Prevalence and distribution of non-tuberculous mycobacteria (NTM) in cattle, African buffaloes (*Syncerus caffer*) and their environments in South Africa

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Abstract

It has been hypothesized that a variety of NTM species to which livestock and wildlife species are naturally exposed, induce broadly cross reactive anti-mycobacterial immune responses which interfere with current standard diagnostic assays. NTM have also been implicated in *M. bovis* specific immune responsiveness, hence potentially the development of tuberculosis. Cattle and African buffaloes are both maintenance hosts of bovine tuberculosis (BTB) in South Africa, yet the effective diagnosis and control in these species may be hampered by adverse effects of NTM. As part of an investigation of the role of NTM in the immune responsiveness of cattle and African buffalo to NTM, we conducted a countrywide survey to establish the prevalent NTM species and their distribution in the natural environments of these animals. A total of 1123 samples (water, soil, nasal and pharyngeal swabs) were

collected for Mycobacterium isolation. In addition, NTM isolated from tissue samples between 1991 and 2011 were included in the analysis. Mycobacteria were isolated from 56% of the samples from the countrywide survey. A total of 420 NTM isolates from soil, water, animal tissues, and animal derived swab samples were genotyped with the following results: 302 belonged to 40 known NTM species, 79 were found to be closely related to 23 known NTM species, and 38 isolates were found to be potential novel species that are not currently listed in the RIDOM and NCBI BLAST databases. The four NTM species or closely related groups most frequently isolated in this survey included *Mycobacterium terrae* (11.2% of isolates), a group of mycobacteria closely related to *Mycobacterium moriokaense* (referred to as *M. moriokaense*-like)(8.1 % of isolates), *Mycobacterium nonchromogenicum* (7.4% of isolates) and *Mycobacterium vaccae/ M. vanbaalenii* (5.2% of isolates). The phylogenetic analysis of the *M. moriokaense* like isolates, based on the 16s rRNA sequences, revealed at least 8 clusters, possibly associated with 8 different NTM species. Our findings provide account of NTM species diversity and associated prevalences in cattle and African buffaloes and their environments in South Africa. The identification of the most prevalent NTM species in this study will allow for a targeted investigation of their effects on host immune responses.

Keywords: NTM, *Mycobacterium nonchromogenicum, Mycobacterium moriokaense, Mycobacterium terrae, Mycobacterium vaccae, M. vanbaalenii*

1. Introduction

It is hypothesized that different NTM species induce broadly cross reactive immune responses in livestock and wildlife species to which they are exposed. These immune responses interfere with current standard diagnostic assays, namely the skin test and the interferon γ-assay (Michel, 2008;

Michel *et al.,* 2010; Schiller *et al.,* 2010), as well as with the efficacy of the *M. bovis* BCG vaccine against and the immune responsiveness to tuberculosis (Brandt *et al.,* 2002; Weir *et a*l., 2006). The use of purified protein derivative (PPD), a crude mixture of proteins that may contain epitopes common in NTM as well as *M. bovis*, in the tuberculin skin test (TST) and the gamma interferon assay, is thought to be the cause of these cross reactive immune responses (Schiller *et al.,* . 2010). The efficacy of *M. bovis* BCG vaccine varies from about 70% to 0% protection in cattle (Berggren, 1981; Vordermeier *et al.,* 2002; Ameni *et al.,* 2010). Variability of the efficacy of *M. bovis* BCG is also observed in humans, ranging from 80% to 0%, with consistently low efficacy in many tropical regions of the world (Fine, 1989; Hart and Sutherland; Ponnighaus *et al.,* 1992; Arbeláez *et al.,* 2000). However, a consistent high efficacy is seen when BCG is used to vaccinate newborns (Rodrigues *et al,* 1991; Colditz *et al.,* 1995). It is hypothesized that exposure to NTM may affect the efficacy of *M. bovis* BCG due to antigens that are common to both BCG and NTM that may provide cross-priming effects with either positive or negative consequences (Kamala *et al.,* 1996; Brandt *et al.,* 2002; Young *et al.,* 2007). Similarly, NTM may influence the development of tuberculosis in animal hosts after natural infection (Buddle *et al.,* 2002; Brandt *et al.,* 2002). NTM distribution in animal populations and the epidemiology of these bacterial species is poorly understood, compared to that of *M. bovis*, but it is assumed that NTM species isolated from animals and their environment in a particular region represent the distribution of the NTM species in that region. This may give the opportunity to study immune responsiveness due to NTM as well as the consequences of these immune responses for experimental vaccination and disease progression after infection with *M. bovis.* Worldwide, cattle are the main hosts of *M. bovis* amongst domestic animals (Cousins, 2001). In South Africa, African buffaloes (*Syncerus caffer*) are considered the most important wildlife maintenance host for *M. bovis* (de Vos *et al.,* 2001; Rodwell *et al.,* 2001), and *M. bovis* has been isolated from buffalo on numerous occasions in South African national parks and private game reserves (Bengis *et al.,* 1996; Michel *et al.,* 2006; 2009, Hlokwe *et al.,* 2011).

Sequencing and phylogenetic analysis of the mycobacterium house- keeping genes, like the 16S rRNA has been used routinely to identify NTM and investigate their diversity (Harmsen *et al.,*2003) . This study aimed to identify the NTM species diversity and associated prevalences in cattle and African buffalo, and their environments in South Africa, by sequencing of the 16S rRNA of the isolated NTM as well as phylogenetic analysis of the very closely related sequences.

