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Molecular characterisation of clinical and environmental isolates of *Mycobacterium kansasii* isolates from South African gold mines

Geoffrey Kwenda, Gavin J. Churchyard, Catherine Thorrold, Ian Heron, Karen Stevenson, Adriano G. Duse and Elsé Marais

ABSTRACT

Mycobacterium kansasii (*M. kansasii*) is a major cause of non-tuberculous mycobacterial pulmonary disease in the South African gold-mining workforce, but the source of infection and molecular epidemiology are unknown. This study investigated the presence of *M. kansasii* in gold and coal mine and associated hostel water supplies and compared the genetic diversity of clinical and environmental isolates of *M. kansasii*. Five *M. kansasii* and ten other potentially pathogenic mycobacteria were cultured mainly from showerhead biofilms. Polymerase chain reaction-restriction analysis of the *hsp65* gene on 196 clinical and environmental *M. kansasii* isolates revealed 160 subtype I, eight subtype II and six subtype IV strains. Twenty-two isolates did not show the typical *M. kansasii* restriction patterns, suggesting that these isolates may represent new subtypes of *M. kansasii*. In contrast to the clonal population structure found amongst the subtype I isolates from studies in other countries, DNA fingerprinting of 114 clinical and three environmental subtype I isolates demonstrated genetic diversity amongst the isolates. This study demonstrated that showerheads are possible sources of *M. kansasii* and other pathogenic non-tuberculous mycobacterial infection in a gold-mining region, that subtype I is the major clinical isolate of *M. kansasii* strain and that this subtype exhibits genetic diversity.

Key words | genetic diversity, infection, *M. kansasii*, showerhead, subtype

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INTRODUCTION

Mycobacterium kansasii (*M. kansasii*) is an emerging, slow-growing non-tuberculous mycobacterial (NTM) pathogen that causes tuberculosis (TB)-like disease, and is often associated with immunocompromised persons or those individuals who have had lung disease previously (Corbett *et al.* 2000; Griffith 2002; Marras *et al.* 2004; Cattamanchi *et al.* 2008). It comprises about seven genetically very similar subtypes, of which subtype I represents the most commonly isolated type (Picardeau *et al.* 1997; Taillard *et al.* 2003; Zhang *et al.* 2004; Yoshida *et al.* 2011). The sources of *M. kansasii* infection are unproven but there is a general notion that *M. kansasii* infections may be acquired from the environment. There is little or no evidence for human-to-human transmission. The organism is recovered almost exclusively from municipal water distribution systems (Engel *et al.* 1980; Kubalek & Mysak 1996; Picardeau *et al.* 1997; Le Dantec *et al.* 2002).

The epidemiology of *M. kansasii* is predominantly urban and has been associated with high density and low income communities, especially in highly industrialised areas (Penny *et al.* 1982; Chobot *et al.* 1997; Iinuma *et al.* 1997; Bloch *et al.* 1998; Churchyard 2000; Santin *et al.* 2004). The organism has been isolated from water distribution systems in the same communities where patients with *M. kansasii* disease have been identified (Steadham 1980; Chobot *et al.* 1997). In the South African gold-mining

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workforce, there is an unusually high incidence of *M. kansasii* infection, and this has been estimated to be about 320 per 100,000 (Corbett *et al.* 2000). These miners have a high prevalence of risk factors for NTM disease, including HIV infection, a high burden of TB and silicosis and extensive use of aerosolised water for dust control by the gold mines (Corbett *et al.* 1999, 2000; Churchyard 2000; Churchyard & Corbett 2001). Despite this, the source of *M. kansasii* infection and the genetic diversity of the South African *M. kansasii* isolates are unknown. The purpose of this study was to determine whether mining and/or residential water were the possible sources of *M. kansasii* infection for the gold miners and to determine the genetic diversity amongst clinical and environmental isolates. We also assessed the prevalence of *M. kansasii* exposure in a coal mine complex, as reports from Europe have reported high incidences of *M. kansasii* infections from coal-mining areas (Kubin *et al.* 1980; Lamden *et al.* 1996; Chobot *et al.* 1997).

METHODS

Study location

Clinical isolates of *M. kansasii* were obtained between November 2005 and December 2007 from two gold mine hospitals (both located in the North-West Province, South Africa) that provide tertiary health care for gold miners, who are mostly black African migrants. Water and biofilm samples were collected from surrounding gold mining operations in the Vaal River area from 31 July to 1 August 2006 and from 28 February to 2 March 2007. Samples were also collected from a coal mine complex near Secunda in Mpumalanga Province from 16 April to 18 April 2008.

Collection and processing of water and biofilm samples

Water and biofilm samples were collected from a convenient sample of water taps in mine hostels, change rooms, kitchens, boreholes, showerheads, piped underground water and dams. The taps and showers were run for about 1 min to clear the service lines prior to sample collection. At each collection site, water samples were collected in 1-L sterile polyethylene bottles each containing 20 mg of sodium thiosulphate to neutralise residual chlorine (Clesceri *et al.* 1999). Biofilms were collected by scraping interior surfaces of either taps or showerheads with a sterile cotton-tipped swab and transferred to 2 ml of Ringer's solution (Diagnostic Media Products, Johannesburg). A total of 27 water and 29 biofilm samples were obtained from a gold-mining region, while 17 water and 15 biofilm samples were obtained from a coal-mining region. Immediately after collection, all samples were placed in a cooler box containing ice packs for transportation to the laboratory where they were stored at 4 °C and processed within 48 h of collection. Briefly, 1 litre of water was filtered through 0.45 μm nitrocellulose membrane filters (Millipore Corporation, Bedford, MA, USA) by vacuum filtration using a Manifold Filtration System (Sartorius AG, Göettingen, Germany). The membranes were transferred into sterile, screw-capped specimen containers with 10 ml of 0.005% cetylpyridinium chloride (CPC) (Sigma-Aldrich, Steinheim, Germany) to reduce background organism levels. The surface of the membranes were abraded vigorously with a sterile platinum inoculating loop and cup-sonicated in a Brunson 1200 Sonifier (Brunson Cleaning Equipment Company, Shelton, CT, USA) at 47 kHz for 5 min. Biofilm samples were vortexed, centrifuged and the pellets resuspended in 10 ml of 0.005% CPC. Both the water and biofilm samples were then exposed to the decontaminant at room temperature for 30 min on a shaking platform. The decontaminated solutions were centrifuged at 2600 *g* for 20 min, the deposits washed twice with 1 ml sterile normal saline and resuspended in 0.5 ml of normal saline.

