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Novel *M. avium* species from Black Wildebeest

Novel *Mycobacterium avium* species isolated from Black Wildebeest (*Connochaetes gnou*) in South Africa.

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ABSTRACT

A study was undertaken to isolate and characterize *Mycobacterium* species from black wildebeest suspected of being infected with tuberculosis in South Africa. This led to the discovery of a new *Mycobacterium avium* species, provisionally referred to as the Gnou isolate from black wildebeest (*Connochaetus gnou*). Sixteen samples from nine black wildebeest were processed for *Mycobacterium* isolation. Following decontamination; samples were incubated in an ordinary incubator at 37°C on Löwenstein-Jensen slants and in liquid medium tubes using the BACTEC™ MGIT™ 960 system respectively. Identification of the isolate was done by standard biochemical tests and using the line probe assay from the GenoType® CM/AS kit (Hain Life Science GmbH, Nehren, Germany). The DNA extract was also analyzed using gene sequencing. Partial gene sequencing and analysis of 16S rRNA gene, 16S-23S rRNA (ITS), *rpoB* and *hsp65* and phylogenetic analyses by searching GenBank using the BLAST algorithm were conducted. Phylogenetic trees were constructed using four methods, namely Bayesian inference, maximum likelihood, maximum parsimony and neighbor-joining methods. The isolate was identified as *Mycobacterium intracellulare* using the GenoType® CM/AS kit and as *Mycobacterium avium* complex (MAC) by gene sequencing. The gene sequence targeting all the genes, ITS, 16S rRNA, *rpoB* and *hsp65* and phylogenetic analyses indicated that this isolate presented a nucleotide sequence different from all currently published sequences, and its position was far enough from other MAC species to suggest that it might be a new species.

Keywords: NTM, MAC, *M. intracellulare*, phylogenetic analyses, black wildebeest.

Background

In late 2006, animals from a commercial game farm reserve in Mpumalanga Province in South Africa were harvested for game meat exportation. During meat inspection, the animal carcasses showed lesions suspicious of tuberculosis which was supported by histopathological results. The exact cause of the disease was not determined and the farm was put under quarantine for suspected bovine tuberculosis.

In February 2009, 158 animals were harvested. A high number of animals (N = 135) showed gross-visible tuberculosis-like lesions and lesions from 6 animals processed for mycobacterial cultures yielded non tuberculous mycobacteria.

Samples (n=16) from 9 animals were submitted to the National Health Laboratory Service (NHLS, Pretoria, South Africa) for mycobacterial isolation and a non-tuberculous mycobacterium (NTM) was isolated and identified as *Mycobacterium intracellulare* using a commercial kit and as *Mycobacterium avium* complex (MAC) by gene sequencing. We submitted the results to the Department of Agriculture, Forestry and Fisheries (DAFF, South Africa) as *M. intracellulare*, and proceeded further with the characterization of the isolate in Japan.

Subsequently, we received the complete history of the herd that included the histopathology report. The report described well developed encapsulated granulomatous lesions observed on the different samples of organs as very suspicious for bovine tuberculosis (BTB). Other lesions observed which were not typical of *M. bovis* (pseudotuberculosis) comprised lack of caseous necrosis and liquefaction in the granulomas. The inspissated material from within the capsules could almost be squeezed out in total, leaving behind an empty "shell". There were also several smaller granulomas with a typical onion ring appearance, but absence of calcification and liquefaction with no gritty sensation on cut section of these capsules. .

The genus *Mycobacterium* contains more than 170 species (http://www.bacterio.net/mycobacterium.html), most of which are classified as NTM or potentially pathogenic mycobacteria (PPM) (Chege et al., 2008; Kim et al., 2014; Malama et al., 2014; Tortoli, 2014) and mycobacteria belonging to the *Mycobacterium tuberculosis* complex (MTC). MTC comprises *M. tuberculosis, M. bovis, M. africanum, M. canetti, M. pinnipedii, M. caprae, M. microti, M. mungi, Dassie bacillus, Oryx bacillus* and the attenuated *M. bovis* Bacille-Calmette-Guerin (BCG) vaccine strain. With the exception of BCG, these species are pathogenic and can cause tuberculosis (TB) in mammalian hosts (Alexander et al., 2010; Pittius et al., 2012; Vos et al., 2001).