2. Materials and Methods

2.1 Sample collection

A countrywide survey was conducted for sample collection for NTM isolation between April 2010 and December 2012. In addition NTM isolates collected from tissue samples between the years 1991-2011 and stored at -20°C were used. Samples from both the country wide survey and the stored isolates were collected at 119 sites distributed across geographic and climatic regions in South Africa. Sampling sites were selected by accordingly identified State Veterinary Offices and largely represented convenience sampling.

A total of 1123 samples (570 nasal and pharyngeal swabs, 143 water samples and 410 soil samples) were collected during the country wide survey (during no particular season) from Kruger National Park, private game reserves, and livestock farms (communal and commercial) across South Africa. The criteria for sample collection during this survey were: 1) Samples included water, soil, and nasal/pharyngeal swabs from cattle at each sampling site; 2) country wide distribution of sampling sites including all geographical and climatic areas; 3) In pertinent cases, isolation of two or more NTM species from the same sample would be considered as separate isolates.

Nasal and pharyngeal swab samples were collected from live buffalo and cattle, as well as soil and water from the habitats of these animals. Soil samples were collected from the cattle grazing camps, crush pens, kraals, commonages, and buffalo capture bomas and around parks where buffalo are seen. In certain cases only environmental samples were collected due to logistical constraints in obtaining samples from animals. Water samples were collected from dams where these animals drink, animal drinking pans, troughs and stagnant river sites. From each site soil samples were collected at 5-25cm depth, and 150ml of water samples were collected in sterile sampling bottles, about 25cm deep. Nasal and pharyngeal swabs were collected using sterile cotton swabs, and then immediately immersed in distilled water or phosphate buffer saline (PBS) (PH= 7) in the field. These were collected from a minimum of 5 randomly selected animals per site. The samples were kept at 4°C until they were processed for NTM isolation.

Tissues samples (with or without visible lesions) from cattle were collected during routine meat inspection by state veterinarians or meat inspectors according to standard operating procedures at abattoirs. Tissue samples from buffalo were collected as part of bovine tuberculosis research projects, using approved sampling protocols, and sent to ARC-OVI Tuberculosis laboratory for diagnosis of mycobacterial diseases by culture. These were: lymph nodes, liver and lungs and spleen.

2.2 Isolation of NTM from different sample types

For nasal or pharyngeal swabs, the samples were soaked in 7 ml of sterile distilled water overnight. The swab was then discarded and the solution centrifuged at 2360 g for 10 min at room temperature. Hydrochloric acid (HCl) at a final concentration of 2% was added to the pellet and the sample was incubated at room temperature for 10 min. The solution was then centrifuged for another 10 min at 2360 g at room temperature, where after the supernatant was discarded. The

pellet was neutralized with 7 ml distilled water and centrifuged again for 10 min at 2360 g at room temperature.

For soil and water samples, a modified protocol by Livanainen, (1995), was used for processing of soil and water samples for mycobacterial isolation. For soil, 20 ml of distilled water was added to 5 g of soil in a 50 ml Falcon tube, and shaken by hand for at least 1 min. The solution was allowed to stand at room temperature for 30 min to allow the sediment to settle. The sample was centrifuged at 800 x g for 5 min. The turbid supernatant was transferred to a clean tube and centrifuged again at 3900 g for 30 min at room temperature. The supernatant was discarded; 20 ml of 2% NaOH was added to the pellet and incubated at room temperature for 10 min. The suspension was centrifuged at 3900 g for 10 min at room temperature. The supernatant was discarded and the pellet was suspended in 20 ml of 5% oxalic acid. The suspension was allowed to stand at room temperature for 15 min and again centrifuged at 3900 g for 10 min. The supernatant was discarded, 20 ml of water was added and the suspended pellet was centrifuged at 3900 g for 10 min at room temperature. Thereafter, the supernatant was discarded, leaving approximately 1ml fluid with the pellet. For water samples, an aliquot of 100 ml from each water sample was allowed to settle at room temperature for 30 min, after which approximately 50 ml of the upper part was transferred to a clean 50 ml Falcon tube and centrifuged at 3900 g for 30 min. The supernatant was discarded and the tube refilled with approximately the remaining 50 ml from the 100 ml aliquot, and centrifuged again at 3900 g for 30 min at room temperature. The supernatant was discarded and the procedure for processing of soil samples as described above was followed.

For all the sample types the sediment was inoculated onto Löwenstein Jensen (LJ) slopes supplemented with glycerol and an antibiotic cocktail of PolymyxinB, AmphotericinB, Carbenicillin and Trimethoprim (PACT) (National Health Laboratories, South Africa, and Becton Dickinson, Germany). Two LJ slopes of each sample were incubated at 37˚C and monitored for colony growth of mycobacteria at 2 days, 7 days, and thereafter weekly for 10 weeks. When growth

of bacteria was observed, based on morphology of mycobacterial colonies (Csillag, 1961) individual colonies were selected for Ziehl Neelsen staining, and suspensions of the acid fast bacteria were prepared for further identification using polymerase chain reaction (PCR) and the remaining cultures were stored at -20°C for further investigations. Tissue samples were processed for mycobacterial isolation according to Bengis *et al*. 1996.

Isolates were classified according to the source categories, i) "environmental" samples from soil, water and, since isolation of NTM from swabs could be an indication of contamination from the environment, also swabs and ii) "animal" the tissue samples.

