB* gene (Table 5.5, strain R2).

RpoB gene analysis performed on available isolates from the Bainsvlei family group revealed that the strain from Brother 2 maintained an isoniazid/rifampin antibiotic resistance profile from 1995 through to 1998. The mutated codon in position 526 also remained the same (Table 5.6). The strains from the sister and the brother's wife reported to have a susceptible phenotype to rifampicin showed no mutations within the 157 bp region of the *rpoB* gene sequence, once again reflecting rifampin resistance developing within a clonal group (Table 5.6). It should be noted that the brother 2 had received a course of intensive therapy in 1996 and theoretically should have been cured from this strain. However, it was later found that some 12-14 workers plus approximately 40 family/friends stayed at the Bainsvlei family home (many of whom were not available for providing sputum samples) that could have harboured the strain. The brother may therefore have been totally cured by the intensive therapy received and been reinfected on his return to the family community with the same multiply-resistant strain type.

5.3 DISCUSSION

The emergence of multi-drug resistant strains of MTB plus the restricted number of efficacious therapeutic agents available to treat patients infected with MDR organisms, poses a serious problem in tuberculosis control and stresses the need for the development of rapid and reliable diagnostic methods for drug susceptibility testing in clinical isolates (Pozzi *et al*, 1999). The renewed effort to define the molecular genetic basis of antimicrobial resistance in mycobacteria is due to the assumption that this will lead to formulation of rapid and unambiguous strategies for the detection of resistant strains (Musser, 1995). It is also thought that knowledge gained from a genetic understanding of resistance mechanisms can be exploited in efforts directed toward rational design of new antimicrobial agents (Musser, 1995).

The Ziehl-Neelsen stain remains the cornerstone of early diagnosis of tuberculosis. Most mycobacteria grow relatively slowly (generation time of TB bacilli is 12 –18 h) and so obtaining results from an acid-fast smear within 24 h is clearly beneficial. The ZN detection level was high at 81% but in the current study ZN was performed by trained laboratory staff that were aware of the parallel investigation. If many acid-fast bacteria are present (> 10 000 bacteria/ml sputum) the ZN stain is easy to interpret, however, recognition of few bacilli requires skill and patience (Wolinsky, 1994). The procedure does have limitations in that microorganisms other than mycobacteria may demonstrate various degrees of acid fastness, in addition the flourochrome and auramine stains have been reported as being more sensitive than ZN (Scrivanos, 1995). Thirty three of the three hundred and thirteen sputum samples were reported as ZN positive. The majority of the laboratories services in South Africa only offer TB microscopy and so rely entirely on the ZN stain for diagnosis of suspected pulmonary tuberculosis. This inexpensive method when performed by skilled persons should ideally lead to high detection levels. Increased accuracy even in proficient laboratories can be achieved by extending testing to include culture

and genetic techniques.

Culturing of MTB using solid LJ agar slopes is more sensitive than staining methods, but results are not available for 4-6 weeks. In the present screening study by culturing sputa on LJ medium an additional 7 positive *M. tuberculosis* cases were identified. Ten percent of LJ slopes were contaminated. The decontamination process of the sputum samples after collection is an important stage. The concentration of the sodium hydroxide used is critical since the procedure involves differential killing of pyogenic organisms (Wolinsky, 1994). The time between collection of samples and processing for culture may also contribute to an increase in contaminants particularly fungi. Reversibly, if the decontamination is too harsh false culture-negative results are obtained. For five samples that were ZN positive *M. tuberculosis* was not detected on LJ media. Further investigation would have confirmed whether a too harsh decontamination stage was the possible cause of this occurrence or whether these strains preferred a liquid media for growth. The risk of cross contamination from digest to digest or from digest to culture media is high during this decontamination step.

