

1 Prudence Ngalula Kabongo

2 University of South Africa

3 South Africa, National Health Laboratory Service

4 Novel *M. avium* species from Black Wildebeest

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

7 P.N. Kabongo^{1, 2*}, C.L. Obi³, C. Nakajima⁴, Y. Suzuki⁴, T. Hattori⁵, J.N. Eloff², J. Wright⁶, N.
8 Mbelle^{7, 8} and L.J. McGaw²

9 ¹*Department of Agriculture and Animal Health, College of Agriculture and Environmental*
10 *Sciences, University of South Africa, Private Bag X04, Florida 1710, South Africa;*
11 kabonpnk@unisa.ac.za

12 ²*Phytomedicine Programme, Department of Paraclinical Sciences, Faculty of Veterinary*
13 *Science, University of Pretoria, Private Bag X04, Onderstepoort 0110, South Africa*

14 ³*Division of Academic Affairs, University of Fort Hare, Alice 5700, South Africa*

15 ⁴*Division of Bioresources, Research Center for Zoonosis Control, Hokkaido University 001-0020,*
16 *Japan*

17 ⁵*Department of Emerging Infectious Diseases, Graduate School of Medicine, Tohoku*
18 *University 1-1 Seiryō-cho, Aobaku, Sendai 980-8574, Japan*

19 ⁶*Inqaba Biotechnical Industries (Pty) Ltd, 525 Justice Mohamed St, Pretoria, 0002, South Africa*

20 ⁷*Department of Medical Microbiology, University of Pretoria*

21 ⁸*National Health Laboratory Service, South Africa*

22 *Corresponding author.

23 E-mail address: kabonpnk@unisa.ac.za (P.N. Kabongo-Kayoka)

24 **ABSTRACT**

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

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

44 **Background**

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

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

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

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

68 The genus *Mycobacterium* contains more than 170 species
69 (<http://www.bacterio.net/mycobacterium.html>), most of which are classified as NTM or
70 potentially pathogenic mycobacteria (PPM) (Chege et al., 2008; Kim et al., 2014; Malama et al.,
71 2014; Tortoli, 2014) and mycobacteria belonging to the *Mycobacterium tuberculosis* complex
72 (MTC). MTC comprises *M. tuberculosis*, *M. bovis*, *M. africanum*, *M. canetti*, *M. pinnipedii*, *M.*
73 *caprae*, *M. microti*, *M. mungi*, *Dassie bacillus*, *Oryx bacillus* and the attenuated *M. bovis*
74 Bacille-Calmette-Guerin (BCG) vaccine strain. With the exception of BCG, these species are
75 pathogenic and can cause tuberculosis (TB) in mammalian hosts (Alexander et al., 2010; Pittius
76 et al., 2012; Vos et al., 2001).

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

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

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

102 Black wildebeest (*Connochaetus gnou*), known in Afrikaans as “Swartwildebees” and in German
103 as “Weisschwanzgnu”, have been hunted in South Africa for meat and hides. The overall
104 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.

111 **2. Materials and Methods**

112 **2.1 Study area**

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

121 **2.2 Study design and sampling**

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

133 **2.3 Sources of Samples**

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

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

150 Table 1

151 2.2. Mycobacterial isolation

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

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

179 **2.3-Mycobacterial identification**

180 **2.3.1 Biochemical profile**

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

184 **2.3. 2 DNA extract and primary molecular identification**

185 DNA extracts were prepared based on the GenoType CM/AS reverse line blot assay (Hain Life
186 Science, Nehren, Germany). DNA was extracted from colonies on LJ slants by heating at 95°C
187 for 20 min in a water bath. Primers provided by the manufacturer's kit (Hain Life Science,
188 Nehren, Germany) were used. The formula for the PCR assay per tube mixture contained 1.1 µl
189 of ultra-pure water, 5 µl of 10 x buffer, 3.6 µl of MgCl₂ (25 mM), 35 µl of primer/nucleotide mix
190 from the kit and 0.3 µl of hot Taq polymerase. The thermocycler was programmed to initiate the
191 PCR as follows: one cycle of 15 min at 95°C, followed by 10 cycles of 30 seconds at 95°C, 2
192 min at 58°C, 25 seconds at 95°C; then 20 cycles of 40 seconds at 53°C and 40 seconds at 70°C
193 with a final cycle of 8 min at 70°C. The hybridization was then followed as per manufacturer's
194 instructions using the strips provided in the kit (DNA strip *Mycobacterium* identification