The *M. avium-intracellulare* complex is the most commonly encountered group of NTM, and the clinically most important members are *M. intracellulare* and *M. avium* (Biet et al., 2005). *M intracellulare* has not been subdivided into subspecies whereas *M. avium* consists of four susbspecies, namely *M. avium* subsp. *avium*, *M. avium* subsp. *hominissuis*, *M. avium* subsp. *silvaticum* and *M. avium* subsp. *paratuberculosis*. Mycobacterium Avium Complex (MAC) includes 10 different species, namely *M. avium*, *M. intracellulare*, *M. colombiense*, *M. bouchedurhonense*, *M. timonense*, *M. arosiense*, *M. chimaera*, *M. vulneris*, *M. yongonense* and *M. marseillense* (Tortoli, 2014).

The importance of NTM has received attention during the past decade, especially in humans. NTM are found in environmental systems (such as various soil and water systems) near human settlements and can be associated with colonization, serious infection or pseudo-outbreaks with a wide variety of presentations (Biet et al., 2005; Kankya et al., 2011; Katale et al., 2014). Indeed in humans, the isolation of NTM from clinical samples of patients presenting with pulmonary symptoms as suspected cases of tuberculosis has increased over the years and has been observed in different countries in Africa, America and Europe (Kankya et al., 2011; Katale et al., 2014; Mirsaeidi et al., 2014; Moore et al., 2010) whereas in animals the clinical significance of NTM has yet to be elucidated in the disease causing process (Chege et al., 2008; Kankya et al., 2011; Katale et al., 2014). The members of the genus *Mycobacterium* are genetically closer to each other than the microorganisms belonging to other genera, making identification a difficult and challenging task. The management, treatment and infection control measures differ significantly between *M. tuberculosis* and NTM infections.

One hundred and fifty species of NTM have been reported worldwide, of which more than 60% are pathogenic to animals or humans (Kim et al., 2014; Tortoli, 2014). In South Africa, reports on the isolation of NTM in animals, humans and environment and their effects in disease-causing processes are limited (Gcebe et al., 2013; Michel et al., 2007; Müller et al., 2011).

Black wildebeest (*Connochaetus gnou*), known in Afrikaans as “Swartwildebees” and in German as “Weisschwanzgnu”, have been hunted in South Africa for meat and hides. The overall research project was mainly on tuberculosis, samples from black wildebeest suspected of being

105 infected with tuberculosis were processed as part of phase I of the project focusing on prevalence
106 and molecular studies of Mycobacteria. The emergence of multidrug and extremely drug
107 resistant *Mycobacterium tuberculosis* strains was one of the main justifications of the project.
108 This work resulted in the reporting of a novel *Mycobacterium avium* complex species from
109 wildebeest in South Africa, which is expected to add to the corpus of knowledge and extend the
110 frontiers of research on NTM.

2. Materials and Methods

2.1 Study area

Mpumalanga province was selected as the area of study based on previous publications reporting TB in wildlife and livestock (Bengis et al., 1996; Michel et al., 2007; Vos et al., 2001) and availability of veterinary staff members experienced in conducting the comparative tuberculin skin test. Mpumalanga is one of the nine provinces in South Africa; it is located in the north-eastern part of the country, bordering Swaziland and Mozambique to the East. It embraces the southern half of the Kruger National Park, a vast nature reserve with abundant wildlife including big game. It has a subtropical foliage supporting about 1 439 000 cattle according to the Trends in the Agricultural sector 2013.

2.2 Study design and sampling

The study was designed as a cross section study sampling animals in the designated area from January 2009 to January 2011. The local municipalities were selected based on the number of commercial farms, proximity to abattoirs, and location at human-wildlife interface and the movement of animals as well. The municipalities selected comprised Malelane, Nelspruit, Lydenburg, Ermelo, Witbank and Standerton. The target population comprised cattle carcasses showing gross tuberculous-like lesions at meat inspection from positive reactors to tuberculin skin test at the municipality abattoirs during the study period. But samples from any other animal species showing gross tuberculous-like lesions were also included as convenience samples. The sampling was purposive to increase the chances of isolating mycobacteria; animals were selected based on positive reaction on tuberculin skin test and suggestive lesions at the abattoir upon meat inspection.