The radiometric culture system BACTEC detects growth of mycobacteria in selective liquid media on the basis of the metabolic release of radioactively labeled CO_2 from ^{14}C palmitic acid (Blumberg, 1995). This liquid media and system was extremely sensitive and helped confirm five positive samples with contaminated LJ slopes. BACTEC produces recovery rates of 99.27% for isolates of the *M. tuberculosis* complex (Tortoli *et al*, 1998). Although reported to shorten time of detection of MTB growth from 4-6 weeks (LJ) to 7-14 day, delays are always encountered as the growth index reaches a maximum reading for sufficient cells to be present to enable ZN stain confirmation.

BACTEC was utilised for confirmation of selected samples when ZN and LJ results did not correlate. Contributing to the selectivity of BACTEC is an antimicrobial supplement which is designed to minimise overgrowth of contaminating flora, as found in the present study confirming five ZN positive samples when LJ slopes were contaminated. Contaminants, however, may be encountered that are resistant to the antimicrobials in the supplement

(BACTEC® PANTA™ Plus Kit protocol, 1996). The large expense of the liquid media and additional kits is a drawback to this culture method being adopted for screening in South Africa.

The shortcomings of expense and time for reliable culture methods are eliminated by the rapid PCR detection technique. Eisanach *et al* (1990) developed a PCR assay for the detection of *M. tuberculosis* DNA that amplifies a 123 bp sequence in IS*6110*. Since PCR techniques detect organism-specific DNA, it can be used in detection of both active and latent disease (Scrivanos, 1995). This genetic technique is therefore not only highly specific but also sensitive. PCR can, however, not be utilised in the capacity that a ZN stain can to confirm a patients sputum sample conversion to negative after treatment, since the PCR will remain positive for 4 weeks or longer (Hill, 1996). In some cases, however, overcoming this 'dead cell' objective can still be achieved by genetic techniques by performing a reverse transcriptase-coupled PCR using mRNA as a template (Hill, 1996). The PCR technique is rapid (1.5 days) with no requirement for the 3-6 weeks to culture the MTB, as DNA template is prepared directly from the decontaminated sputum samples. PCR also has the advantage over BACTEC since no radio-active material is used so safety precautions and the problems of radio-active waste are avoided (Blumberg, 1995).

PCR like BACTEC was employed as confirmation when ZN and LJ results did not correlate. The PCR testing detected a high number of positive amplification products from sputum samples with ZN negative / LJ negative results. Twenty-one sputum samples were screened plus nine samples from batches that did not contain positive LJ/ZN sputa. Thirteen of the 21 samples produced positive PCR results, eight of which were weak, while five of the nine clean batch samples produced positive PCR results, four of which were weak. Under-detection with ZN/LJ, primary localised transient infection or amplicon carry over / cross contamination could explain the weak PCR results. Commercial PCR kits are available that will allow confirmation of such amplicon transfer and could greatly benefit such circumstances.

Only 10 of the 27 persons (new cases) that received treatment returned to the clinic after 2 months. Therefore 63% of patients were seen not to be adhering to treatment or reassessment. A single return sample was ZN positive, PCR and BACTEC confirmed this positive result. Also evident was an old case that had a ZN positive result for 6 months. The *M. tuberculosis* strain from this case tested sensitive to the four antituberculosis drugs and one can only assume the person did not comply with treatment. The return sputum sample collection is essential to monitor the conversion of the ZN stain to negative. If ZN positivity persists after 1-2 months only then is susceptibility testing performed. The lack of proper records of persons with MTB and the government policy of no tracing is seriously hampering TB cure rates.

Fingerprinting based on IS*6110* has already shown great potential in strain differentiation and its usefulness in outbreak and various epidemiological investigations (van Embden *et al*, 1993). The drug-resistant strains isolated in 1997 also showed diverse profiles but five clonal groups were evident. Comparing fingerprint profiles can aid in revealing compliance. This information is vital as it has a direct effect on the development and transfer of antibiotic resistant strains (Yang *et al*, 1994). Excluding the cross-contaminated sample, the fingerprinting profiles from 30 sputum samples (26 persons) in 1998 again showed 25 unique profiles indicating the presence of various diverse strain types in Bloemfontein. Non-adherence to treatment was evident as well as the emergence of resistant clones. The 13 rifampicin-resistant strains (1998) also had diverse profiles indicating resistance had developed in different *M. tuberculosis* populations. Two samples collected from the same hospital ward had the same IS*6110* fingerprint profiles, showing possible dissemination of this certain strain type between the patients.