Bacterial strains

All the clinical isolates of *M. kansasii* were obtained in the course of routine patient care between November 2005 and December 2007 from two hospitals in the Vaal River Region in the North-West Province as indicated above. The bacteria were single patient isolates from gold miners suspected of having chest infections and were confirmed as *M. kansasii* by the AccuProbe Assay (GenProbe Incorporated, San Diego, CA, USA). Environmental mycobacteria were isolated from water and biofilm samples as described above. Control strains of *M. kansasii* representing genotypes I, II, III, IV, V and VI (Steadham 1980; Picardeau *et al.* 1997;

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Zhang *et al.* 2004) were kindly provided by Veroniqué Vincent and Cristina Gutierrez of the National Reference Centre for Mycobacteria, Pasteur Institute, Paris, France and Petra de Haas of the National Institute of Public Health and the Environment, Bilthoven, The Netherlands. An ATCC 12478 strain was obtained from the National Health Laboratory Service (NHLS) Central TB Laboratory in Johannesburg.

Isolation and identification of environmental mycobacteria

One hundred microlitres of each of the resuspended decontaminated samples were inoculated in triplicate on the surface of Middlebrook 7H10 agar medium (BD-Difco Laboratories, Sparks, MD, USA) supplemented with 10% oleic acid-albumin dextrose-catalase (OADC) (Merck Chemicals, Darmstadt, Germany) and 0.05% Tween 80. The plates were sealed with Parafilm (Pechiney Plastic Packaging Company, Chicago, IL, USA) and incubated at 25, 30 and 37 °C. The plates were examined every 2 days for the first 10 days and once a week thereafter for 2 months after which time if there was still no sign of growth they were considered negative. The number of colonies per litre or per swab of the original sample and colony type were noted. Single colonies of putative acid-fast bacteria, stained by the Ziehl–Neelsen (ZN) method, were picked and subcultured on Löwenstein–Jensen slopes (Diagnostic Media Products, Johannesburg).

Identification to species level was done by biochemical tests (Leão *et al.* 2004), the AccuProbe assay (Gen-Probe, San Diego, CA, USA), polymerase chain reaction (PCR)-restriction analysis and DNA sequencing. To confirm the identity of *M. kansasii* strains obtained from both clinical and environmental sources, a *M. kansasii*-specific PCR identification method was employed utilising primers, Fw1 and Rv1 (Table 1). Bacterial lysates were prepared according to Leão *et al.* (2004). The amplification mixture consisted of 5 µl supernatant DNA, 200 µM of each deoxynucleoside triphosphate (dNTP), 1 µM of each primer, 1× Mastermix (50 mM KCl, 10 mM Tris-HCl [pH 8.3], 1.5 mM $MgCl_2$, 0.001 [wt/vol] gelatin and 1.25 U of *Taq* polymerase [Fermentas Life Sciences, Glen Burnie, MD, USA]) in a total volume of 25 µl. The reaction tube was heated for 5 min at 94 °C, followed by 35 cycles of amplification (1 min at 94 °C, 1 min at 57 °C and 1 min at 72 °C) and a 10 min extension step at 72 °C. The specificity of the primers was assessed by including DNA samples from other mycobacterial strains: *M. gastri*, *M. tuberculosis*, *M. bovis*, *M. scrofulaceum*, *M. gordonae*, *M. avium*, *M. intracellulare*, *M. xenopi*, *M. fortuitum*, *M. abscessus*, *M. peregrinum* and *M. chelonae*.

hsp65 PCR-restriction analysis of environmental and clinical isolates of *M. kansasii*

For subtyping of *M. kansasii* isolates, PCR-restriction analysis (PRA) was employed and carried out as described previously (Telenti *et al.* 1993). The *hsp65* gene was amplified from the cell supernatants by PCR using primers Tb11 and Tb12 (Table 1), which result in a 441 bp amplicon

Table 1 | Primers and target genes used for PCR and sequencing

Primer set	Primer sequence	Target gene or sequence	Size of amplicon	Source
Fw1	5′-CGGCCATTGTTCTACAGTCT-3′		167bp	Yoshida *et al.* (2011)
Rv1	5′-TAGAGATCCTCGCTTTGGT-3			
Tb11	5′-ACCAACGATGGTGTGTCCAT-3′	*hsp*65	441bp	Telenti *et al.* (1993)
Tb12	5′-CTTGTCGAACCGCATACCCT-3′			
ITS1	5′-GATTGGGACGAAGTCGTAAC-3′	16S-23S (ITS)		Roth *et al.* (2000)
ITS2	5′-AGCCTCCCACGTCCTTCATC-3′	spacer		
RPO5	5′-TCAAGGAGAAGCGCTACGA-3	*gyrB*		Lee *et al.* (2003)
RPO3	5′-GGATGTTGATCAGGGTCTGC-3′			
MtuF	5′-GACAGYGAGTGGATGGGYCGSGTFCACCG-3′	*secA1*		Zelazny *et al.* (2005)
MtuR	5′-ACCACGCCCAGCTTGTAGATCTCGTGCAGCTC-3′			