2.3 *In vitro* amplification and sequencing of the 16s rRNA for identification of mycobacteria

Individual colonies from cultures were picked and boiled culture suspensions were prepared as DNA templates for PCR amplification of the 16S rRNA of mycobacteria. A 25 µl PCR reaction mixture (Qiagen HotStar Taq, Germany) was prepared, containing 12.9 µl deionised water, 2.5 µl of 10x PCR buffer {Tris.Cl, KCl, $(NH_4)_2$, SO_4}, 2 µl $MgCl_2$, 1 µl dNTP's (10 mM), 0.1 µl Taq Polymerase, 5 µl of 5x Q-solution, 1 µl of each forward and reverse primers (50 pmol) and 1-2 µl of DNA template. Primers 16S-F (5'-AGA GTT TGA TCM TGG CTC AG-3') and 16S-R (5' - GCG ACA AAC CAC CTA AGA G -3') were used for the 16S ribosomal RNA amplification (Harmsen *et al.,* 2003). The PCR cycling parameters were as follows: initial denaturation at 95°C for 15 min, followed by 35 cycles of denaturation at 95°C for 30 sec, annealing at 60 °C for 30 sec and elongation at 72°C for 30 sec, and a final extension at 72°C for 10 min. The PCR products of 577bp were sent to the DNA sequencing unit at the Central Analytical Facility of Stellenbosch University, South Africa for sequencing of the forward strands using an ABI sequencer (Applied Biosystem Inc.). Sequences were then analysed using the RIDOM 16S ribosomal RNA database

(RIDOM-www.ridom-rdna.de) and NCBI BLAST (www.blast.ncbi.nlm.nih.gov/Blast.cgi) for mycobacterial speciation.

2.4 Phylogenetic analyses of the NTM isolates

Phylogenetic analyses of isolates based on the partial 16S rRNA (577bp) of a variety of *M. moriokaense*-like isolates were performed using Molecular Evolutionary Genetics Analysis (MEGA) platform (www.megasoftware.net) (version 5.05). Two *M. moriokaense* isolates were also included in the analysis, and a *Nocadia* spp sequence retrieved from Genbank (www.ncbi.nlm.nih.gov) was used as an out-group species. Sequence alignments were performed using Clustalw (Thompson *et al.,* 1994). Phylogenetic trees were constructed using the neighbor-joining method (Saitou and Nei, 1987). The p-distance parameters were used as a substitution model. A thousand bootstrap replicates were run. The neighbour-joining trees were also compared to trees constructed using the maximum likelihood method and Kimura-2 was used as a substitution model.

2.5 Results

2.5.1 Sample collection and NTM isolation

Samples were collected from 114 cattle farms, 2 wildlife conservation areas (Kruger National Park, and Hluhluwe Imfolozi Park) and 3 private game farms, during the country wide survey (fig 1). At 8 of the 114 cattle farms, only soil and water samples were collected due to difficulty in accessing animals.