Infection is high among close contacts of sputum and patients. This was demonstrated by the identical fingerprint profiles of a Bainsvlei family. The two brothers had contracted the same strain of MTB, which was resistant to isoniazid and rifampicin. The strains isolated from the sister and wife were susceptible to isoniazid. Strain persistence was evident as the drug resistance profile plus fingerprint profile of brother 2 remained the same in 1996 and 1997. In 1998 his

drug resistance profile and fingerprint profile was again maintained.

DNA sequencing was used to characterise mutations associated with rifampin resistance in a 69 bp region of the *rpoB* gene, encoding the β subunit of RNA polymerase in MTB. It is only relatively recently that the molecular basis of rifampicin resistance was identified (Telenti *et al,* 1993), therefore examining the range of mutations, particularly from a non-US source, is important. In addition, sequencing provides the most convincing confirmation that a particular band is in fact the specific region thought to be amplified (Hill, 1996).

The two rifampicin/isoniazid-resistant strains from the same clonal group (based on shared fingerprint profiles) isolated from different locations in the Free State had different mutated codons (positions 531 and 516 respectively). This showed the occurrence of antibiotic resistance gene evolutionary divergence at the clonal level. Two samples (ZN and culture-positive, 1998) contained missense mutations in positions 533 and 516 respectively. Efficacy of initial treatment would be reduced for these persons as they were treated with the first line combination of antituberculosis drugs including rifampicin while infected with rifampicin-resistant strains. Equal numbers of the 11 rifampicin resistant strains had codon mutation in positions 516, 526 and 531. The most common amino acid substitution of serine to leucine in position 531 (Pozzi *et al*, 1999; Musser, 1995) being evident in this study. The relative frequency of mutations in certain positions, however, is directly related to geographic location (Pozzi *et al*, 1999). The two multi-drug resistant strains isolated from the same hospital ward, which possessed the same fingerprint and drug resistance profiles, shared the same codon mutation site; position 531. This is to be expected if the strains are "truly" clonally related. The Bainsvlei family member returning infected with a multi-drug resistant strain had the same variant *rpoB* allele (Tyr526) in 1995, 1997 and 1998, confirming the persistence of the resistant gene type.

One strain from 1997 and 1 from 1998 were found to have mutated *rpoB* alleles but rifampicin-susceptible phenotypes indicating possible inaccuracies with susceptibility testing. Where the opposite case occurred, in other words no

mutations found with rifampicin-resistant phenotypes reported, possible explanations are 1) mutation(s) occurring outside the amplified *rpoB* gene region or 2) incorrect susceptibility testing.

FINAL DISCUSSION

The objective of this study was the surveillance of community and environmentally acquired infections utilising different genetic techniques. Pathogenic microorganisms studied were waterborne pathogens, *N. gonorrhoeae* and *M. tuberculosis*. Investigating genetic techniques and microorganisms at the molecular level helps broaden the knowledge that can be obtained from conventional methods.

The aged and very young are at risk for the diarrhoea causing bacteria, *E. coli* 0157:H7, *Shigella, Salmonella* and *Listeria monocytogenes*. PCR has provided an inexpensive (approximately R8 per test) and rapid means of screening large numbers of water samples and has the advantage over conventional culture methods of detecting viable but non-culturable pathogenic waterborne bacteria. If few such bacteria are present in association with large numbers of microorganisms in environmental waters, genetic techniques will provide a more accurate picture of the presence/recent presence of potentially harmful bacteria. PCR testing involved important primers which targeted genes responsible for pathogenicity, toxin encoding and plasmid antigen genes.