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(Telenti *et al.* 1993). For analysis and interpretation of PRA patterns, Quantity One Software version 4.6.8 (Bio-Rad, Hercules, CA, USA) was used. The results for the restriction fragments were evaluated with the help of a published algorithm (Devallois *et al.* 1997; Chimara *et al.* 2008) and compared to PRASITE (http://www.app.chuv.ch/prasite/index.html), an internet database maintained by the Swiss National Centre for mycobacteria. Any restriction fragment below 50 bp was disregarded in order to avoid confusion with primer-dimer bands.

To confirm the identity of the mycobacteria detected, DNA sequencing of the mycobacterial *hsp*65, *secA1*, *gyrB* genes or 16S-23S rDNA Internal Transcribed Sequence (ITS) was performed. The primers used and their sources are given in Table 1. Sequencing was performed on both strands at the Inqaba Biotechnical Industries Sequencing Facility (Pretoria, South Africa) by the dye terminator method using an ABI PRISM 3730XL DNA analyser (Applied Biosystems, Foster City, CA, USA). The DNA sequence reads were edited using Ridom TraceEdit Software (Ridom Bioinformatics GmbH, Würzburg, Germany) and used to search the National Center for Biotechnology Information (NCBI) RefSeq database using BLASTN software (http://www.ncbi.nlm.nih.gov/BLAST). A distance score of 0.00% to less than 1.00% was used as the criterion for species identity.

Macrorestriction analysis of *M. kansasii* subtype I isolates

DNA for pulsed-field gel electrophoresis (PFGE) was prepared from stirred broth cultures as previously described (Hughes *et al.* 2001) with some modifications. Briefly, bacterial cells were grown in 10 ml of Middlebrook 7H9 broth supplemented with OADC (Difco, Becton-Dickinson, Sparks, MD, USA), 0.5% glycerol and 0.05% Tween 80 in 30 ml sterile plastic disposable universal containers with continuous stirring using magnetic bars on a 15 Point IKAMAG Magnetic Stirrer (IKA-Werke GmbH & Co.KG, Staufen, Germany). Cultures were incubated at 37 °C for about 7 days or until turbid (OD_{550nm} 2.0). Two millilitres of bacterial cells were harvested at room temperature by centrifugation at 2600 *g* for 20 min. The cells were resuspended in 500 μl spheroplasting buffer (containing 20 mM citrate phosphate buffer, pH 5.6 [0.2 M citric acid, 0.5 M disodium hydrogen phosphate], 50 mM EDTA and 0.1% (w/v) Tween-80), warmed to 55 °C for 1 min in a waterbath, an equal volume of 1.5% low melting agarose (Bio-Rad Laboratories, Hercules, CA, USA) in 50 mM EDTA added, the mixture poured into pre-cooled plug moulds (Bio-Rad Laboratories, Hercules, CA, USA) and allowed to set for ~20–30 min. The agarose plugs were incubated in freshly prepared Tris-EDTA (TE) buffer (pH 8.0) containing 4 mg/ml of lysozyme (Sigma-Aldrich, Steinheim, Germany) for 48 h at 37 °C with the enzyme refreshed after 24 h.

The plugs were then exposed to freshly prepared ESP solution (2 mg/ml proteinase K and 1% N-lauroyl sarcosine [Sigma-Aldrich, Steinheim, Germany] in 0.5 M EDTA, pH 8.0) for 7 days (refreshed after 4 days) at 55 °C. Five millimetre slices were cut from the plugs, washed seven times in TE buffer (pH 8.0) (refreshed hourly), pre-equilibrated in 300 μl 1× restriction buffer containing 0.1 mg/ml BSA (Fermentas Life Sciences, Glen Burnie, MD, USA) for 1 h at room temperature and then incubated in 100 μl restriction buffer containing 0.1 mg/ml BSA and 30 U *Dra*I (Fermentas Life Sciences, Glen Burnie, MD, USA). After overnight incubation at 37 °C, the restriction solution was refreshed and the samples were incubated for a further 4–6 h to ensure complete digestion. Electrophoresis was performed using a 1% (wt/vol) low-meting point agarose gel (Bio-Rad Laboratories, Hercules, CA, USA) in 0.5× TBE (10× stock: 89 mM Tris, 89 mM boric acid and 2 mM EDTA [pH 8.0]). A PFG MidRange II DNA marker (New England BioLabs, Ipswich, Glen Burnie, USA) was included on each gel. The gels were pre-equilibrated for 1 h at 14 °C in a CHEF-DRIII electrophoretic tank (Bio-Rad Laboratories, Hercules, CA, USA) in 0.5× TBE buffer. Electrophoresis was performed for 40 h at 14 °C using the following parameters: angle, 120°; gradient, 6 v/cm; pump setting, 70 pmb; ramp, linear; and switch times, 6.75–35.38 s. The parameters were selected to separate fragments in the range 30–400 kb. The gels were stained with 0.5 μg/ml ethidium bromide for 30 min, and after destaining for 30 min, the gels were imaged on a Gel Doc XR Documentation System (Bio-Rad Laboratories, Hercules, CA, USA).