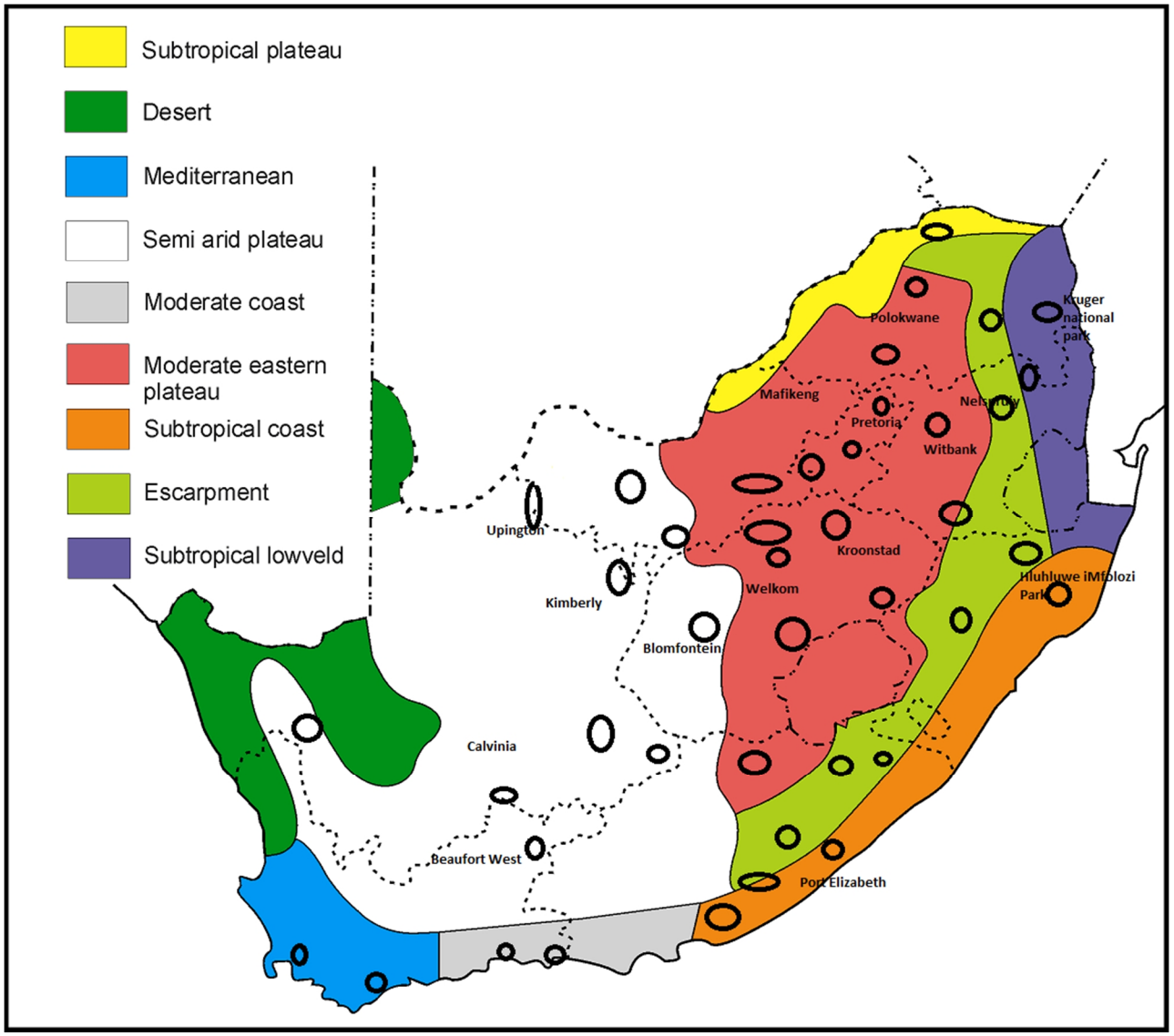

Fig 1: Geographical and climatical distribution of sampling sites for country wide survey in South Africa. Sampling sites are indicated by (○).

Six hundred and twenty nine isolates of acid fast bacteria were detected from 1123 samples (including all sampling sites) collected during the countrywide survey (56% detection rate). These were 285 isolates from 570 samples from nasal and pharyngeal swabs (50 % detection rate) , 262 from 410 soil samples (66% detection rate) and 82 from 143 water samples (57% detection rate). Hundred and five (9.3%) cultured samples were contaminated before growth of mycobacteria. Stored NTM isolates from tissue (n=91) and soil (n=2), were derived from samples in the Hluhluwe Imfolozi Park, Kruger National Park, 1 game farm and 32 cattle farms during routine diagnostic isolation of mycobacteria. The NTM isolates from the tissue samples were isolated in pure culture.

2.5.2 Characterisation of NTM isolates

From 420 out of the 629 mycobacterium isolates 16S rRNA was sequenced for species identification. Of these 420 isolates 160 were recovered from soil (n=160) from water (n=66) from swabs (n=103) from tissue (n=91) (Table 1).

Table 1 A: Number of NTM isolates across the nine provinces of South Africa

Table 1: NTM species identified per sample type from environmental and animal sources					
NTM species	**Environmental source**			**Animal source**	**Total**
	soil :n (%)*	water: n (%)*	swabs : n (%)*	tissue :n (%)*	total: n (%)**
M. nonchromogenicum	16(10)	11(16.7)	2(1.9)	2(2.2)	31 (7.4)
M. terrae	33(20.6)	7(10.6)	5(4.8)	2(2.2)	47 (11.2)
M. vaccae/ M. vanbaalenii	3(1.9)	0(0)	17(16.3)	2(2.2)	22 (5.2)
M. duvalii	2(1.25)	0 (0)	3(2.9)	3(3.3)	8(1.9)
M. triviale	1(0.625)	1(1.5)	0(0)	1(1.1)	3 (0.7)
M. interjectum	1(0.625)	0(0)	0(0)	1(1.1)	2(0.48)
M. goodii	1(0.625)	0(0)	0(0)	2(2.2)	3 (0.7)
M. palustre	1(0.625)	0 (0)	0(0)	2(2.2)	3 (0.7)

M. acapulcensis	2(1.25)	0(0)	9 (8.65)	8(8.83)	19 (4.5)
M. intermedium	4(2.5)	2(3)	0(0)	1(1.1)	7 (1.7)
M. flavesence/ M. novocastrense	0(0)	0(0)	1(0.96)	3 (3.3)	4(0.95)
M. simiae	1(0.625)	4(6)	0(0)	5 (5.55)	10 (2.4)
M. pulveris/ M. elephantis	0(0)	0(0)	1(0.96)	3(3.3)	4 (0.95)
M. paraffinicum	14(8.75)	3(4.5)	0 (0)	0(0)	17 (4.05)
M. wolinsky	0(0)	0 (0)	0(0)	1 (1.1)	1 (0.2)
M. gordonae	2(1.25)	0 (0)	0 (0)	1(1.1)	3 (0.7)
M. moriokaense	4(2.5)	3(4.5)	9 (8.65)	2(2.2)	18 (4.3)
M. fortuitum complex	8(5)	0 (0)	0 (0.)	8(8.7)	16 (3.8)
M. avium complex	6 (3.8)	3(4.5)	0 (0)	16(17.77)	25 (6.2)
M. septicum/ M. peregrinum	7 (4.4)	0 (0)	1 (0.96)	1(1.1)	9 (2.1)
M. thermoresistible	1(0.6)	0 (0)	0(0)	0(0)	1 (0.24)
M. asiaticum	1(0.625)	0 (0)	2 (1.9)	3(3.3)	6 (1.43)
M. chitae	0(0)	0 (0)	1 (0.96)	3(3.3)	4 (0.95)
M. szulgai	0(0)	2 (3)	0 (0)	1(1.1)	3 (0.7)
M. confluentis	2(1.25)	0 (0)	0 (0)	1(1.1)	3 (0.7)
M. parafortuitum	1(0.625)	0 (0)	4 (3.8)	0(0)	5 (1.2)
M. kumamotonense	3(1.9)	0 (0)	0 (0)	0(0)	3 (0.7)
M. arupense	1(0.625)	0 (0)	0(0)	0(0)	1 (0.2)
M. holsaticum	0(0)	0 (0)	2 (1.9)	0(0)	2 (0.48)
M. astroafricunum	1(0.625)	1 (1.5)	0 (0)	0(0)	2 (0.48)
M. neoaurum	3(1.9)	0 (0)	3(2.9)	0(0)	6 (1.43)
M. senuense	1(0.625)	0 (0)	0 (0)	0(0)	1 (0.24)
M. flouroanthenivorans	1 (0.625)	0(0)	0 (0)	0(0)	1 (0.24)
M. monasence	0(0)	0 (0)	1(0.96)	0(0)	1 (0.24)
M. engbackii	3(1.9)	2 (3)	0(0)	0(0)	5 (1.2)
M. triviplex/ M. monteferionse	2(1.25)	1(1.5)	0 (0)	0(0)	3 (0.7)
M. madagascariense	0(0)	1(1.5)	0 (0)	0(0)	1 (0.24)
M. nebraskense/ M. gastri/ M. bohemicum/ M. kansasii/ M. malmoense	0(0)	1 (1.5)	0 (0)	0(0)	1 (0.24)
M. lacticola	0(0)	0 (0)	0 (0)	1(1.1)	1 (0.24)
M. chelonae/ abcessus	0(0)	0 (0)	0 (0)	1(1.1)	1 (0.24)
M. moriokaense-like	6(3.8)	6 (9)	13 (12.5)	9(10)	34 (8.1)
Unidentified NTM	28(17.5)	18 (27.3)	29 (27.9)	8(8.83)	83 (19.8)
Total	160 (38.1)	66(15.7)	103(24.5)	91(21.66)	420 (100)