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

197 **2.4. Gene sequencing**

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

201 **2.4.1 16S ribosomal RNA gene and ITS**

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

215 **2.4.2 *rpoB***

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

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

226 **2.4.3 *hsp65***

227 Partial *hsp65* gene was amplified and sequenced with primers Myco-hsp65F (565-585): 5'-
228 AGGGTATGCGGTTTCGACAAG and MAC hsp65R (Turenne et al., 2006). Amplification was
229 performed with an initial denaturation at 96°C for 1 min, 38 cycles of denaturation at 96°C for
230 10 seconds, annealing at 53°C for 10 seconds and extension at 72°C for 45 seconds followed by
231 a final extension at 72°C for 5 min in the same reaction mixture content described in *rpoB*
232 section. The band with a size of 1067 bp was excised and sequencing was performed by the
233 same procedure as in *rpoB* section.

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

236 **2.5 Phylogenetic analyses**

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

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

262 **2.6 Statistical analyses**

263 The agreement between databases was calculated using Cohen's Kappa. To compare the results
264 obtained from each gene and combination of two genes, only the databases identified with better
265 performance in the first analysis were used. One thousand bootstrap replications were used for
266 testing the reliability of all the groupings within the various trees (Hallgren, 2012; Joao et al.,
267 2014).

268 **2.7 GenBank accession numbers**

269 The sequences generated in this study were deposited in the GenBank database under accession
270 number KR856202 for *hsp65*, KR856203 for *rpoB*, KR856204 for 16SrRNA and KR856205 for
271 16S-23S rRNA ITS.

272

273 **3 Results**

274 **3.1 Mycobacterial isolation**

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

279 **3.2 Biochemical characteristics**

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

286 **Table 2**

287 **3.3 Primary molecular identification**

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

290 **3.4 Phylogenetic analyses**

291 **ITS sequences**

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

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

299 **16S rRNA, hsp65 and rpoB analyses.**

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

306 **Figure 1**

307 **4. Discussion and Conclusion**

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

327 presence of a small number of a specific pathogen does not correlate with the virulence of the
328 pathogen nor its economic importance. Organisms act in synergy, potentiating the colonization
329 by other bacteria. Although mixed infection with NTM has been reported, attention should be
330 given to NTM in the future as several studies have recognized the significance of NTM as a
331 major public health issue around the world (Kankya et al., 2011; Kim et al., 2014; Malama et al.,
332 2014; Moore et al., 2010; Temmerman et al., 2014). Furthermore, NTM have been found to
333 interfere with the diagnosis of TB in cattle; indeed, some cross reactions between the antigens of
334 NTM with those used for diagnostic purposes such as *M. avium* and *M. fortuitum* may
335 compromise the diagnosis and control of bovine tuberculosis (De la Rúa-Domenech et al., 2006;
336 Gcebe et al., 2013).

337 To our knowledge, this is the first report of this new isolate of NTM, “Gnou isolate”, sampled
338 from black wildebeest (*Connochaetes gnou*) in South Africa. The diagnosis of NTM should be
339 standardized as the isolate was identified as *Mycobacterium intracellulare* using DNA strip
340 according to Hain’s method at National Health Laboratory Service and confirmed as a new
341 species of MAC by gene sequencing at Research Center for Zoonosis Control at Hokkaido
342 University in Japan. Tortoli et al. (2010) reported the misidentification of NTM using
343 commercial kit assays as we have also experienced with this study. The partial sequencing of
344 16s rRNA gene alone is not sufficient to fully identify NTM to species level; algorithm analysis
345 combining all four genes should be considered as well as biochemical identification. One of the
346 characteristics of this isolate was slow growth. The role of NTM, in particular slow growing
347 MAC, should be further investigated in the disease causing process as they could potentiate
348 colonization by other rapidly growing microorganisms. The isolation and identification at species
349 level of some NTM should be performed to establish their clinical relevance in animals and
350 humans. Countries should also be encouraged to register new species on the international data
351 bank for purposes of adding to the epidemiological knowledge of this genus.