Phylogenetic analysis

A TIFF image of each gel was exported to the GelCompar Version 6.0 Software (Applied Maths, Kortrijk, Belgium)

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for analysis. For inter-gel comparisons, the two outermost and centre lanes of the DNA markers on each gel were used. Due to problems associated with DNA degradation and fungal contamination only 114/157 subtype I strains were analysable. The genetic relationships amongst the *M. kansasii* isolates was determined by dividing the generated macrorestriction profiles into clusters based on the best similarity index (in this case 80%). Acceptability of the clusters was assessed by visual inspection of gels to check for similarities or identities of the patterns. Similarity of macrorestriction patterns of the isolates within each cluster was confirmed if they differed by a maximum of six bands, which is suggested to correspond to two mutations and is considered to represent a significant level of genetic relatedness (Tenover *et al.* 1995). Isolates with seven or more distinct bands were designated 'unrelated'.

Ethics approval

Ethics approval for this study was granted by the University of the Witwatersrand Human Research (Medical) Ethics Committee.

RESULTS

Isolation and identification of environmental mycobacteria

A total of 57 (27 water and 30 biofilm) samples from 35 sites in the Vaal River gold-mining region, and 32 (16 water and 16 biofilm) samples from 18 sites in the Secunda coal-mining complex were examined. Culture results showed that 37/57 (64.9%) of the samples from the gold-mining region were positive for NTM disease (Table 2). Out of these, 29/37 (78.4%) were isolated from biofilm samples, while 8/37 (21.6%) were isolated from water. For samples from the coal-mining region, 17/32 (53.1%) of the samples were positive for NTM (Table 2). Sixteen (94.1%) of these NTM were isolated from biofilm samples and only one (5.9%) from a water sample. Only one underground sample (biofilm) from the gold-mining region was positive for NTM, while no samples from the coal-mining region were positive. Dam water did not yield any mycobacterial colonies. For both regions, the majority of the NTM were isolated from biofilm samples obtained from residential water distribution systems, and were isolated at 30 °C.

Based on culture, biochemical tests, PCR and DNA sequencing results, several NTM species, including potentially pathogenic and saprophytic species, were isolated from samples obtained from the two regions (Table 2). Potentially pathogenic mycobacteria from the gold-mining region included *M. kansasii*, *M. avium*, *M. fortuitum*, *M. peregrinum*, *M. chelonae*, *M. abscessus*, *M. parascrofulaceum*, *M. setense*, and *M. montefiorense*, while those from the coal-mining region included *M. avium*, *M. intracellulare*, *M. peregrinum*, *M. chelonae* and *M. tusciae*. No *M. kansasii* were isolated from the coal-mining region. In both regions, these potentially pathogenic NTM represented the majority of the isolates, 67.6% (25/37, gold-mining region) and 70.6% (12/17, coal-mining region). These mycobacteria were mainly isolated from showerhead biofilms.

The isolation of five *M. kansasii* isolates from the environment was confirmed using biochemical tests and the *M. kansasii*-specific PCR assay. None of the other mycobacterial species tested (*M. gastri*, *M. tuberculosis*, *M. bovis*, *M. scrofulaceum*, *M. gordonae*, *M. avium*, *M. intracellulare*, *M. xenopi*, *M. fortuitum*, *M. abscessus*, *M. peregrinum* and *M. chelonae*) was positive with this PCR assay. DNA sequencing of the 16S-23S rDNA spacer region, the *gyrB* and *secA1* genes also confirmed all the five isolates to be *M. kansasii*.

hsp65 PRA of environmental and clinical isolates of *M. kansasii*

To investigate the distribution of subtypes among the environmental and clinical isolates of *M. kansasii*, five environmental isolates and 191 single patient isolates were analysed by PRA. Consistent with previous findings (Alcaide *et al.* 1997; Zhang *et al.* 2004), subtype I was found to be the predominant subtype (3 [60%] amongst the environmental isolates [i.e. LRRSH-1, KPUGW and WSQR; Figure 1] and 157 [82.2%] amongst the clinical isolates), followed by subtype II (8, 4.2%) and subtype IV (1 [0.2%] amongst the environmental isolates [i.e. LRR] and 5 [2.6%] amongst the clinical isolates) (Table 3). Subtypes III, V, VI and VII were not detected in this study. Unexpectedly, two sets of

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Table 2 | Distribution of samples positive for NTM species isolated from water and biofilm samples obtained from gold- and coal-mining regions