n= number of isolates belonging to the respective NTM species, *= percentage of isolates per sample type, ** percentage per total number of isolates analysed (420).. *M. avium* complex include *M. avium* subp *avium/ paratuberculosis, M. vulneris and M. intracellulare and M. colombiense. M. fortuitum complex* include: *M. fortuitum fortuitum* and *M. fortuitum acetamidolyticum.*

2.5.3 NTM species diversity and associated prevalence

Identification of NTM to species level was performed by sequencing of a partial region of their 5' 16S rDNA sequence. Forty species were identified based on their availability on the NCBI and RIDOM databases. In addition, 79 NTM isolates did not belong to any species represented in these databases but were closely related to 23 known species. Finally 38 isolates were identified as potentially novel species as they were not related to any species represented in the databases. Of the 40 characterised NTM species, 37 were from environmental sources (water, soil and swabs), and 25 from animal tissue samples. Most of the NTM species recovered from the environmental sources were also found in animal tissue, except for *M. parafortuitum, M. kumanotonense, M. arupense, M. holsaticum, M. paraffinicum, M. austroafricanum, M. neoaurum, M. senuense, M. floroanthenivorans, M. monasence, M. engbackii, M. triplex/ M. montefirionse, M. thermorestistible, M. madagascariense* and *M.nebraskense/M. gastri/ M.bohemicum, M. malmoense* which were not isolated from animal tissues. These species were, however, detected at very low frequencies of <1% of total isolates from the environmental source. Isolates identified as *M. wolinsky, M. lacticola and M. chelonae or M. abcessus* were isolated from animal sources but not from the environment. These isolates were also detected at low frequency of <1.1% of total isolates from the animal sources. Twenty eight of the known NTM species were recovered from soil, 14 from water, and 16 from animal swabs. Ten NTM species were isolated from both soil and water samples and these are *M. nonchromogenicum, M. terrae, M. triviale, M. intermedium, M. paraffinicum, M. moriokaense, M. austroafricanum, M. engbackii, M. triplex* or *M. monteferionse* and *Mycobacterium avium* complex (MAC)), including *M. avium* subsp *avium, M. colombiense, M. vulneris* and *M. intracellulare*. Except for *M. fortuitum* complex (*M. fortuitum* subsp *fortuitum* and *M. fortuitum* subsp *acetamidolyticum*) and *M. septicum or M. peregrinum* whose detection rates were 5% and 4.4%, respectively (percentage per sample type), the NTM isolates detected from

either soil or water occurred at very low frequencies of <1.9% (percentage per sample type). Ten NTM species and *M. moriokaense* –like isolates were detected from both swab samples and either soil and water. These are *M. nonchromogenicum, M. terrae, M. vacce* or *M. vanbaalennii, M. duvalii, M. acapulcensis, M. morioakense, M. septicum, M. asiaticum, M. parafortuitum* and *M. neoaurum.* Of the isolates not belonging to species represented on the databases, 34 were derived from soil, 24 from water, 42 from swabs and 17 from tissue. Some isolates belonging to the closest related species could not be differentiated from each other by partial sequencing of the 16S rRNA gene, including *M. triplex* and *M. montefiorense; M. septicum* and *M. peregrinum; M. vaccae* and *M. vanbalenii; M. flavesence and M. novocastrense; M. pulveris* and *M. elephantis, M. chelonae* and *M. abcessus* as well as *M. nebraskense M. kansassi, M. gastri, M. bohemicum* and *M. malmoense.* The species identified by the RIDOM and NCBI databases and their prevalence are shown in figure 2; their distribution across sample types (water, soil, nasal and pharyngeal swabs and animal tissue) from different sample sources (environmental and animal) are shown in Table 1. In cases where samples were collected from both the animals and their environments the same NTM species were occasionally isolated from both sources. For example, 3 *M. moriokaense* and 3 *M. moriokaense* –like isolates were isolated from 16 buffalo in Hluhluwe iMfolozi Park (HiP), and 1 isolate of each NTM species was isolated from 8 soil samples collected from the same Park. Isolates from the four most commonly isolated species are in decreasing order: *M. terrae* (n=47)*;* a group of isolates belonging to species closely related to *M. moriokaense:* referred to as *M. moriokaense*-like isolates (n=34); *M. nonchromogenicum* (n=31); and *M. vaccae / M. vanbaalenii* (n=22). The distribution of these isolates across sample sources is summarised in Tables 2. All four of these NTM species/groupings were isolated from both environmental as well as animal sources. Members of *Mycobacterium avium* complex (MAC) represented 6.2% of the isolates and were isolated from both sample sources, while *M. fortuitum* complex isolates represented 3.8% of the isolates and were also isolated from both sample sources.