The detection of strong PCR bands with clinical *Salmonella* and *L. monocytogenes* strains showed that the corresponding primers were suitable for South African strains. The PCR assay using TaKaRa primers all followed the same cycling parameters, allowing for large numbers of samples to be amplified with the different range of primers. The time to perform PCR was approximately 1.5 days compared to the 3-5 days needed for conventional methods, which required several stages before pure cultures were ready to be confirmed by biochemical tests.

PCR detection revealed four cytotoxin containing *E. coli,* seven *ipaH* containing *Shigella* sp., ten enterotoxin containing *Salmonella* species, and thirteen

listeriolysin containing *L. monocytogenes*. A percentage of the positives were, however, weak and so the reproducibility of these products needs to be considered or a two cycle PCR method should be recommended. Culture methods and API 20E (bioMérieux) testing confirmed a single *Salmonella* sp. Differentiating between the *Proteus* colonies and *Salmonella* colonies on selective XLD agar was difficult. An additional *Salmonella* sp. not detected by PCR was isolated.

Confirming the *E. coli* PCR-positive samples by culture took the form of screening for the presence of a particular *E. coli* serovar, whereas the PCR and corresponding primers were developed to detect any verocytotoxin producing EHEC. Not all *E. coli* 0157:H7 isolates produce the cytotoxin, which is the mode of pathogenesis of the organism (Lab M Culture media manual). Furthermore other serovars of *E. coli* can produce verocytotoxins and can be pathogenic for humans (Lab M Culture media manual). Therefore, although the latex agglutination test confirmed there to be no culturable *E. coli* 0157:H7, the PCR could have detected other harmful serovars and so is still relevant to be noted. The specificity of the immunomagnetic separation kit was poor, as the magnetic particles bound to *E. coli* other than serovar 0157 or possible contaminants.

The detection of the *L. monocytogenes* specific listeriolysin gene from water sample 39 followed by the inability to culture this same bacterium emphasises the ability of PCR to detect microorganisms that are non-culturable.

Identifying and differentiating between the pathogenic and non-pathogenic *Neisseria* sp. is key to diagnosis and treatment. Monitoring intra-species and inter-species diversity can be achieved by RAPDs. The convenience and reproducibility of the RAPD analysis beads was clearly evident. The non-clonality of *N. gonorrhoeae* was reflected in the complex genetic profiling obtained. On applying the RAPD techniques to the identification of the *Neisseria* species investigated, the non-pathogenic *Neisseria* were in fact more genetically variable than *N. gonorrhoeae*, *N. meningitidis* or *M. catarrhalis*. The specific RAPD bands present confirmed that RAPD bead analysis could assist in definitive identification of *N. gonorrhoeae*, *N. meningitidis* or *M. catarrhalis.*

Study of the *N. gonorrhoeae* plasmids and susceptibility testing was performed to monitor plasmid mediated tetracycline resistance. The conjugative (24.5 MDa) plasmid was present in 29/102 (28.4%) strains while the *tetM*-conjugative (25.2 MDa) plasmid was present in 48/102 (47%) strains. Seventy percent of strains showed increased tetracycline resistance (≥ 2 µg/ml) while 42% of strains exhibited high-level (16-128 µg/ml) resistance.

The restriction of *tetM*-conjugative and conjugative plasmids isolated in 1996 revealed different profiles from those previously described showing that these plasmid types are continuing to evolve.

Amplification of a fragment of the *tetM* gene provided a simple and quick method for predicting high-level tetracycline resistance. On restricting the 43 high-level tetracycline-resistant genes, strains with tetracycline MICs ≥ 16 µg/ml all were found to contain the American-type *tetM* gene and 25.2 MDa plasmids were demonstrated. The establishment of *tetM*-conjugative plasmids containing the American-type *tetM* gene is increasing in Bloemfontein, 2% in 1994 to 47% in 1997.

