DOI: 10.7589/2010-07-178 *Journal of Wildlife Diseases*, 48(4), 2012, pp. 849–857

INFECTION OF AFRICAN BUFFALO (*SYNCERUS CAFFER*) BY ORYX BACILLUS, A RARE MEMBER OF THE ANTELOPE CLADE OF THE *MYCOBACTERIUM TUBERCULOSIS* COMPLEX

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ABSTRACT: *Mycobacterium tuberculosis* complex species cause tuberculosis disease in animals and humans. Although they share 99.9% similarity at the nucleotide level, several host-adapted ecotypes of the tubercule bacilli have been identified. In the wildlife setting, probably the most well-known member of this complex is *Mycobacterium bovis*, the causative agent of bovine tuberculosis. The recently described oryx bacillus is an extremely rare slow-growing member of the antelope clade of the *M. tuberculosis* complex and is closely related to the dassie bacillus, *Mycobacterium africanum* and *Mycobacterium microti*. The antelope clade is a group of strains apparently host adapted to antelopes, as most described infections were associated with deer and antelope, most specifically the Arabian oryx (*Oryx leucoryx*). In this study, oryx bacillus was isolated from a free-ranging adult female African buffalo (*Syncerus caffer*), in good physical condition, which tested strongly positive on three consecutive comparative intradermal tuberculin tests. Upon necropsy, a single pulmonary granuloma and an active retropharyngeal lymph node was found. Comprehensive molecular genetic assays were performed, which confirmed that the causative microorganism was not *M. bovis* but oryx bacillus. Oryx bacillus has never been reported in Southern Africa and has never been found to infect African buffalo. The identification of this microorganism in buffalo is an important observation in view of the large and ever-increasing epidemic of the closely related *M. tuberculosis* complex species *M. bovis* in some African buffalo populations in South Africa.

Key words: African buffalo, antelope clade, *Mycobacterium tuberculosis* complex, oryx bacillus, Southern Africa.

INTRODUCTION

Mycobacterium tuberculosis complex species cause tuberculosis in animals and humans. Species in the complex are characterized by 99.9% similarity at the nucleotide level and identical 16S rRNA DNA sequences (Brosch et al., 2002). The oryx bacillus is a recently described species of the *M. tuberculosis* complex belonging to the antelope clade, a group of strains apparently host adapted to antelopes (van Soolingen et al., 1994; Kremer et al., 1999; Mostowy et al., 2005; Smith et al., 2006). Infections with oryx bacillus have rarely been reported, although it is likely that a significant number of infections might have been incorrectly diagnosed as either *Mycobacterium africanum* or *Mycobacterium bovis* before the advent of genomic molecular typing (van Soolingen et al., 1994; Kremer et al., 1999; Mostowy et al., 2005; Rahim et al., 2007). Most infections that have been described were associated with artiodactyl hosts, most specifically the Arabian oryx (*Oryx leucoryx*), hence, the name oryx bacillus (Kremer et al., 1999; Mostowy et al., 2005; Smith et al., 2006). However, reports indicate that this microorganism is able to infect humans, and it has been isolated from lungs, lymph node, abscess, ascitic

fluid, and urine of nine patients born in India but resident in Australia (Fyfe, pers. comm.).

In this study, a free-ranging adult female African buffalo (*Syncerus caffer*) in good physical condition, was strongly positive on three consecutive comparative intradermal tuberculin tests. The animal was subsequently euthanized, and upon necropsy, a single pulmonary granuloma and an active retropharyngeal lymph node was found. The causative microorganism was found not to be *M. bovis* but oryx bacillus. We performed comprehensive molecular assays to investigate this microorganism. Given the rarity of oryx bacillus infection, the physiologic and molecular information produced in this study is of great importance for our understanding of this species of the *M. tuberculosis* complex and its infection characteristics in wildlife.

MATERIALS AND METHODS

Study area and sample collection

Samples were collected on a large private game farm in KwaZulu-Natal, South Africa. This game farm consisted of an amalgamation of four former neighboring game farms and covered a large area consisting of dense bush and hilly country. During June 2007, 20 free-ranging buffalo were captured for testing for bovine tuberculosis on this farm. One adult female was strongly positive on three consecutive comparative intradermal tuberculin tests. Blood was taken for interferon-gamma assays, and the animal showed a strong reaction to bovine tuberculin. It was euthanized, a detailed necropsy was carried out, and samples were taken for further analyses.