Fig 2: Pie chart diagram indicating NTM prevalence

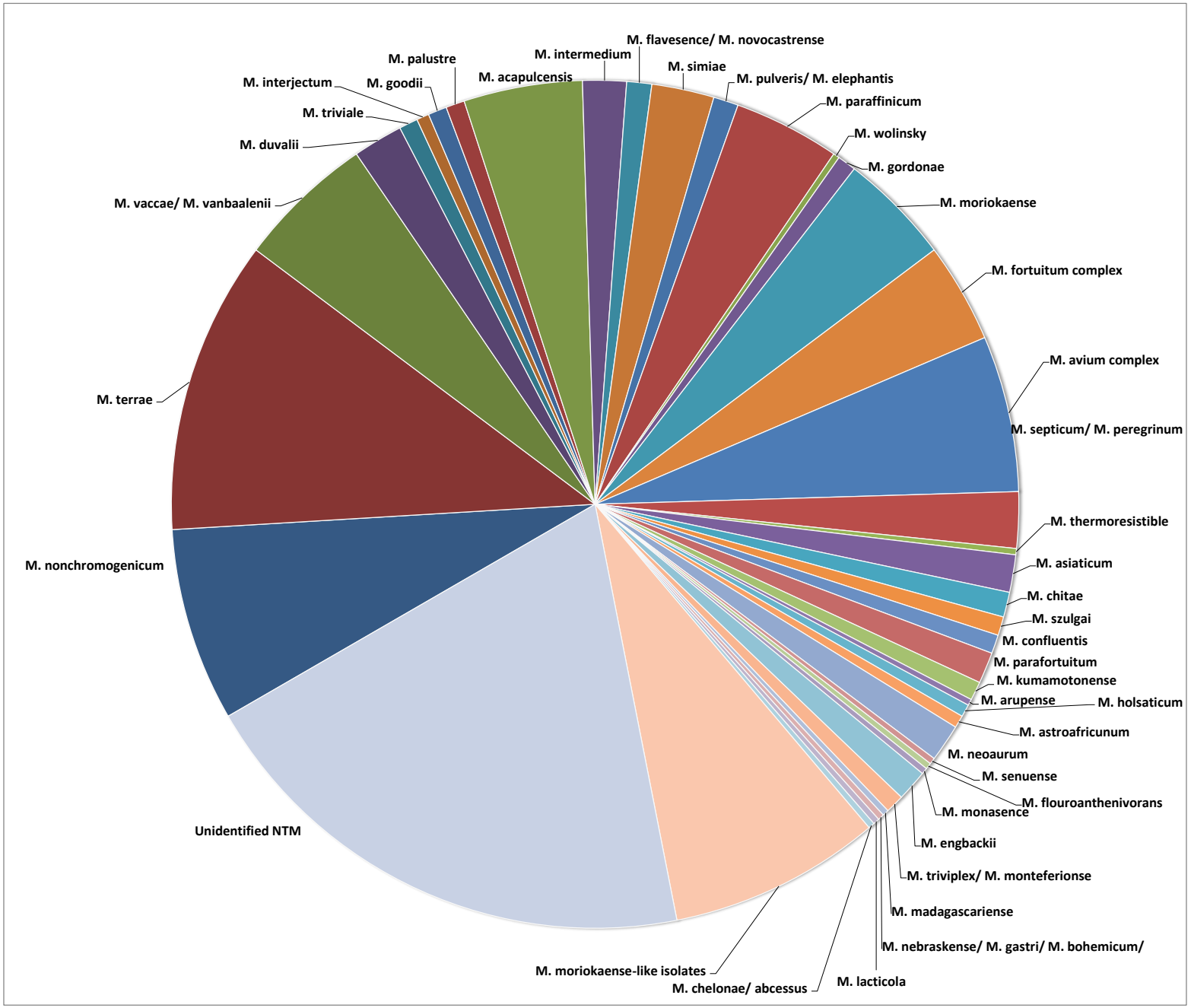

Table 2: Sample source distribution of the four most frequently isolated NTM species				
NTM species	**Sample sources**			
	Environmental source: n (%)*	Animal source: n(%)*	total: n (%)**	
M. nonchromogenicum	29(8.7)	2(2.2)	31 (7.4)	
M. terrae	45 (13.6)	2(2.2)	47 (11.2)	
M. vaccae/ M. vanbaalenii	20(6.1)	2(2.2)	22 (5.2)	
M. moriokaense-like isolates	25 (7.6)	9(10)	34 (8.1)	

n= number of isolates belonging to the respective NTM species, *= percentage of isolates per sample source, ** percentage of isolates per total number of isolates (420).

2.5.4 Phylogenetic analysis of multiple *M. moriokaense*-like isolates.

Phylogenetic classification of *M. moriokaense*- like isolates is shown in Figure 3. The neighbour joining tree shows genotypes belonging to 8 or more different clusters. One of the *M. moriokaense* isolates (*M. moriokaense* C-22) grouped with the one cluster consisting of 13 (38%) *M. moriokaense* –like isolates. These groupings were also supported by analysis using a maximum likelihood tree (results not shown).