Mycobacterial isolates	Underground water No of positives *n* (%)	Underground pipe Biofilms No of positives *n* (%)	Hostel tap Water No of positives *n* (%)	Hostel tap water Biofilms No of positives *n* (%)	Hostel showerhead Biofilms No of positives *n* (%)	All samples No of positives *n* (%)
Gold-mining region	0 (0.0)	1 (25.0)	7 (18.9)	21 (72.4)	8 (27.6)	37 (64.9)
Potentially pathogenic NTM	0 (0.0)	1 (2.7)	5 (13.5)	12 (32.4)	7 (18.9)	25 (67.6)
M. kansasii	0 (0.0)	1 (2.7)	0 (0.0)	0 (0.0)	4 (18.8)	5 (13.7)
M. avium	0 (0.0)	0 (0.0)	0 (0.0)	4 (10.8)	0 (0.0)	4 (10.8)
M. fortuitum	0 (0.0)	0 (0.0)	4 (10.8)	3 (8.1)	0 (0.0)	7 (18.9)
M. peregrinum	0 (0.0)	0 (0.0)	1 (2.7)	1 (2.7)	0 (0.0)	2 (5.4)
M. chelonae	0 (0.0)	0 (0.0)	0 (0.0)	2 (5.4)	1 (2.7)	3 (8.1)
M. abscessus	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (2.7)	1 (2.7)
M. parascrofulaceum	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (2.7)	1 (2.7)
M. montefiorense	0 (0.0)	0 (0.0)	1 (2.7)	0 (0.0)	0 (0.0)	1 (2.7)
M. setense	0 (0.0)	0 (0.0)	1 (2.7)	0 (0.0)	0 (0.0)	1 (2.7)
Saprophytic NTM	0 (0.0)	0 (0.0)	1 (2.7)	9 (24.3)	2 (5.4)	12 (32.4)
M. goodii	0 (0.0)	0 (0.0)	1 (2.7)	0 (0.0)	0 (0.0)	1 (2.7)
M. gordonae	0 (0.0)	0 (0.0)	1 (2.7)	8 (21.6)	2 (5.4)	11 (29.7)
Coal-mining complex	0 (0.0)	0 (0.0)	1 (5.9)	9 (52.9)	7 (41.2)	17 (53.1)
Potentially pathogenic NTM	0 (0.0)	0 (0.0)	1 (5.9)	4 (23.5)	7 (41.2)	12 (70.6)
M. avium	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	2 (11.8)	2 (11.8)
M. intracellulare	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (5.9)	1 (5.9)
M. peregrinum	0 (0.0)	0 (0.0)	0 (0.0)	2 (11.8)	1 (5.9)	(17.6)
M. chelonae	0 (0.0)	0 (0.0)	0 (0.0)	1 (5.9)	3 (17.6)	4 (23.5)
M. tusciae	0 (0.0)	0 (0.0)	0 (0.0)	1 (5.9)	0 (0.0)	1 (5.9)
Saprophytic NTM	0 (0.0)	0 (0.0)	1 (5.9)	5 (29.4)	0 (0.0)	6 (35.3)
M. gordonae	0 (0.0)	0 (0.0)	1 (5.9)	5 (29.4)	0 (0.0)	6 (35.3)

Notes: Total number of samples from a gold-mining region = 57 (biofilms = 30 [1 underground pipe and 29 hostel taps and hostel showerheads]; water in distribution systems = 23; underground water = 4). Culture positive, 37/57 (64.9%). Of these positive samples, 29/37 (78.4%) were recovered from biofilms and 8/37 (21.6%) were recovered from water.
Total number of samples from a coal-mining region = 32 (biofilms = 16; surface water = 14; underground water = 2). Culture positive, 17/32 (53.1%). Of these positive samples, 16/17 (94.1%) were recovered from biofilms and 1/17 (5.9%) were recovered from water. NTM, non-tuberculous mycobacteria.

isolates did not produce any of the typical *M. kansasii* PRA patterns. One set comprised 1 (0.2%) environmental isolate (NCHSH) and 16 (8.4%) clinical isolates (unique isolate 1 in Table 3) and the other five (2.6%) isolates (unique isolate 2 in Table 3). The *Bst*EII digestion pattern for a group of 16 isolates resulted in a pattern identical to that of subtype IV (240, 120 and 85 bp), while the *Hae*III digestion gave a unique pattern (165, 115 and 60 bp). The *Bst*EII digestion pattern for the set of five isolates was identical to that of subtype I isolates (240 and 210 bp), while that of *Hae*III was also unique (130 and 105 bp) (Table 3). The two sets of isolates were further confirmed as belonging to the *M. kansasii* species by DNA sequencing of the *gyrB* and the *secA1* genes, and 16S-23S rDNA spacer region. It was of interest to ascertain whether these new isolates could be detected by the current detection system in our TB laboratory. Interestingly, the AccuProbe Assay (GenProbe Incorporated, San Diego, CA, USA) used in the laboratory was able to identify all of them as being *M. kansasii* species.

Macrorestriction analysis of *M. kansasii* isolates

Previous studies have indicated that *M. kansasii* subtype I strains are predominantly clonal in nature (Alcaide *et al.*

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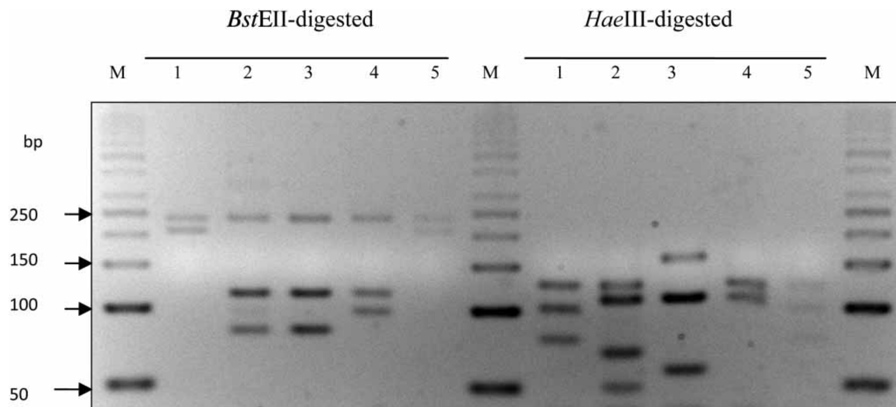

Figure 1 | PRA patterns of representative environmental isolates of *M. kansasii*. Lane M, 50-bp DNA marker; lane 1, subtype I (LRRSH-1); lane 2, subtype IV (LRR); lane 3, unique isolate 1 (NCHSH); lane 4, subtype II (clinical isolate); and lane 5, subtype I (WSQR). bp, base pair.