Postmortem examinations

Selected lymph nodes were aseptically removed from the carcass and visually examined for macroscopic tuberculous lesions. Sections with suspicious lesions were placed in 10% buffered formalin for histopathologic examination. Paraffin wax sections were routinely stained with hematoxylin and eosin, and selected sections with the Ziehl-Neelsen (ZN) acid-fast stain for microscopic examination for acid-fast bacilli. The lymph nodes examined were 1) head: mandibular, retropharyngeal, parotid, and tonsils; 2) thorax: mediastinal and tracheobronchial complex; 3) abdomen: hepatic and mesenteric; 4) peripheral: prescapular, axiliary, prefemoral, popliteal, sacral, and supramammary.

Microorganism culture and identification

Affected lung tissue was prepared and cultured in triplicate in the BACTEC MGIT culture system (Becton Dickinson, Franklin Lakes, New Jersey, USA) as described (Warren et al., 2006). Heat-killed culture lysates were subjected to a 5′-16S rDNA PCR-sequencing assay, which is able to identify specific *Mycobacterium* spp. (Harmsen et al., 2003). Subsequently, an array of PCR-based assays was performed to further characterize the organism. These included the *M. tuberculosis* complex-specific multiplex-PCR test (Warren et al., 2006), as well as further tests based on regions of difference (RD; Mostowy et al., 2005) and single-nucleotide polymorphisms (Huard et al., 2006).

Spacer-oligotyping and mycobacterial interspersed repetitive unit-variable-number tandem-repeat typing

Spacer-oligotyping (spoligotyping) was done on heat-killed culture lysates, using an internationally standardized method as described by Kamerbeek et al. (1997). Mycobacterial interspersed repetitive unit-variable-number tandem-repeat (MIRU-VNTR) typing was done on the full set of 24 repeats on heat-killed culture lysates, using an internationally standardized method (Supply et al., 2006).

RESULTS

Necropsy

With the exception of a single pulmonary granuloma and an active retropharyngeal lymph node, no pathologic changes were seen in any of the organs examined. The focal, chronic, and fairly well-encapsulated granuloma was in the lower half of the dorsocaudal portion of the right cranial lobe (Fig. 1). It contained a small, weakly defined area of apparent calcification immediately below the capsule. On cut section, it contained a central caseous necrotic mass with a partial liquefaction in the center. The right retropharyngeal lymph node was slightly enlarged revealing a nonspecific inflammatory reaction on cut section. Histologically, all

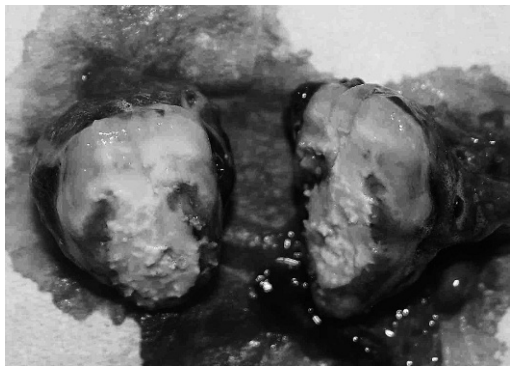

FIGURE 1. Single, focal, chronic, and fairly well-encapsulated pulmonary granuloma, 18×13×13 mm, in the lower half of the dorsocaudal portion of the right cranial lobe, with a small and weakly defined area of apparent calcification immediately below the capsule. The granuloma contained a central caseous necrotic mass with partial liquefaction in the center.