352 **Acknowledgements**

353 The research protocol was approved by the Ethical Research Committee at University of South
354 Africa (UNISA) Reference number: CAES/087; the National Research Foundation (NRF) unique
355 numbers: 66141 and 86458, and the Department of Agriculture, Forestry and Fisheries (DAFF).

356 This research study was supported by NRF under the aegis of the bilateral research collaboration
357 between South Africa and the Japan Society for the Promotion of Science (JSPS). Financial
358 support was also provided by the University of South Africa. We would like to thank DAFF for
359 approving this research project and National Health Laboratory Service for making the facilities
360 available. The State Veterinarians namely Dr Jacoba Wessels and Dr Melissa Nel (nee Hansen)
361 are immensely thanked for providing the samples as well as the history and histopathological
362 results. We acknowledge the technical assistance of the following people: Fukujima and Horuka
363 Suzuki from the Research Center for Zoonosis Control at Hokkaido University in Japan; Kathy
364 Lindeque, Buki Onwuebuna and Charlotte Kekana from National Health Laboratory Service
365 South Africa.

366

367 **References**

- 368 Alexander, K. A., P. N. Laver, A. L. Michel, M. Williams, P. D. Helden, R. M. Warren and N. C. G.
369 Pittius, 2010: Novel *Mycobacterium tuberculosis* complex pathogen, *M. mungi*. *Emerg. Infect. Dis.*
370 16, 1296–1299.
- 371 Altschul, S. F., W. Gish, W. Miller, E. W. Myers and D. J. Lipman, 1990: Basic local alignment search
372 tool. *J. Mol. Biol.* 215, 403–410.
- 373 Bengis, R. G., N. P. J. Kriek, D. F. Keet, J. P. Raath, V. Vos, and H. F. A. K. Huchzermeyer, 1996: An
374 outbreak of bovine tuberculosis in a free-living African buffalo (*Syncerus caffer-Sparrman*)
375 population in the Kruger National Park: a preliminary report. *Onderstepoort J. Vet. Res.* 63, 15–18.
- 376 Biet, F., M. L. Boschioli, M. F. Thorel and L. A. Guilloteau, 2005: Zoonotic aspects of *Mycobacterium*
377 *bovis* and *Mycobacterium avium-intracellulare* complex (MAIC). *Vet. Res.*, Special issue:
378 Emerging or re-emerging bacterial zoonoses. 36, 411–436.
- 379 Chatterjee, M., S. Bhattacharya, K. Karak and S. G. Dastidar, 2013: Effects of different methods of
380 decontamination for successful cultivation of *Mycobacterium tuberculosis*. *Indian J. Med. Res.* 138,
381 541–548.
- 382 Chege, G. K., R. M. Warren, N. C. G. Pittius, W. A. Burgers, R. J. Wilkinson, E. G. Shephard and A. L.
383 Williamson, 2008: Detection of natural infection with *Mycobacterium intracellulare* in healthy
384 wild-caught Chacma baboons (*Papio ursinus*) by ESAT-6 and CFP-10 IFN- γ ELISPOT tests
385 following a tuberculosis outbreak. *BMC Microbiol.* 8, (07 February 2008).
- 386 Cook, V. J., C. Y. Turenne, J. Wolfe, R. Pauls, and A. Kabani, 2003: Conventional methods versus 16S
387 ribosomal DNA sequencing for identification of nontuberculous mycobacteria: cost analysis. *J.*
388 *Clin. Microbiol.* 41, 1010–1015.
- 389 Darriba, D., G. L. Taboada, R. Doallo, and D. Posada, 2012: jModelTest 2: more models, new heuristics
390 and parallel computing. *Nat. Methods* 9, 772.
- 391 De la Rúa-Domenech, R., T. Goodchild, M. Vordermeier and R. Clifton-Hadley, 2006: Ante mortem
392 diagnosis of Bovine Tuberculosis: the significance of unconfirmed test reactors. *Gov. Vet. J.* 16,
393 65–71.
- 394 Felsenstein, J., 1985: Phylogenies and the Comparative Method. *Am. Nat.* 125, 1–15.
- 395 Gcebe, N., V. Rutten, N. C. G. Pittius and A. L. Michel, 2013: Prevalence and distribution of non-
396 tuberculous mycobacteria (NTM) in cattle, African buffaloes (*Syncerus caffer*) and their
397 environments in South Africa. *Transbound. Emerg. Dis.* 60, 74–84.
- 398 Guindon, S., J. F. Dufayard, V. Lefort, M. Anisimova, W. Hordijk and O. Gascuel, 2010: New
399 algorithms and methods to estimate maximum-likelihood phylogenies: assessing the performance of
400 PhyML 3.0. *Syst. Biol.* 59, 307–321.