The initial investigations into *M. tuberculosis* were based on stain and culture screening to monitor community response. Thirty three out of 313 sputum samples were ZN positive. The radiometric BACTEC system was very sensitive but the high cost is a drawback. PCR assisted in confirming the ZN results of 5 sputum samples where the LJ culture slope was contaminated. In such cases where contamination is evident results would be delayed until a further sample could be received and cultured, this can be avoided with PCR. One of nine ZN/LJ negative initial sputum samples produced a strong PCR positive result and an additional 4 produced weak bands. PCR therefore detected 11% MTB over ZN and LJ questioning the lack of additional diagnostic methods in the South African DOTS programme.

Community response was poor with only 63% of persons returning for reassessment. Results of new and old cases varied considerably. One of the 10

persons returned with a ZN positive result confirmed by PCR and BACTEC. Susceptibility testing revealed an isoniazid-susceptible strain from this person's initial sputum sample while an isoniazid-resistant strain was isolated from the return sample. A single patient (old case) had a maintained ZN positive result for 6 months and a strain was isolated that was fully susceptibility to all antibiotics tested. Conventional susceptibility testing, although simplified with the BACTEC system and accompanying drug kits, is expensive and results may take up to 1 week.

The diversity at the present day of MTB strains in Free State communities was evident with the IS*6110* fingerprinting profiles. The fingerprinting of 50 INH - and/or RIF-resistant strains from 1997 revealed 32 diverse profiles. Five clonally related clusters were evident. The samples collected from different districts in the Free State showed the spreading of these clonal groups. Non-adherence and the emergence of resistant clonal groups were evident. Of 26 person's tuberculosis samples investigated in 1998 25 diverse fingerprint profiles were found. Comparing profile patterns lead to confirmation that cross-contamination between 2 samples occurred. The cross contamination was reflected not only with the shared fingerprint profile, but with the ZN/LJ results. Strain 5 (ZN+) had 3+ growth on LJ agar while strain 4 (ZN–) showed 1 colony growth on LJ agar. Fingerprinting of 11 rifampicin-resistant strains (1998) showed the emergence of diverse resistant strains. The possible spread of TB in a hospital ward was revealed through shared fingerprint profiles of 2 samples.

Detecting missense mutations in the *rpoB* gene, which is responsible for resistance to one of the fundamental antituberculosis drugs rifampicin, was achieved by a combination of DNA amplification and sequencing. Previously reported *rpoB* mutation sites were evident in this study; at positions 516, 526, 531 and 533. The two local clonal groups (identified by fingerprint profile, 1997) did not share mutated *rpoB* alleles. This could possibly be explained by clonally related susceptible strains independently developing sub-clones bearing distinct *rpoB* alleles.

From 13 MTB strains (1998) screened for the *rpoB* gene that were subsequently

sequenced, it was found that two ZN/LJ positive samples had missense mutation at positions 516 and 526. A reduced therapeutic outcome would result with these patients, especially if they were tested to be resistant to isoniazid as well. Eleven rifampin-resistant strains (1998) revealed only one strain without *rpoB* gene mutations. The same mutated codon was evident with two strains (with shared fingerprint profile) isolated from the same location. This is indicative of same strain infection between patients in a hospital. Sequencing can not only be performed to screen for mutated genes responsible for rifampicin resistance, but for the other key antituberculosis drug isoniazid.

The Bainsvlei family member's samples from 1995, 1997 and 1998 revealed the same fingerprint profile (shared by other family members in 1995) and same mutated *rpoB* codon indicating the persistence of a rifampin/isoniazid resistant strain. Subsequent information on the brother's past MTB infections and treatment showed that possible reinfection could have occurred from his immediate community.

Because of the long incubation time needed for definitive laboratory diagnosis of TB, the tracing of contact infected individuals is a major strategy for limiting the dissemination of MTB (Hermans *et al*, 1990). Unfortunately no such contact tracing is performed in known infected communities in South Africa. Informal settlements and formal housing accommodating several temporary lodgers constitutes just one of the specific problems in communities affecting TB control in South Africa.