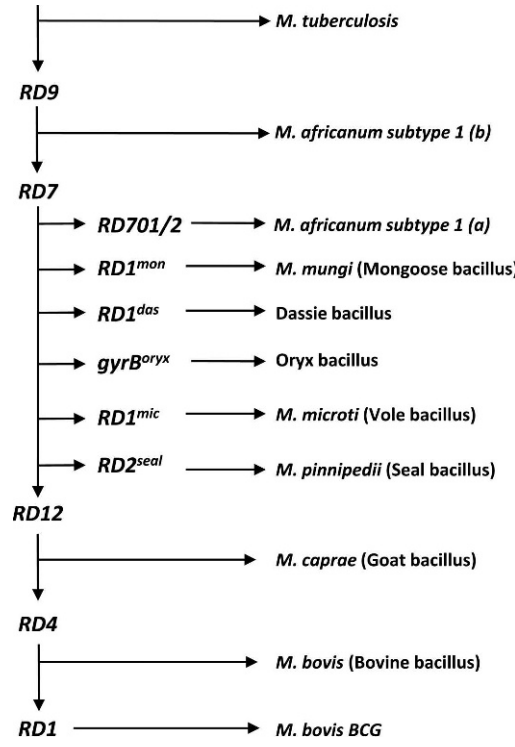

FIGURE 2. Phylogenetic tree of the members of the *Mycobacterium tuberculosis* complex with differentiating markers used for species identification by polymerase chain reaction and sequencing (deletion regions [regions of difference] and single-nucleotide polymorphism $gyrB^{oryx}$ indicated).

lymph nodes were moderately active. Some showed red blood cells, fibroblasts, and a few neutrophils within the medullary sinuses, and there was moderate sinus histiocytosis. The tonsils showed highly active lymphoid tissue. No clear granulomas were visible histologically, and ZN staining did not yield any clear acid-fast bacilli in the lymph node sections examined.

Culture and identification

Sequencing of PCR products identified the microorganism as a member of the *M. tuberculosis* complex with RD4 and $RD1^{BCG}$ intact and RD9 and RD12 deleted. These genomic features are consistent with a species with an evolutionary position between *M. africanum* West African-1 and *Mycobacterium caprae* on the phylogenetic tree of the *M. tuberculosis* complex, and excludes *"Mycobacterium canettii," M. tuberculosis*, and *M. bovis* (Fig. 2). Further RD testing (Mostowy et al., 2005) showed that the $RD1^{mic}$, $RD1^{das}$, and $RD2^{seal}$ regions were intact (confirming that this isolate is not a member of the *M. microti*, *Mycobacterium pinnipedii*, or the dassie bacillus species) and that the RD7 region was deleted, but RD701 and 702 were intact (confirming that the microorganism was not *M. africanum* West African-1 or *M. africanum* West African-2). The PCR assays confirmed the absence of RDoryx_wag22 and RD12oryx, which are markers for the oryx bacillus (Mostowy et al., 2005). To confirm the identity of this microorganism, the *gyrB* gene was sequenced (Fig. 3), revealing the oryx bacillus-specific $gyrB^{oryx}$ G to a single-nucleotide polymorphism at position 1113 (Huard et al., 2006).

Spoligotyping and MIRU-VNTR typing

Spoligotyping showed a pattern exactly matching that of a previous isolate of the oryx bacillus (no. SB0319) from the *M. bovis* spoligotype database (Mbovis.org, 2008) and dissimilar to the most frequently