2.3 Sources of Samples

Samples from Black Wildebeest were received as part of phase I of a research project related to "Prevalence and molecular studies of Mycobacteria". The samples were processed at the National Health Laboratory Service (NHLS) as part of a joint collaboration between University of Pretoria and NHLS. The history of the case was provided by the state veterinarian in charge.

During a hunting period in February 2009 on a commercial game reserve located in Mpumalanga (South Africa), game animals (n=158) were randomly harvested and processed in the local abattoir according to standard operating procedures. These animals comprised black wildebeest (*Connochaetes gnou*) (n=137), blesbok (*Damaliscus dorcas phillipsi*) (n=15), blue wildebeest (*Connochaetes taurinus*) (n=2), red hartebeest (*Alcelaphus buselaphus caama*) (n=2) and springbok (*Antidorcas marsupialis*) (n=2). The animals lagging behind were the main target as well as females. During routine meat inspection of these animals, a high number of black wildebeest (n=135) showed granulomatous lesions in one or more lymph nodes or organs, reason why samples were selected from this antelope species. Sixteen samples randomly selected from nine black wildebeest showing fresh lesions suggestive of tuberculosis infection were submitted at NHLS for isolation and identification of *Mycobacterium* sp. The samples included different organs and lymph nodes transported on ice **(see Table 1).**

Table 1

2.2. Mycobacterial isolation

Samples were frozen at –20 °C until processing at NHLS. Direct impression smears were made from lesions and smears were stained using the Ziehl-Neelsen method. Tissue samples taken in a sterile manner from the inside of granulomatous lesions at the border between healthy and pathological tissues were finely cut using a sterile scalpel blade and homogenized using sterile glass beads by vortexing as described by Bengis et al. (1996) and Warren et al. (2006) with some modifications. To maximize the mycobacterial yield, specimens were subjected to a digestion and decontamination procedure using N-acetyl-L-cysteine-sodium hydroxide (NALC-NaOH) solution with NaOH at a final concentration of 2% (Chatterjee et al., 2013). The specimens were left at room temperature for 15 min during the decontamination process and thereafter neutralized with phosphate buffer, centrifuged (Beckman Coulter) at 3000 x g for 15 min at 4°C, and the supernatant decanted and pellet suspended into 1 ml of phosphate buffer. The sediment was inoculated onto two LJ slants supplemented with pyruvate and glycerol and an antibiotic mixture of polymyxin B, amphotericin B, carbenicillin and Trimethoprim (PACT) (National Health Laboratories, south Africa, and Becton Dickinson, Germany) using a 0.01ml calibrated loop. A further 0.5 ml of the sediment was inoculated with a graduated Pasteur pipette into a

prepared liquid medium tube (BBL™ MGIT™ Mycobacterium Growth indicator tubes) enriched with OADC and containing 800 µl of PANTA™ antibiotic mixture (BD™). This was incubated in the BACTEC™ MGIT™ 960 mycobacterial detection system at 37°C (Warren et al., 2006). The system was monitored for a maximum period of 42 days for bacterial growth whereas LJ slants were observed for colony growth and any other contaminant at two week intervals for 10 weeks. Tubes detected as positive within that period were further processed using Ziehl-Neelsen staining and examined microscopically for the presence of acid fast organisms and morphology thereafter subcultured on LJ slant supplemented with glycerol and pyruvate. For identification purposes, a single colony was subcultured on a fresh LJ slant to obtain pure colonies. The same was repeated with two other colonies on different LJ slants to rule out the possibility of missing a different organism. Reference cultures of *M. avium* (ATCC 25291), *M. bovis* BCG and *M. tuberculosis* (ATCC 25177) were used as positive controls.

2.3-Mycobacterial identification

2.3.1 Biochemical profile

Colonies were suspended in Middlebrook 7H9 (M7H9) broth enriched with OADC and inoculated into the different biochemical substrates according to the manufacturer's instruction (Mycobacterium identification kit, Kyokuto Pharmaceutical Industrial Co., Ltd., Japan).