Increased funding into TB control programmes and clinics/laboratories would constitute an important start towards reducing the mortality rate due to *M. tuberculosis* in South Africa. The management of side effects caused by the antituberculosis drugs by health care workers is also needed so that persons do not interrupt their treatment. Long term control of the TB problem does not look promising unless a fully integrated health system is established. The implementation of genetic techniques such as rapid direct resistance gene screening will aid in the monitoring of strain persistence and dissemination. Genetic techniques such as PCR are not always at a higher cost than

conventional methods and so should be considered. A successful TB control programme is reliant on accurate and rapid diagnosis, therefore any techniques able to assist with this step require serious consideration. Education of the community needs to take place emphasising the importance of compliance with antituberculosis drug regimens and returning for reassessment.

The TB control programme implemented in many countries including South Africa, DOTS, has reported a predicted cure rate of 85%. With the slow implementation of the DOTS programme in Bloemfontein, during the 1998 study period a cure rate of only 23% was achieved. To worsen the problem, an environment that is conducive to the spread and continued development of multi-drug-resistant TB is being maintained.

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APPENDIX

Wizard ™PCR Preps DNA Purification System (Boehringer Mannheim)

1. 100 µl of Direct Purification Buffer was added together with 100 µl of PCR product in microcentrifuge tubes. This was mixed briefly on a vortex.
2. One ml of resin was added and mixed on a vortex shaker over 1 min.
3. The Syringe Barrels were attached to the Luer-Lok® extension of each Minicolumn. The Minicolumn/Syringe Barrel assembly was inserted into the vacuum manifold.
4. The resin/DNA mix was added to the Syringe Barrel. A vacuum was applied to draw the resin/DNA mix into the Minicolumn before the vacuum was broken.
5. Two millilitres of 80% isopropanol was added to wash the column and the vacuum was re-applied.
6. The resin was dried by continuing to draw the vacuum for 30 seconds after the solution had been pulled through the Minicolumn.
7. The Minicolumn was transferred into a microcentrifuge tube and centrifuged (10000 x**g**, 2 min).
8. The DNA was eluted with 50 µl of water. The Minicolumn was centrifuged finally for 20 sec at 10000 x**g**.
9. The purified DNA was stored at 4°C.

Primary Wash Buffer (Southern blot)

The primary wash buffer consisted of the following: (1 litre)

Urea (120 g)	2 M
SDS (1 g)	0.1%
0.5 M Na phosphate pH7.0 (100 ml)	50 mM
NaCl (8.7 g)	150 mM
1.0 M $MgCl_2$ (10 ml)	10 mM
Blocking reagent (2 g)	

Secondary Wash Buffer (Southern blot)

The secondary wash buffer (20 x stock, 1 litre) consisted of the following:

Tris base (121 g)	1 M
NaCl (112 g)	2 M

The pH was adjusted to 10.0 with HCl.

Secondary Wash buffer (Southern blot)

The secondary wash buffer (20 x stock) was diluted 1:20 and 2 ml/l of 1M MgCl (final concentration of 2 mM magnesium) was added to produce a working dilution.

AlkPhos Direct, Gene Images Labelling & Detection (Amersham Life Science)

Preparation of labelled probe was as follows:

1. Cross-linker solution (20 μl) was diluted with water (80 μl) to give the working concentration.
2. The DNA to be labelled was diluted with water to a concentration of 10 ng/μl.
3. The diluted DNA (10 μl) was denatured in a thermocycler (98°C/5 min).
4. The DNA was then cooled on ice (5 min) and spun briefly in a microcentrifuge to collect the contents at the bottom of the tube.
5. Reaction buffer (10 μl) was added and mixed gently.
6. Labelling reagent was added followed by the cross-linker working solution (10 μl).
7. The probe mixture was then incubated for 30 min at 37°C.
8. After incubation the labelled probe was used immediately or stored on ice for up to 2 h.

Chemiluminescent signal generation and detection with CDP-*Star* (Amersham Life Science).