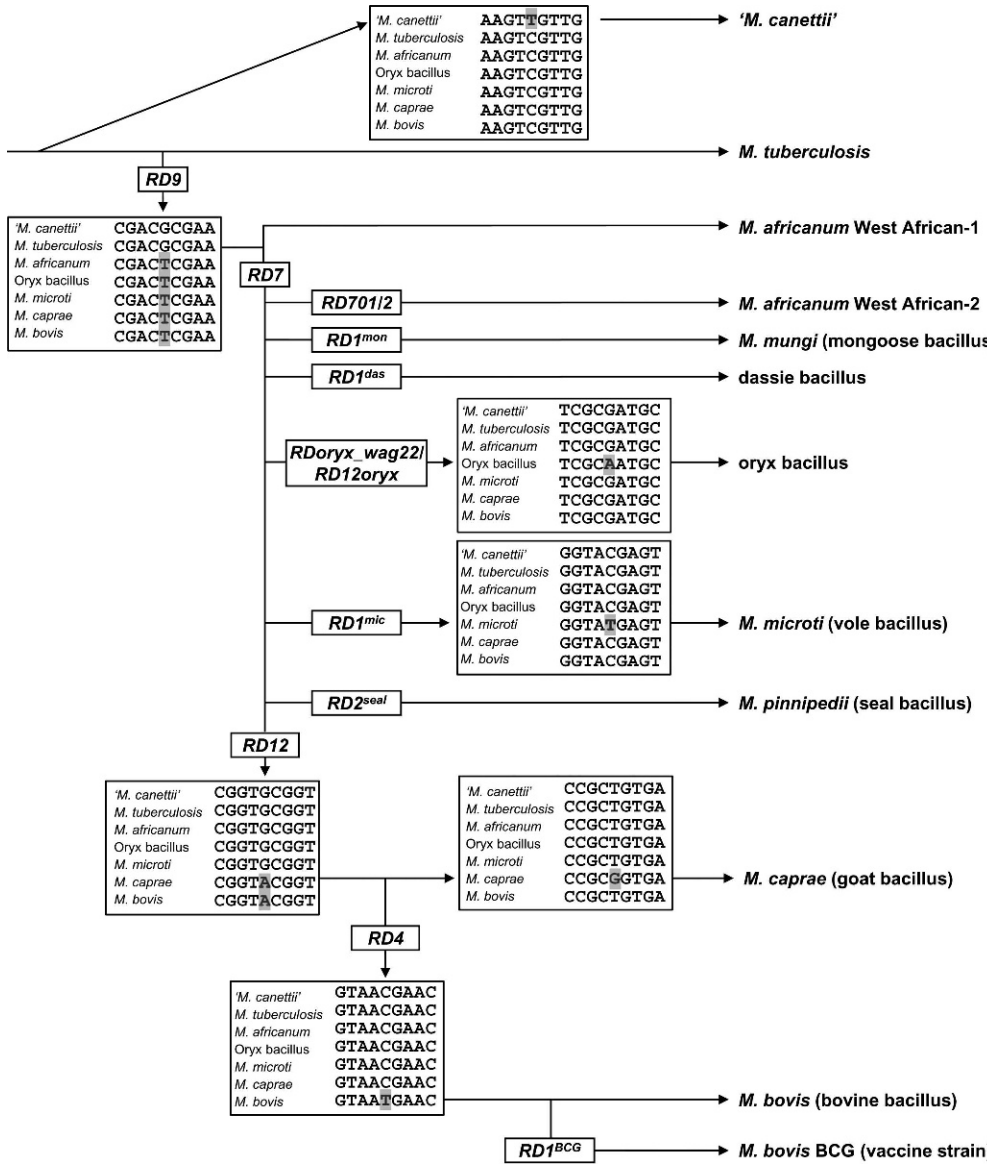

FIGURE 3. Single-nucleotide polymorphism detection in *gyrB* gene through polymerase chain reaction and sequencing provides the ability to differentiate six of the nine *Mycobacterium tuberculosis* complex species.

observed patterns for other members of the complex (Fig. 4). It also matched the international spoligotyping database SpolDB4 type ST701, annotated as *M. africanum* (Brudey et al., 2006). SpolDB4 does not contain an annotated spoligotype pattern for the oryx bacillus. However, a comparison of the spoligotypes for oryx bacillus from previously published papers (Kremer et al., 1999; Mostowy et al., 2005), which conforms with the oryx bacillus spoligotypes (SB0319 and SB0422) in the *M. bovis* spoligotype database (Mbovis.org, 2008), showed clearly that SpolDB4 types ST701 and ST587 are annotated incorrectly in this database as *M. africanum* and actually represent oryx bacillus (Fig. 4). This is confirmed by a recent unpublished