2.3. 2 DNA extract and primary molecular identification

DNA extracts were prepared based on the GenoType CM/AS reverse line blot assay (Hain Life Science, Nehren, Germany). DNA was extracted from colonies on LJ slants by heating at 95°C for 20 min in a water bath. Primers provided by the manufacturer's kit (Hain Life Science, Nehren, Germany) were used. The formula for the PCR assay per tube mixture contained 1.1 µl of ultra-pure water, 5 µl of 10 x buffer, 3.6 µl of $MgCl_2$ (25 mM), 35 µl of primer/nucleotide mix from the kit and 0.3 µl of hot Taq polymerase. The thermocycler was programmed to initiate the PCR as follows: one cycle of 15 min at 95°C, followed by 10 cycles of 30 seconds at 95°C, 2 min at 58°C, 25 seconds at 95°C; then 20 cycles of 40 seconds at 53°C and 40 seconds at 70°C with a final cycle of 8 min at 70°C. The hybridization was then followed as per manufacturer's instructions using the strips provided in the kit (DNA strip *Mycobacterium* identification

species). Part of each DNA extract was also stored at -20°C prior to further investigations in Japan.

2.4. Gene sequencing

The DNA extract obtained during the primary identification was processed further by sequencing analysis, targeting *hsp65*, *rpoB*, 16S rRNA genes and 16S-23SrRNA internal transcribed spacer (ITS) for identification of the bacterial species.

2.4.1 16S ribosomal RNA gene and ITS

The 16S ribosomal RNA gene and flanking 16S–23S rRNA ITS region was amplified with a primer pair of Bact-rrs-F: 5'-AGAGTTTGATCCTGGCTCAG and myco ITS-23S-Rv: 5'-CGGTTGACAGCTCCCCGAGGC. Amplification reaction mixture consisted of 0.5 μM of each primers, 1× ExTaq buffer (Takara Bio Inc., Japan), 0.5 M betaine, 0.25 mM each of dNTPs, 1 U of ExTaq DNA polymerase (Takara Bio Inc.), 1 μL of target DNA solution and milli-Q water to adjust the final volume to 20 μL. Amplification was performed in a thermal cycler with a pre-heat step at 98°C for 1 min, 38 cycles of denaturation at 98°C for 10 seconds, annealing at 55°C for 10 seconds and extension at 72°C for 110 seconds followed by a final extension at 72°C for 5 min. The amplicon was electrophoresed in a 1% agarose gel and a band of approximately 1.9 kbp was excised and purified with Wizard SV Gel and PCR Clean-Up System (Promega Corp., USA). The sequence of the 5' region of the 16S ribosomal RNA gene and ITS were read with primers Bact-rrs-F and myco ITS-23S-Rv, respectively, by ABI PRISM 3130*xl* Genetic Analyzer (Life Technologies Corp., CA, U.S.A.) according to the manufacturer's instructions

2.4.2 *rpoB*

Partial *rpoB* gene was amplified and sequenced with primers Myco-F and Myco-R designed by (Ben Salah et al., 2008). PCR reaction mixture was comprised of 0.5 μM of each primers, 1× GoTaq buffer (Promega Corp., USA), 0.5 M betaine, 0.25 mM each of dNTPs, 1 U of GoTaq DNA polymerase (Promega Corp.), 1 μL of target DNA solution and milli-Q water to adjust the final volume to 20 μL. Amplification was performed with an initial denaturation at 96°C for 1 min, 38 cycles of denaturation at 96°C for 10 seconds, annealing at 60°C for 10 seconds and extension at 72°C for 45 seconds followed by a final extension at 72°C for 5 min. The amplicon

was electrophoresed in a 1% agarose gel and a band with a size of 761bp was excised and purified. The sequence was read by ABI PRISM 3130*xl* Genetic Analyzer (Life Technologies Corp.) according to the manufacturer's instructions.

2.4.3 *hsp65*

Partial *hsp65* gene was amplified and sequenced with primers Myco-hsp65F (565-585): 5'-AGGGTATGCGGTTCGACAAG and MAC hsp65R (Turenne et al., 2006). Amplification was performed with an initial denaturation at 96°C for 1 min, 38 cycles of denaturation at 96°C for 10 seconds, annealing at 53°C for 10 seconds and extension at 72°C for 45 seconds followed by a final extension at 72°C for 5 min in the same reaction mixture content described in rpoB section. The band with a size of 1067 bp was excised and sequencing was performed by the same procedure as in rpoB section.