401 Hall, T. A., 199: BioEdit: a user-friendly biological sequence alignment editor and analysis program for
402 Windows 95/98/NT, in: *Nucleic Acids Symposium Series*. 95–98.

403 Hallgren, K. A., 2012: Computing Inter-Rater Reliability for Observational Data: An Overview and
404 Tutorial. *Tutor. Quant. Methods Psychol.* 8, 23–34.

405 Janda, J. M. and S. L. Abbott, 2007: 16S rRNA gene sequencing for bacterial identification in the
406 diagnostic laboratory: pluses, perils, and pitfalls. *J. Clin. Microbiol.* 45, 2761–2764.

407 Joao, I., P. Cristovao, L. Antunes, B. Nunes and L. Jordao, 2014: Identification of nontuberculous
408 mycobacteria by partial gene sequencing and public databases. *Int. J. Mycobacteriology* 3, 144–
409 151.

410 Kankya, C., A. Muwonge, B. DjAnne, M. Munyeme, J. Opuda-Asibo, E. Skjerve, J. Oloya, V.
411 Edvardsen and T.B. Johansen, 2011: Isolation of non-tuberculous mycobacteria from pastoral
412 ecosystems of Uganda: Public Health significance. *BMC Public Health* 11, 320.

413 Katale, B. Z., E. V. Mbugi, L. Botha, J. D. Keyyu, S. Kendall, H. M. Dockrell, A. L. Michel, R. R.
414 Kazwala, M. M. Rweyemamu, P. van Helden and M. I. Matee, 2014: Species diversity of non-
415 tuberculous mycobacteria isolated from humans, livestock and wildlife in the Serengeti ecosystem,
416 Tanzania. *BMC Infect. Dis.* 14, 10.

417 Katoh, K., K. Kuma, H. Toh and T. Miyata, 2005: MAFFT version 5: improvement in accuracy of
418 multiple sequence alignment. *Nucleic Acids Res.* 33, 511–518.

419 Katoh, K., K. Misawa, K. Kuma and T. Miyata, 2002: MAFFT: a novel method for rapid multiple
420 sequence alignment based on fast Fourier transform. *Nucleic Acids Res.* 30, 3059–3066.

421 Kim, B. R., J. M. Kim, B. J. Kim, Y. Jang, S. Ryoo, Y. H. Kook and B. J. Kim, 2014: Identification of
422 nontuberculous mycobacteria isolated from Hanwoo (*Bos taurus coreanae*) in South Korea by
423 sequencing analysis targeting hsp65, rpoB and 16S rRNA genes. *Vet. Microbiol.* 173, 385–389.

424 Malama, S., M. Munyeme, S. Mwanza and J. B. Muma, 2014: Isolation and characterization of non
425 tuberculous mycobacteria from humans and animals in Namwala District of Zambia. *BMC Res.*
426 *Notes* 7, 10.

427 Michel, A. L., L. M. de Klerk, N. C. G. van Pittius, R. M. Warren and P. D. van Helden, 2007. Bovine
428 tuberculosis in African buffaloes: observations regarding *Mycobacterium bovis* shedding into water
429 and exposure to environmental mycobacteria. *BMC Vet. Res.* 3, 23.