2.6 Discussion and conclusion

NTM have been isolated ubiquitously in nature, i.e. in the environment: in soil (Covert *et al.,* 1999; Martin-Casabona *et al.,* 2004), and water (Collins *et al.,* 1984; Shitaye *et al.,* 2009), as well as in animals and humans (Kazwala *et al*., 1998; Berg *et al.,* 2009). Some NTM species have occasionally been isolated from animals in the Kruger National Park and the Hluhluwe iMfolozi Park in South Africa (van Helden *et al.,* 2005; Michel, 2007; Michel *et al.,* 2008) but no countrywide NTM survey was conducted. The species diversity and prevalence of NTM detected

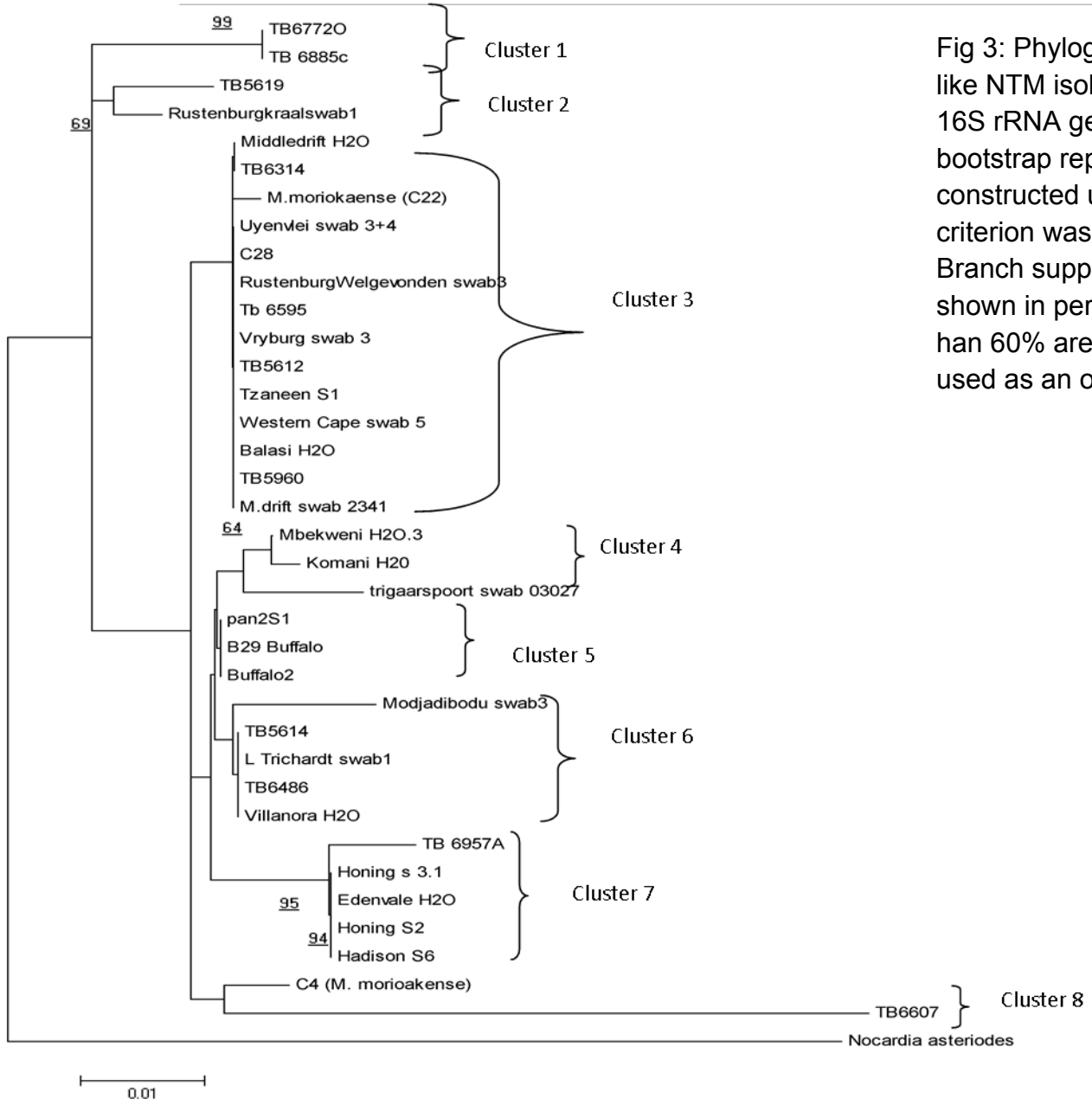

Fig 3: Phylogenetic tree of M. moriokaense like NTM isolates based on the partial 16S rRNA gene sequences. One thousand bootstrap replicates were run. The tree was constructed using MEGA V5.05 and the criterion was neighbour joining.
Branch support values on the nodes are shown in percentage and the values less t han 60% are not shown. Norcadia spp was used as an out group sequence