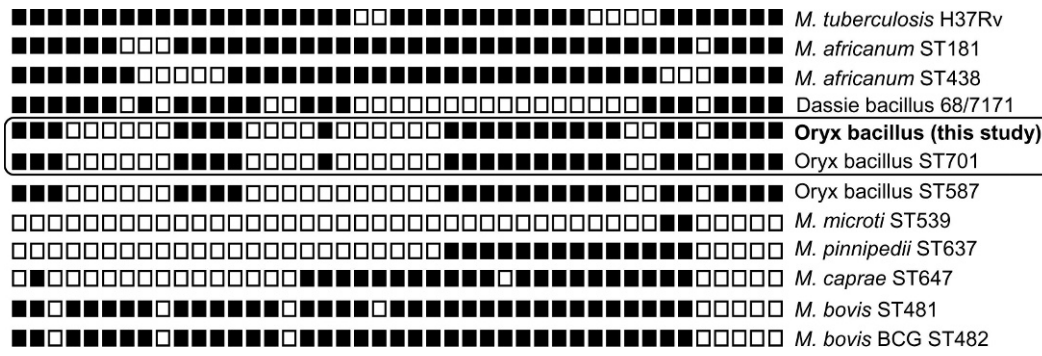

FIGURE 4. Spoligotype pattern for the oryx bacillus isolate is similar to a previously reported oryx bacillus spoligotype pattern (Kremer et al., 1999; Mostowy et al., 2005) and distinct from the most frequently observed patterns for *Mycobacterium tuberculosis* H37Rv, *Mycobacterium africanum*, dassie bacillus *Mycobacterium microti*, *Mycobacterium pinnipedii*, *Mycobacterium caprae*, *Mycobacterium bovis*, and *Mycobacterium bovis* bacille Calmette et Guerin (Brudey et al., 2006).

study that showed that nine oryx bacillus isolates from human origin, with similar but not identical IS*6110* restriction fragment length polymorphism profiles; all had spoligotype pattern ST587 (Fyfe, pers. comm.).

Complete MIRU-VNTR typing of the oryx bacillus has never before been published, although a study reported exact tandem-repeat (ETR-) A to -E values of 22544 and 32544 for a Saudi-Arabian and Netherlands isolate of oryx bacillus, respectively (Kremer et al., 1999). Supply et al. (2006) used these same isolates for complete typing but did not publish the final results. The oryx bacillus results in our study compared very well with these isolates, having exactly the same MIRU-VNTR results as sample 24 isolated in Saudi Arabia (Table 1). Similar to the finding by Supply et al. (2006), QUB11b also did not amplify in our study of the oryx bacillus isolate.

DISCUSSION

The *M. tuberculosis* complex consists of species infecting a wide range of mammals. For example, *M. microti* infects field voles (*Microtus agrestis*); *M. pinnipedii* infects seals (including Australian sea lions [*Neophoca cinerea*], New Zealand fur seals [*Arctocephalus forsteri*], Australian fur seals [*Arctocephalus pusillus doriferus*], southern sea lions [*Otaria flavescens*], wild South American fur seals [*Arctocephalus australis*], and wild subantarctic fur seals [*Arctocephalus tropicalis*]); *M. caprae* infects goats (*Capra hircus*); and *M. bovis* infects cattle (*Bos taurus*) and a wide range of other mammals (Smith et al. 2006). Infections with oryx bacillus have rarely been reported, although it is likely that many infections might have been incorrectly diagnosed as either *M. africanum* or *M. bovis* before the advent of genomic molecular typing for this microorganism around 2002 (Brosch et al., 2002). Unpublished reports indicate that this microorganism is able to infect humans as it has been isolated from lungs, lymph node, abscess, ascitic fluid, and urine of nine patients born in India but resident in Australia (Fyfe, pers. comm). Oryx bacillus infection in camelids has been reported in the United Arab Emirates (Kinne et al., 2006; Wernery et al., 2007). A dromedary (*Camelus dromedarius*) diagnosed with pulmonary tuberculosis caused by oryx bacillus was severely emaciated and died 2 mo after the onset of the disease. It exhibited typical tuberculous lesions in both lungs and lung lymph nodes. A guinea pig (*Cavia porcellus*) inoculated with lung

TABLE 1. Comparison of the full 24 set of mycobacterial interspersed repetitive unit-variable-number tandem-repeat (MIRU-VNTR) results of oryx bacillus isolates from three countries.