430 Mirsaeidi, M., M. Farshidpour, M. B. Allen, G. Ebrahimi and J. O. Falkinham, 2014: Highlight on
431 advances in nontuberculous mycobacterial disease in North America. *Biomed Res Int* 2014, 10.

432 Moore, J. E., M. E. Kruijshaar, L. P. Ormerod, F. Drobniewski and I. Abubakar, 2010: Increasing
433 reports of non-tuberculous mycobacteria in England, Wales and Northern Ireland, 1995-2006. *BMC*
434 *Public Health* 10, 612.

435 Müller, B., L. Klerk-Lorist, M. M. Henton, E. Lane, S. Parsons, N. C. G. Pittius, A. Kotze, P. D.

436 Helden and M. Tanner, 2011: Mixed infections of *Corynebacterium pseudotuberculosis* and non-
437 tuberculous mycobacteria in South african antelopes presenting with tuberculosis-like lesions. *Vet.*
438 *Microbiol.* 147, 340–345.

439 Pittius, N.C.G., K. D. Perrett, A. L. Michel, D. F. Keet, T. Hlokwe, E. M. Streicher, R. M. Warren and
440 P. D. Helden, 2012. Infection of African buffalo (*Syncerus caffer*) by *oryx bacillus*, a rare member
441 of the antelope clade of the *Mycobacterium tuberculosis* complex. *J. Wildl. Dis.* 48, 849–857.

442 Ronquist, F., M. Teslenko, P. van der Mark, D.L. Ayres, A. Darling, S. HÅhna, B. Larget, L. Liu, M.
443 A. Suchard and J. P. Huelsenbeck , 2012: Mr. Bayes 3.2: Efficient bayesian phylogenetic inference
444 and model choice across a large model space. *Syst. Biol.* 61, 539–542.

445 Tamura, K., D. Peterson, N. Peterson, G. Stecher, M. Nei and S. Kumar, 2011: MEGA5: Molecular
446 evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum
447 parsimony methods. *Mol. Biol. Evol.* 28, 2731–2739.

448 Temmerman, S., L.Vandekerckhove, E. Sermijn, D. Vogelaers, G. Claeys, M. Vanechoutte, P. Cools
449 and S. Callens, 2014: Disseminated infection with *Mycobacterium tilburgii* in a male
450 immunocompromised patient. *J. Clin. Microbiol.* 52, 1777–1779.

451 Tortoli, E., 2014: Microbiological features and clinical relevance of new species of the genus
452 *Mycobacterium*. *Clin. Microbiol. Rev.* 27, 727–752.

453 Tortoli, E., M. Pecorari, G. Fabio, M. MessinÅ and A. Fabio, 2010: Commercial DNA Probes for
454 *Mycobacteria* incorrectly identify a number of less frequently encountered Species. *J. Clin.*
455 *Microbiol.* 48, 307–310.

456 Turenne, C.Y., M. Semret, D. V. Cousins, D. M. Collins and M. A. Behr, 2006: Sequencing of hsp65
457 distinguishes among subsets of the *Mycobacterium avium* Complex. *J. Clin. Microbiol.* 44, 433–
458 440.

459 Vos, V., R. G. Bengis, N. P. J Kriek, A.L. Michel, D. F. Keet, J. P. Raath and H. F. K. A.
460 Huchzermeyer, 2001: The epidemiology of tuberculosis in free-ranging African buffalo (*Syncerus*
461 *caffer*) in the Kruger National Park, South Africa. *Onderstepoort J. Vet. Res.* 68, 119–130.

462 Warren, R. M., N. C. G. Pittius, M. Barnard, A. Hesseling, E. Engelke, M. Kock, M. C. Gutierrez, G.
463 K. Chege, T. C. Victor, E. G. Hoal and P. D. Helden, 2006: Differentiation of *Mycobacterium*
464 tuberculosis complex by PCR amplification of genomic regions of difference. *Int. J. Tuberc. Lung*
465 *Dis.* 10, 818–822.

466