Table 3 | PRA patterns of the *M. kansasii* DNA after *Bst*EII and *Hae*III digestion

M. kansasii subtype	*Bst*EII fragment size (bp)	*Hae*III fragment size (bp)	Number of isolates in this study (*n*, %)
1	240/210	130/105/ 80	157 (82.2)
2	240/130/85	130/105	8 (4.2)
3	240/130/85	130/95/70	–
4	240/120/85	130/115/70/50	5 (2.6)
5	325/120	130/95/75/60	–
6	240/130/85	130/100/75	–
7	240/130/85	130/95/80	–
Unique isolate 1	240/120/85	165/115/60	16 (8.4)
Unique isolate 2	240/210	130/105	5 (2.6)

1997; Picardeau *et al*. 1997; Zhang *et al*. 2004). To investigate whether this was the case with the subtype I strains isolated in our study, macrorestriction analysis was performed on the strains with *Dra*I, which generated fragments ranging in size from 50 to 400 kb (Figure 2). The PFGE analysis demonstrated diversity amongst the isolates. When electrophoretic profiles were compared, 12 clusters, A–L, could be observed (Figure 3). Cluster B contained the largest number of isolates (42) which shared 82.2–100% similarity, followed by clusters A (19) and F (15), each with strains sharing 81.0–100% and 80.4–100% similarities, respectively; clusters E and H were represented by seven isolates each, showing similarities of more than 81.3%; clusters C and K each represented by five isolates showing a similarity of at least 80.7%; clusters D and L contained two isolates, each exhibiting at least 87.0 and 88% similarity, respectively; cluster G was represented by four isolates with at least 84.6% similarity; and clusters I and J with three isolates each, exhibited at least 82.1% similarity. Two isolates, MK033 and MK178, and the *M. kansasii* strain ATCC 12478 were outside these clusters. The most interesting cluster was F, which comprised 14 clinical and three environmental isolates. Particularly striking was the observation that LRRSH-1, an isolate from a mine hostel showerhead, showed 100% identity with clinical isolate MK010. The other two isolates, KPUGW and WSQR, obtained from a mine underground water pipe biofilm and a mine hostel showerhead, respectively, were also 100% identical to each other, but were only 86.1% identical to LRRSH-1 and a number of clinical isolates. These environmental isolates were obtained from three different sites. Generally, there was no evidence of clustering or association between PFGE pattern and the location of the hospital attended by patients.

DISCUSSION

Data presented in this study demonstrate the occurrence of a variety of culturable NTM obtained from water distribution systems in gold- and coal-mining regions in South Africa. The majority of the mycobacteria were potentially pathogenic species such as *M. kansasii*, *M. avium*, *M. peregrinum*,

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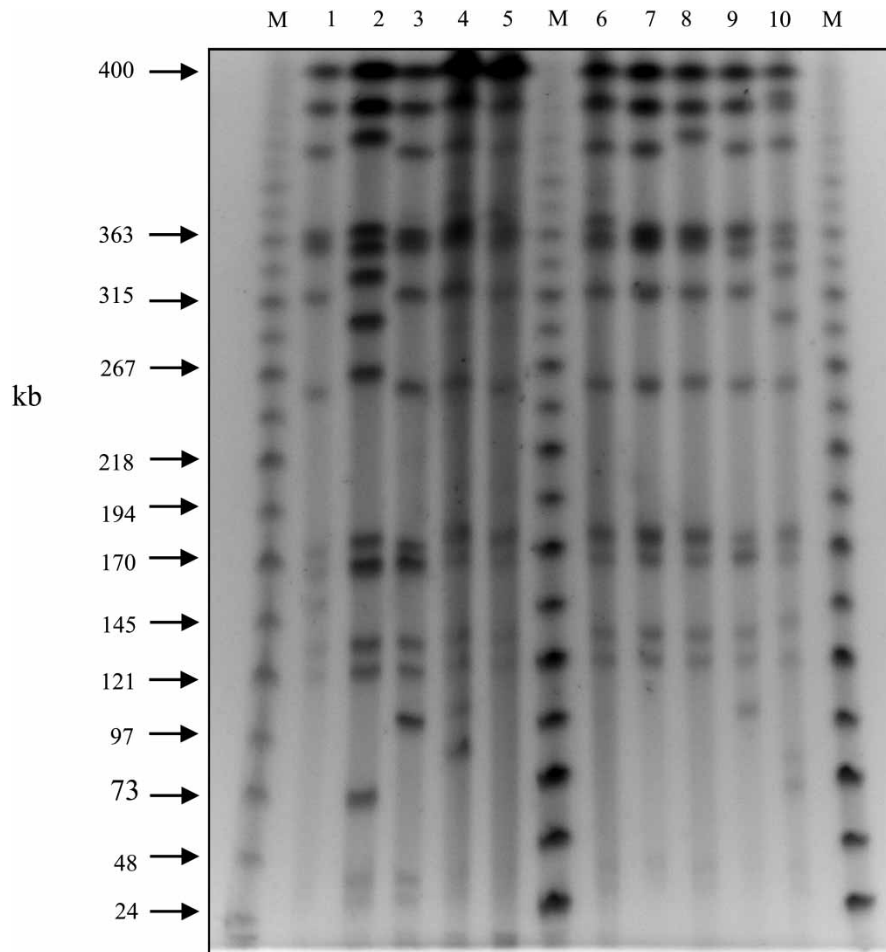

Figure 2 | Representative gel of macrorestriction analysis, using PFGE, of *M. kansasii* genomic DNA after *Dra*I digestion. Lane M, PFG MidRange II marker (New England BioLabs); lane 1, MK194; lane 2, MK029; lane 3, MK002; lane 4, MK017; lane 5, MK031; lane 6, MK026; lane 7, MK147; lane 8, MK150; lane 9, MK104; lane 10, MK117. Strains MK002, MK017, MK026, MK031, MK147 and MK194 belong to the same cluster (Figure 3, cluster B), while strains MK029, MK104 and MK117 belong to separate clusters, H, A and I, respectively. kb, kilobase.