in this study in the environments of cattle and free-ranging buffaloes in South Africa were defined. . In this study NTM species were recovered from samples collected from geographically and climatically different sites in the natural environments of cattle and African buffaloes in South Africa, indicating their distribution in the environment. Twenty seven per cent of known NTM species isolated from environmental samples were found to be shared between soil and water on one hand and animal swabs on the other hand , suggesting that NTM are readily exchanged between these two environments (water and soil) and the animals' mucous membranes. When comparing isolates from animal tissues, and from environmental sources (water and soil and animal swabs), we found that 55% of the known NTM species occurred in both animal and environmental samples. This number is likely to be higher due to additional isolates from both sources which were classified as unknown species. This confirms the well accepted fact that the main niche of NTM is the environment, and animals are thought to be infected from environmental sources through inhalation or ingestion (Primm *et al*., 1994). Furthermore isolates detected only from either the environmental or animal samples were detected at very low frequencies (<1.1%). The isolation of NTM from animal tissues and the correlation between the isolates from tissue of animals and those from environmental samples, also suggests that most species in this study have the ability to be transmitted from the environment to animals, where they either cause colonisation without causing pathological changes or they may, in some cases, lead to disease. The four most frequently isolated NTM species in this study were *M. terrae, M. nonchromogenicum, M. moriokaense* –like isolates and *M.vaccae/ M. vanbaleenii,* whose effects on the immune responsiveness of animals and humans are unknown. *M. vaccae* has been suggested to enhance immune function, hence making it helpful in treatment of TB patients (Yang *et al.,* 2011). NTM closely related to *M. moriokaense* have been isolated previously in a buffalo and environmental samples in South Africa (Michel *et al.,* 2007). The occurrence of these *M. moriokaense*-like species, as well as the discovery of several other unknown NTM in South Africa suggest the occurrence of a number of uncharacterised

mycobacterium species in these environments and warrants further characterisation of these species. The abundance of *M. moriokaense*-like isolates prompted us to investigate their phylogenetic relatedness especially in view of their potential immune modulating role in cattle and buffaloes. The phylogenetic analysis of these isolates based on their 16S rRNA revealed at least 8 groups, suggesting that we may have isolated at least 8 different NTM species that are closely related to *M. moriokaense*. Studies on isolation of NTM from bovine tissue sources in Africa have mainly focussed on either cattle from slaughter houses or on NTM that were coincidentally isolated from animal lesions while looking for *M. bovis* (Kazwala *et al.,* 1998; Tschopp *et al.,* 2010). In one of these studies in Ethiopian cattle, more than 40% of culture positive isolates were NTM and *M. nonchromogenicum* was isolated as a predominant species (Berg *et al.,* 2009). A study from Chad (2006), found *M. nonchromogenicum* together with MAC and *M. fortuitum* to be common in humans and cattle (Diguimbaye-Djaibé *et al.,* 2006). *M. terrae* was isolated as a frequently occurring species in a study on Ethiopian wildlife (Tschopp *et al.,* 2010), and was isolated from cattle in Tanzania (Kazwala, *et al.,* 1998; Cleaveland *et al*, 2005), and in the wildlife environment in South Africa (Michel *et al.,* 2007). In Uganda Kankya *et al.,* 2011 isolated non tuberculous mycobacteria in 15.5% of environmental samples, they identified 15 NTM species, from 48 samples, and *M. nonchromogenicum, M. fortuitum* complex, *M. avium* complex and *M. gordonae* were identified as most frequently detected species. This suggests that *M. nonchromogenicum* and *M. terrae* are ubiquitous NTM species in Africa although percentages differ in different countries. Identification of 40 known NTM species, 79 unknown isolates related to other NTM species in the RIDOM and NCBI databases, as well as 38 unknown, potential novel, species and a 56% overall mycobacterium detection rate in environmental samples served to demonstrate that the diversity of NTM species in South Africa and perhaps Africa is higher than in other parts of the world. For instance in studies performed in central, south and north India, even though their NTM detection rates (43% , 54%, 40% respectively) were comparable to that observed in this study, only 7 , 20

and 10 different characterised NTM species were recovered from 60 ,717 and 291 environmental samples respectively. (Kamala *et al.,* 1994; Narang *et al.,* 2009; Parashar *et al.,* 2009). Similarly in another study conducted in Ohio (1999), 33% NTM detection rate led to identification of 18 NTM species from 139 environmental samples (Covert *et al.,* 1999). In another study in Iran (2004) 10 NTM species were identified from 72 NTM isolates recovered in 490 environmental samples (Rahbar *et al.* 2010). In a study conducted in Czech Republic between 2003 and 2004, 12 characterised NTM species were detected from 400 isolates that were recovered in 1389 environmental samples (Beran *et al.,* 2006).

M. avium PPD is used mostly for application in both the comparative TST and the gamma interferon test for TB in most countries based on the knowledge that *M. avium* is among the most frequently isolated NTM in both animals and humans and has been shown to prime immune responsiveness (Prichard, 1988; Wood and Jones, 2001; Palmer *et al.,* 2006). *M. fortuitum* PPD is used in the modified gamma interferon assay based on the isolation of *M. fortuitum* from IFNg test positive buffaloes in the Kruger National Park (Michel *et al.,* 2008; 2011). Although potentially cross reactive among NTM, members of the *Mycobacterium avium* complex (MAC) (6.2%) as well as *M. fortuitum* (3,8%) complex, were not isolated as frequent as the abundant species defined here, therefore, their exclusive use as environmental controls in TST and gamma interferon in South Africa may be reconsidered to include further investigation of the prevalent NTM. The identification of these abundant and common NTM species now allows a targeted investigation of their effects on host immune response and BCG vaccination

In conclusion, we have for the first time shown that NTM are highly abundant and more diverse in the environment of South African cattle and buffalo than in many other parts of the world, and potential novel NTM species exist in South Africa for which no information is yet available on the

international databases. We have shown that NTM are readily exchanged between water or soil and animals via their mucous membranes. Even though we have classified the swabs as environmental samples, they may have colonised stimulated the host's immune response in some cases. We have also shown that certain NTM species occur more frequently in the environment than others viz *M. terrae, M. nonchromogenicum, M. vaccae/ M. vanbaalenii* and *M. moriokaense* –like isolates. This suggests that cattle and buffalo are not only exposed to a variety of NTM species, but they are exposed more to some species than to others, with potential implications for TB diagnosis, vaccination and *M.bovis* infection due to cross reactive immune responsiveness

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