	24[b]	69[b]	African buffalo[c] (this study)
Sample No.[a]	Saudi Arabia	The Netherlands	South Africa
MIRU 02 (154)	2	2	2
VNTR 0424/Mtub04 (424)	2	2	2
VNTR 0577/ETR-C (577)	5	5	5
MIRU 04/ETR-D (580)	3	3	3
MIRU 40 (802)	2	2	2
MIRU 10 (960)	7	7	7
MIRU 16 (1644)	4	4	4
VNTR 1955/Mtub21 (1955)	3	3	3
MIRU 20 (2059)	2	2	2
VNTR 2163b/QUB11b (2163b)	**?**	**?**	**?**
VNTR 2165/ETR-A (2165)	*2*	*3*	*2*
VNTR 2347/Mtub29 (2347)	3	3	3
VNTR 2401/Mtub30 (2401)	4	4	4
VNTR 2461/ETR-B (2461)	2	2	2
MIRU 23 (2531)	4	4	4
MIRU 24 (2687)	1	1	1
MIRU 26 (2996)	4	4	4
MIRU 27 (3007)	3	3	3
VNTR 3171/Mtub34 (3171)	3	3	3
MIRU 31/ETR-E (3192)	4	4	4
VNTR 3690/Mtub39 (3690)	4	4	4
VNTR 4052/QUB26 (4052)	2	2	2
VNTR 4156/QUB4156 (4156)	3	3	3
MIRU 39 (4348)	2	2	2

[a,b] ETR = exact tandem repeat. Data kindly supplied by Supply et al. (2006). Boldface question marks indicate unamplifiable VNTR; italic text indicates VNTR showing differences between isolates.

[c] *Syncerus caffer*.

tissue from this camel died after 3 wk from typical tuberculosis, indicating that the microorganism is able to cause severe disease under the right circumstances. This microorganism was also able to spread to other members of the herd, and two other camels were confirmed postmortem to have had tuberculosis due to oryx bacillus. The authors speculated that the camels may have become infected by ingesting excretions of infected Arabian oryx, since dromedaries are coprophagous.

There is, to our knowledge, only one example of oryx bacillus infecting bovids. In this recently reported case, *M. tuberculosis* complex mycobacteria were isolated from the lung tissue of four cows that died of unknown disease at a dairy farm in Bangladesh (Rahim et al., 2007). Histopathologic examination of the lung tissue demonstrated prominent granulomas, caseating necrosis, and calcification indicative of tuberculosis infection. Although these mycobacteria were identified by the authors as *M. africanum*, three of the four isolates had spoligotypes exactly matching that of oryx bacillus, while the fourth spoligotype was a slight variation thereof. It seems that the bacillus was able to infect and cause death in the cattle.

Oryx bacillus isolated from animals has been shown to harbor 18–20 copies of the insertion element IS*6110* (Kremer et al., 1999), with up to 26 copies reported from human isolates (Fyfe, pers. comm.). Strains with such high numbers of IS*6110* copies are exceptional among *M. tuberculosis* complex isolates from animals (van Soolingen et al., 1994). Six strains with 20 copies of IS*6110* (thought to be *M.*

bovis) were previously isolated from various artiodactyls (two waterbuck [*Kobus ellipsiprymnus*], two oryx [species name not provided in publication], and two antelope [species name not provided in publication]) in a single zoo in the Netherlands (van Soolingen et al., 1994). It is highly likely that this represented an outbreak of oryx bacillus, given that these were all isolated from antelope species and all contained high numbers of IS*6110*. Investigators that screened the Netherlands National Institute of Public Health and the Environment spoligotype database identified nine strains with spoligotypes closely matching that of the oryx bacillus (Smith et al., 2006). These strains were all isolated from antelopes (one gazelle, one deer, two oryx, two antelope, and three waterbuck [species names not provided in publication]), and all contained over 19 copies of IS*6110* similar to the previously isolated strains of oryx bacillus. Probably the oldest isolate with characteristics similar to oryx bacillus was used in a study in 1994, with the isolate from an unknown source originating about 20 yr earlier (van Soolingen et al., 1994). Another outbreak of tuberculosis described in a captive herd of Arabian oryx in Saudi Arabia, caused the death of a quarter of the animals (Flamand et al., 1994) and was possibly also due to oryx bacillus. Tuberculosis was reported in East African oryx (*Oryx gazella beisa*) as early as 1976 at a municipal zoo in the USA (Lomme et al., 1976). Microscopic examination revealed granulomas containing acid-fast bacilli in the lungs and liver of the infected animals, as well as in the uterus and mediastinal lymph nodes of one animal. "*M. tuberculosis*" was isolated from the tissues of both oryx and from fluid aspirated from the mammary gland of one oryx. Given the level of species differentiation at the time, it is possible that these infections were also due to oryx bacillus.