M. chelonae, *M. fortuitum*, *M. parascrofulaceum M. intracellulare M. montefiorense*, *M. setense* and *M. tusciae*. Saprophytic mycobacteria were also isolated and these included *M. goodii* and *M. gordonae*. The potentially pathogenic mycobacteria were isolated from biofilms obtained from showerheads or water taps. Additionally, *M. kansasii* was isolated from biofilms from a mine underground water pipe. Biofilms are significant sources of mycobacteria in water distribution systems (Falkinham *et al.* 2001; Feazel *et al.* 2009). This has been attributable to the enrichment effect showerheads have on bacterial populations and to the waxy content of mycobacteria that imparts resistance to shear forces generated by shower operation (Feazel *et al.* 2009). A South African study reported the presence of NTM in 18% of the 78 biofilm samples collected from urban and semi-urban sources (September *et al.* 2004). Nearly all the NTM strains isolated were potential pathogens, which included *M. fortuitum*, *M. abscessus* and *M. septicum*, but no *M. kansasii*. Several other studies have also reported NTM to occur in biofilms of water distribution systems (Falkinham *et al.* 2001, 2008; Le Dantec *et al.* 2002). Thus, exposure to water from water distribution systems, especially showerheads, could pose a health risk, especially to immunocompromised individuals (Falkinham *et al.* 2001, 2008; Le Dantec *et al.* 2002; Torvinen *et al.* 2004; Feazel *et al.* 2009).

Environmental *M. kansasii* strains were only recovered from the gold-mining region in this study. A unique environmental isolate, not showing a typical *M. kansasii* PRA

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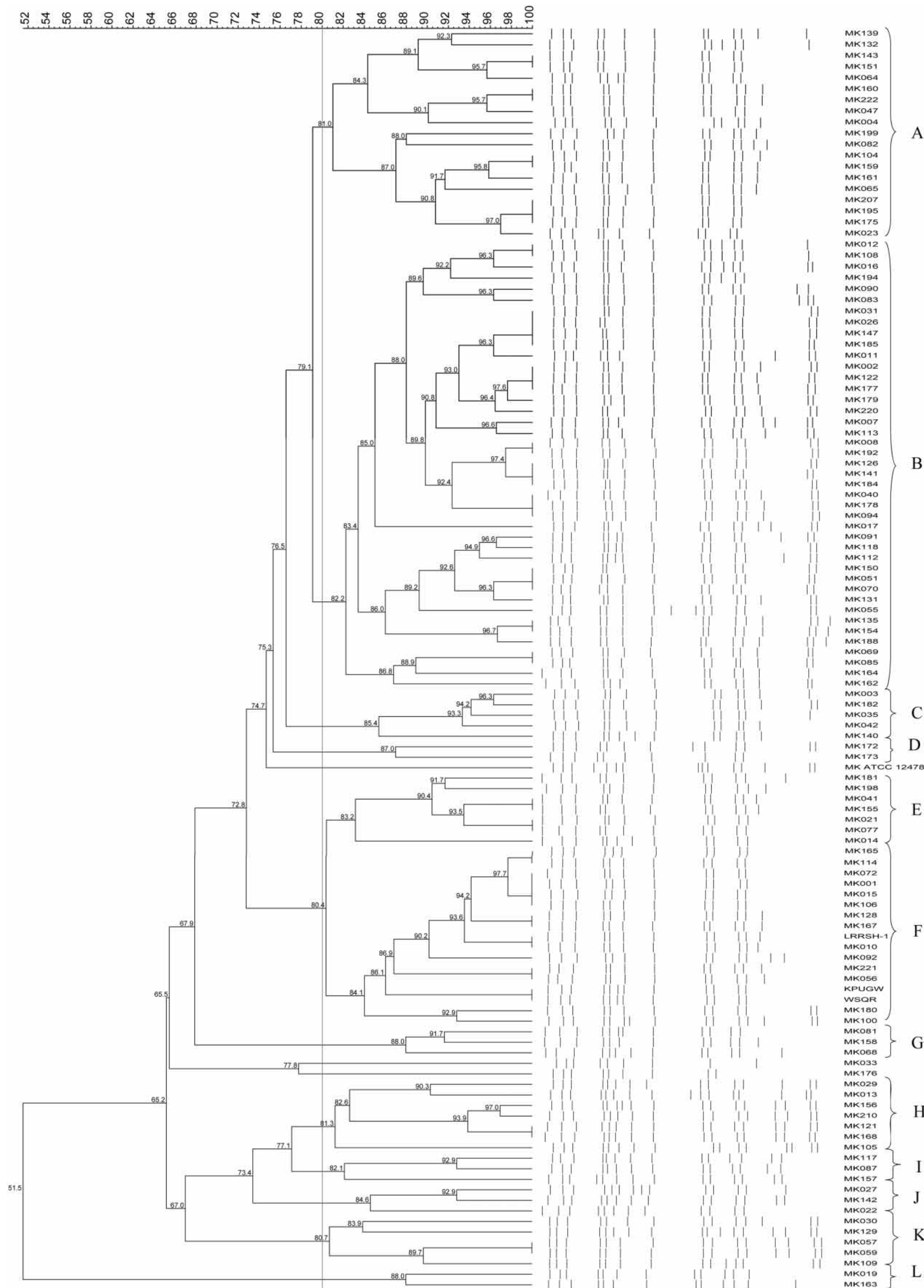

Figure 3 | Dendrogram showing the genetic relationships amongst the macrorestriction (using PFGE) profiles of *M. kansasii* isolates analysed in this study. An 80% similarity index (SI) (grey vertical line) was chosen as it provided best discrimination of the isolates.