The obtained sequences were compared with the GenBank nucleotide database by the blastn program (National Center for Biotechnology Information: http://www.ncbi.nlm.nih.gov).

2.5 Phylogenetic analyses

The datasets for the different genes (ITS, 16S, *hsp65* and *rpoB*) were collated by searching GenBank (http://www.ncbi.nlm.nih.gov/) using the BLAST algorithm, an acronym for the Basic Local Alignment Search Tool (Altschul et al., 1990). BLAST searches various databases, as specified by the user, to look for similar sequences, and then uses a similarity matrix to measure the similarity between sequences and the possibility that the similarity could be due to chance based on the nucleotide sequence of the query versus its target. There are several types of searches available but for these analyses "blastn" was used which searches nucleotide databases with a nucleotide query. These sequences were then downloaded into a local database using BioEdit version 7.2.1 (Hall, 1999). Sequences were aligned using MAFFT version 7 (Katoh et al., 2005, 2002); http://www.mafft.cbrc.jp/alignment/server/) with default parameters. Multiple Alignments by Fast Fourier Transformation (MAFFT) utilizes an iterative algorithm, unlike previous progressive alignment methods. Where necessary, small adjustments were made to the alignments manually to minimize hypothesized insertion/deletion events, again using BioEdit software (Hall, 1999). The programme jModelTest 2.1.6 (Darriba et al., 2012) was run for each

gene separately, as well as the concatenated dataset, to determine the best evolutionary model to use for each dataset. Phylogenetic trees were constructed for all data sets by four different methods, Bayesian inference (BI), maximum likelihood (ML), maximum parsimony (MP) and neighbor-joining (NJ). This not only allowed independent confirmation of results, but also the benefit of different methods with different strengths, weaknesses and sensitivities. NJ and MP analyses were conducted with MEGA 5 (Molecular Evolutionary Genetic Analysis) (Tamura et al., 2011). PhyML (Guindon et al., 2010) was used to carry out ML analyses on all the datasets and Mr. Bayes v3.2.5 (Ronquist et al., 2012) was used on the concatenated dataset. Both NJ and ML analyses used the relevant evolutionary model as given in jModelTest. All analyses, except those by Bayesian inference, consisted of 1000 bootstrap replications (Felsenstein, 1985), a statistical method for testing the reliability of all the groupings within the various trees.

2.6 Statistical analyses

The agreement between databases was calculated using Cohen's Kappa. To compare the results obtained from each gene and combination of two genes, only the databases identified with better performance in the first analysis were used. One thousand bootstrap replications were used for testing the reliability of all the groupings within the various trees (Hallgren, 2012; Joao et al., 2014).

2.7 GenBank accession numbers

The sequences generated in this study were deposited in the GenBank database under accession number KR856202 for *hsp65*, KR856203 for *rpoB*, KR856204 for 16SrRNA and KR856205 for 16S-23S rRNA ITS.

3 Results

3. 1 Mycobacterial isolation

A mediastinal lymph node (from 1 animal) out of 16 samples (from 9 animals) yielded similar smooth colonies of slow growing microorganisms on Löwenstein-Jensen (LJ) slants inoculated with the sediment from positive MGIT tubes. The acid-fast rods were observed under the microscope.

3.2 Biochemical characteristics

The phenotypic characteristics, including growth rate (fast/slow), production of pigment, growth at different temperatures and biochemical reactions with relevant substrates were evaluated. Colonies were identified as MAC slow growers with no pigment on specific substrates. They were positive for tellurite reduction, stimulation by pyruvate and heat-stable catalase but negative for niacin production, nitrate reduction, urease, acid phosphatase activity, Tween 80 hydrolysis (7 and 14 days) and urease (Table 2); (Cook et al., 2003).

Table 2

3. 3 Primary molecular identification

The isolate was identified as *Mycobacterium intracellulare* with a commercial kit routinely used at NHLS, the GenoType Mycobacterium CM/AS (Hain Lifesciences GmbH, Nehren, Germany).

3. 4 Phylogenetic analyses

ITS sequences

Alignment of the 16S-23S rRNA ITS of Mycobacterium “Gnou isolate” with the other sequevars of the MAC show that the Gnou isolate is most closely related to the MAC-T sequevar with only three nucleotide differences (results not shown).