No oryx bacillus infection has been reported in Southern Africa, despite the extremely high infection rates of *M. tuberculosis* in humans and *M. bovis* in animals in parts of this region. Closely related *M. africanum* has also been rarely encountered in this region, although the other closely related species dassie bacillus seems to be endemic (Parsons et al., 2008).

For *M. bovis*, the aerosol route of infection is predominant, and most macroscopic lesions are found in the lymph nodes of the head and thorax and the lungs. Whether this is also true for oryx bacillus remains to be determined; however, the location and macroscopic appearance of the oryx bacillus pulmonary granuloma in this case corresponds to that described in the vast majority of *M. bovis*-infected buffalo and are indicative of aerosol transmission.

The original buffalo herd on this farm partly consisted of a few animals originating from a zoo in Portugal with the rest originating from the Addo Elephant Park in the Cape Province of South Africa. In 1990, suspect lesions in organs from a clinically unwell buffalo from this farm were cultured, and *M. bovis* diagnosed. This animal was one of the consignment of six buffalo from the zoo in Portugal and had apparently arrived in a crate along with three other buffalo. The time between arrival in South Africa and necropsy is unknown. Although this happened around 20 yr before the isolation of the oryx bacillus, it is possible, given the apparent absence of oryx bacillus in the region and the fact that oryx bacillus has been isolated from European zoo animals in the past, that the bacterium was imported into the region from the zoo and has become latently established in this particular herd. No proof for this hypothesis can be provided, though, given the time that lapsed since the importation. The original culture from the diseased zoo buffalo from 1990 could also not be located for retyping, and it is, thus, impossible to say whether this animal also had oryx bacillus infection. No other positive reactors have been reported in

these buffalo or any of the cattle in the surrounding farms over the years, and we have not been able to identify any other possible source for the infecting microorganism. If this microorganism did arrive with the imported buffalo from the zoo, it illustrates the need for vigilance in preventing the translocation of mycobacterial pathogens during the movement of species.

The identification of the first case of oryx bacillus infection in wild African buffalo is of great importance in view of the current *M. bovis* epidemic in parts of Southern Africa. *M. bovis* is also considered a nonindigenous microorganism within Southern African ecosystems. In the Kruger National Park in South Africa, the disease probably originated from infected domestic cattle and is believed to have entered the park in the early 1960s (De Vos et al., 2001). It has since survived and propagated in the favorable conditions supplied by a fully susceptible and immunologically naive buffalo population and spread from the south of the park to the northernmost parts within only 15 yr after its first identification (De Vos et al., 2001). It has also spilled over from buffalo into nine other wildlife species, namely lion (*Panthera leo*), leopard (*Panthera pardus*), cheetah (*Acinonyx jubatus*), hyena (*Crocuta crocuta*), kudu (*Tragelaphus strepsiceros*), baboon (*Papio ursinus*), warthog (*Phacochoerus africanus*), honey badger (*Mellivora capensis*), and genet (*Genetta genetta*). Buffalo are considered to be the primary maintenance host, and large predators of buffalo, especially lions, seem to be highly vulnerable to infection with *M. bovis*. Thus, finding another closely related nonindigenous *M. tuberculosis* complex microorganism infecting African buffalo is an important observation as this has the potential to also provide favorable conditions for the maintenance and spread of this microorganism throughout the wildlife populations of Southern Africa. There are reports that oryx bacillus can cause fatal disease in animals and humans. Therefore, it is not only a risk to other wildlife but also to humans. This study highlights the need for genomic molecular diagnosis of isolates of the members of the *M. tuberculosis* complex to ensure the correct identification of the species causing the disease.

ACKNOWLEDGMENTS

We thank Debbie Cooke and Marianna de Kock for technical assistance.

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Submitted for publication 6 July 2010.
Accepted 4 April 2012.