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pattern, was detected, which may represent a new *M. kansasii* subtype. It is not clear whether the detection of *M. kansasii* in this region is unusual as systematic studies of other South African sites need to be done to assess the local prevalence. A previous attempt at isolating the organism from water distribution systems in the gold-mining complex studied failed (G.J. Churchyard, personal communication). Data from our study show that *M. kansasii* is found both in underground mine water and in the domestic water supply of the mine residences. To our knowledge, this is the first study in South Africa to report the isolation of *M. kansasii* from the environment.

Molecular characterisation of the clinical *M. kansasii* isolates from the Vaal River gold-mining region revealed a predominance of subtype I in line with other studies (Alcaide *et al.* 1997; Gaafar *et al.* 2003; Taillard *et al.* 2003; Santin *et al.* 2004; Zhang *et al.* 2004). The predominance of subtype I may represent an association with virulence, conferring upon it enhanced capacity for colonisation and pathogenic activity for humans (Alcaide *et al.* 1997; Taillard *et al.* 2003; Goy *et al.* 2007). A number of studies have shown that this subtype is the predominant subspecies regardless of patients' HIV status (Chimara *et al.* 2004; Santin *et al.* 2004). Three of the environmental *M. kansasii* isolates from the gold-mining region were also classified as subtype I by PRA. Other subtypes detected among clinical isolates included subtypes II, IV and two sets of isolates with unique PRA restriction patterns not matching any of the typical *M. kansasii* restriction patterns. The two sets of isolates may represent new subtypes of *M. kansasii* which may reflect the existence of a unique ecological niche for the bacterium that influences its evolution (Taillard *et al.* 2003). One of the environmental *M. kansasii* isolates was also subtype IV and another matched one of the new PRA restriction patterns.

Macrorestriction analysis by PFGE allowed further discrimination of subtype I isolates and demonstrated genetic diversity amongst the isolates. Several previous studies have demonstrated a tight clonal structure amongst subtype I strains (Alcaide *et al.* 1997; Picardeau *et al.* 1997; Zhang *et al.* 2004; Wu *et al.* 2009). The underlying reasons for the observed diversity of the strains in our study are unclear. It could possibly be attributed to the high prevalence of risk factors for NTM disease in the South African miners (Corbett *et al.* 1999, 2000; Churchyard 2000; Churchyard & Corbett 2001), high rates of NTM diseases in the miners that suppress their immune systems, or unique local ecological factors that could influence the adaptation of the strains (Feil *et al.* 2001; Feil & Spratt 2001; Coenye & LiPuma 2003). The diversity of the clinical isolates would support an environmental origin such as water.

It was initially thought that underground water could have been the possible source of infection since the gold mines use aerosolised water extensively for settling dust (Churchyard 2000). However, macrorestriction analysis showed a clonal relationship between an environmental isolate from a mine hostel showerhead biofilm and a clinical isolate of *M. kansasii*. This observation may suggest that potable water may be a possible source of *M. kansasii* infection for the miners in the gold-mining region. Aerosols generated from activities such as bathing and showering may put the miners at risk of acquiring infection caused by *M. kansasii*, particularly those with chronic lung disease due to prior TB or silicosis. However, these data do not establish that water is the source of the infection, but implicates it as a possible source of infection. Secondly, although the macrorestriction patterns for some clinical isolates showed clonal identity, it was impossible to assess retrospectively whether transmission had occurred amongst the patients or whether the patients had been exposed to a common source. In the absence of an experimental infection model, it is difficult to prove that the miners acquired the infection from water.

The study had a number of limitations. Some of the negative findings, especially with water samples, may be attributed to the limited number of samples and sites studied. Only one gold-mining area was studied. In addition, the decontamination procedure for the samples, prior to plating on solid media, may have reduced the number of culturable NTM since this has been shown to kill bacteria (Brooks *et al.* 1984). Many NTM are difficult to culture and grow very slowly, making them vulnerable to overgrowth by other organisms (Brooks *et al.* 1984). A problem observed in this study was with fungal overgrowth, especially with water samples. Another limitation of the study was the lack of epidemiological data, which were, unfortunately, not available due to a lack of access to patient records. Thus, it was not possible for us to correlate our data

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with the incidence and severity of *M. kansasii* disease in the gold-mining region studied.

Further studies are needed to demonstrate transmission of *M. kansasii* from water to humans and to determine the distribution of *M. kansasii* strains isolated from other parts of South Africa. A national survey of *M. kansasii* isolates will give an accurate picture of the *M. kansasii* strains circulating in the country. A strategy to decrease exposure and risk of developing NTM disease may need to be implemented.

CONCLUSION

This study has shown that most of the NTMs isolated from the two mining regions studied were primarily potentially pathogenic mycobacteria and that exposure to water in water distribution systems, especially showerheads, poses a risk for NTM disease. It also showed that subtype I is the predominant *M. kansasii* strain causing infection in the gold-mining workforce studied. In contrast to the clonal relationships amongst isolates from other countries, South African *M. kansasii* subtype I isolates exhibited a greater degree of diversity. To our knowledge this is the first report from South Africa to document the isolation of *M. kansasii* from the environment and to show a clonal relationship between an environmental and a clinical isolate of *M. kansasii*.

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