The following steps were performed after the blot underwent hybridisation and stringency washes:

1. The excess secondary wash buffer was drained from the blot that was then placed on a flat, non-absorbent tray.
2. Detection reagent was pipetted onto the blot (12ml) and left for 3 min.
3. A sheet of autoradiography film was placed on top of the blot in a darkened room.
4. The cassette was closed and exposed for 1 h at room temperature.

Nucleon GX kit (Amersham Life Science, Buckinghamshire, England)

1. The cut gel slice mass was converted to DNA volume (1 mg equivalent to 1 µl)
2. Sodium perchlorate (4.5 volumes) was added to the sample volume and incubated at 55°C until gel slice had melted.
3. Resuspended resin (15 µl) was added to the melted gel slice.
4. The DNA/resin solution was incubated for 1 min at room temperature, followed by centrifugation for 30 sec at 14000 x**g.**
5. The supernatant was discarded and 1 ml of 1 x wash was added. The pellet was resuspended and spun for 30 sec at 14000 x**g**.
6. The wash solution was discarded and any remaining wash solution was spun down. The samples were left at room temperature for 5 min.
7. Sterile distilled water (10 µl) was added and incubated (25°C, 1 min).
8. The eluted DNA was collected by centrifugation (30 sec, 14000 x**g**).
9. A further 10 µl of sterile distilled water was added and spun again.

ThermoSequenase Kit (Amersham Life Science)

1. The DNA template was diluted to the recommended quantity (0.1-1 µg of single stranded DNA) with sterile distilled water to a total volume of 11 µl.
2. For each reaction, 8 µl of pre-mix was added to the DNA plus 1 µl of primer (5 pmols) to a final volume of 20µl.

3. The cycling program in the thermocycler (Perkin Elmer) was as follows:
1 min initial denaturation at 96°C, 30 cycles of 96°C for 30 sec, 45°C for 15 sec and 60°C for 4 min.
4. Ammonium acetate (7.5 µM) was added to each reaction tube.
5. Cold (-20°C) ethanol (100%) was added, mixed and tubes placed on ice for 20 min to precipitate the DNA.
6. The tubes were then centrifuged (16000 x**g**, 16 min).
7. The supernatant was removed.
8. Cold 70% ethanol was added to wash the pellet.
9. The reaction mixes were briefly centrifuged (16000 x**g**, 2 min).
10. The supernatant was again drawn off.
11. Placing in the tubes in the thermocycler for 3 min at 60°C dried the pellet.
12. The dry pellet was resuspended by adding 3 µl loading dye to each reaction tube, mixed well and then centrifuged.
13. The samples were then sequenced and analysed with an ABI Prism™ 377 (Perkin Elmer).

DYEnamic™ ET terminator cycle sequencing premix kit (Amersham Pharmacia Biotech)

1. The DNA template was diluted to the recommended quantity (0.1-1 µg of single stranded DNA) with sterile distilled water to a total volume of 11 µl.
2. For each reaction, 8 µl of pre-mix was added to the DNA plus 1 µl of primer (5 pmols) to a final volume of 20µl.
3. The cycling program in the thermocycler (Perkin Elmer) was as follows:
30 cycles of 95°C for 20 sec, 50°C for 15 sec and 60°C for 1 min.
4. When cycling was complete 2 µl of sodium acetate/EDTA buffer was added to each sample.
5. Ethanol (95%) was added (80 µl/tube), mixed and tubes placed on ice for 20 min to precipitate the DNA.
6. The tubes were then centrifuged (16000 x**g**, 16 min).
7. The supernatant was removed.
8. 70% Ethanol was added to wash the pellet.

9. The reaction mixes were briefly centrifuged (16000 x**g**, 2 min).

10. The supernatant was again drawn off.

11. Placing in the tubes in the thermocycler for 3 min at 60°C dried the pellet.

12. The dry pellet was resuspended by adding 3 µl loading dye to each reaction tube, mixed well and then centrifuged.

13. The samples were then sequenced and analysed with an ABI Prism™ 377 (Perkin Elmer).