Phylogenetic analyses of these data by NJ, MP and ML, placed the Gnou isolate within the MAC and more closely related to MAC-T, with MAC-M, MAC-I and MAC-L forming a sister group. All three methods placed the Gnou isolate in the same place with varying degrees of bootstrap support.

16S rRNA, hsp65 and rpoB analyses.

All three datasets by every method grouped the Gnou isolate with *M. vulneris, M. bouchedurhonense,* and *M. colombiense* with *M. arosiense* included in the group by just the MP analysis of HSP65. However all other analyses placed *M. arosiense* close to the Gnou isolate grouping. With the similarity between the various trees the four genes were combined into one dataset to give a concatenated tree (Figure 1). Generally the groupings all have good bootstrap support. All the species above *M. mantenii* in this phylogeny are members of the MAC.

Figure 1

4. Discussion and Conclusion

This is the first report in South Africa of the discovery of a novel *Mycobacterium avium* complex species from black wildebeest which has been named "Gnou isolate" to reflect the species name of the wildebeest from which it was isolated. Our isolate was identified using analysis of 16S rRNA gene, *hsp65*, *rpoB* and ITS. A number of studies suggest that 16S rRNA gene sequencing provides genus identification in most cases (>90%) but less so with regard to species (65 to 83%) (Janda and Abbott, 2007; Joao et al., 2014). Although 16S rRNA gene sequencing is highly useful in regards to bacterial classification, it has low phylogenetic power at the species level and poor discriminatory power for some genera (Janda and Abbott, 2007). It has also been reported that analysis of 16S rRNA gene alone is insufficient for the accurate identification of NTM (Joao et al., 2014); it was proposed that a stepwise algorithm combining 16S rRNA and *hsp65* gene analysis by multiple public databases could be used to identify NTM at the species level. In some cases, 16S rRNA gene sequence data cannot provide a definitive answer since it cannot distinguish between recently diverged species. While it is impossible to be completely accurate when modeling all evolution that has occurred between a set of sequences, several parameters appear to be particularly important. These are corrections for substitution patterns (nucleotide substitution matrices) and correction for different evolutionary rates at different sites, most accurately corrected using a gamma distribution model, the shape parameter α of which is calculated by jModelTest. It has been established that NTM cause disease (Kim et al., 2014), but the significance of NTM in the disease processes in animals should be investigated further as the

presence of a small number of a specific pathogen does not correlate with the virulence of the pathogen nor its economic importance. Organisms act in synergy, potentiating the colonization by other bacteria. Although mixed infection with NTM has been reported, attention should be given to NTM in the future as several studies have recognized the significance of NTM as a major public health issue around the world (Kankya et al., 2011; Kim et al., 2014; Malama et al., 2014; Moore et al., 2010; Temmerman et al., 2014). Furthermore, NTM have been found to interfere with the diagnosis of TB in cattle; indeed, some cross reactions between the antigens of NTM with those used for diagnostic purposes such as *M. avium* and *M. fortuitum* may compromise the diagnosis and control of bovine tuberculosis (De la Rua-Domenech et al., 2006; Gcebe et al., 2013).

To our knowledge, this is the first report of this new isolate of NTM, "Gnou isolate", sampled from black wildebeest (*Connochaetes gnou*) in South Africa. The diagnosis of NTM should be standardized as the isolate was identified as *Mycobacterium intracellulare* using DNA strip according to Hain's method at National Health Laboratory Service and confirmed as a new species of MAC by gene sequencing at Research Center for Zoonosis Control at Hokkaido University in Japan. Tortoli et al. (2010) reported the misidentification of NTM using commercial kit assays as we have also experienced with this study. The partial sequencing of 16s rRNA gene alone is not sufficient to fully identify NTM to species level; algorithm analysis combining all four genes should be considered as well as biochemical identification. One of the characteristics of this isolate was slow growth. The role of NTM, in particular slow growing MAC, should be further investigated in the disease causing process as they could potentiate colonization by other rapidly growing microorganisms. The isolation and identification at species level of some NTM should be performed to establish their clinical relevance in animals and humans. Countries should also be encouraged to register new species on the international data bank for purposes of adding to the epidemiological knowledge of this genus.

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