Concomitant fungal and *Mycobacterium bovis* infections in beef cattle in Kenya

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Bovine tuberculosis is an important zoonosis and accurate diagnosis is important for its surveillance. Post-mortem diagnosis may, however, be compromised by lesions caused by other pathogens. In an investigation on its prevalence in slaughter cattle in Kenya, *Mycobacterium bovis* and dimorphic fungi were inadvertently identified separately or concurrently in tuberculous lesions. Beef carcasses were inspected for lesions in two abattoirs in Nairobi. Tissues with lesions were collected and transported to the laboratory. Smears of lesions were stained by acid-fast procedure and examined microscopically. Lesions were cultured in Löwenstein-Jensen (LJ) and in BBL™ *Mycobacterium* growth indicator tubes (MGIT) media. Mycobacteria isolates in LJ medium were identified by DNA typing. Smears of BBL™ MGIT cultures were acid-fast stained and examined microscopically. Tissue sections were stained with periodic acid-Schiff reagent before examination. Of the 929 carcasses examined, 176 had granulomatous lesions. Dimorphic fungi were detected as acid-fast negative cells in 58 (32.9; 33.5%) of the lesion smears, either alone (29.0; 16.4%) or concurrently with acid-fast bacilli (29.0; 16.4%). The fungi were also detected in some BBL™ MGIT-culture smears and lesioned tissue sections. The fungi were identified, by means of cellular morphology, as *Paracoccidioides brasiliensis* and *Blastomyces dermatitidis*. A total of 64 isolates of mycobacteria were recovered in LJ medium, 19 of which were identified as *M. bovis*. The present report documents native *P. brasiliensis* infections outside the presumed endemic region and *B. dermatitidis* infections in a livestock animal. The findings further indicate the importance of dimorphic fungi as a differential diagnosis of bovine tuberculosis in the region.

Introduction

Bovine tuberculosis is caused by *Mycobacterium bovis* and manifests in cattle as granulomatous lesions mainly in the lungs and lymph nodes of the thorax (Liebana *et al.* 2008; Office International des Epizooties [OIE] 2009). Although post-mortem examination and culture are effective procedures for the diagnosis of bovine tuberculosis (OIE 2009), the sensitivity of the procedure is affected by the presence of non-tuberculous parasitic, bacterial or mycotic granulomas and bacterial abscesses, which may be indistinguishable macroscopically from tuberculous granuloma (Liebana *et al.* 2008; OIE 2009). A study in Mali cattle revealed that 72% of tuberculous lesions detected during standard meat inspections were due to pathogens other than *M. bovis* (Muller *et al.* 2009).

Dimorphic fungi, which include *Blastomyces dermatitidis* and *Paracoccidioides brasiliensis*, are known causative agents of endemic systemic mycoses. Although *B. dermatitidis* has a worldwide distribution, *P. brasiliensis* is geographically restricted to South and Central America (Chakrabarti & Shivaprakash 2005; McEwen *et al.* 1995; Restrepo 1985). *Paracoccidioides brasiliensis* is mainly a human pathogen, causing chronic granulomatous pulmonary or disseminated infection (Borges-Walmsley *et al.* 2002). However, infections have been reported in domestic and captive wild animals (Bagagli *et al.* 2003; Costa & Fara-Netto 1978; Costa *et al.* 1995; Ricci *et al.* 2004). Human paracoccidioidomycosis cases reported outside the endemic region are considered imported (Onda *et al.* 2011; Van Damme *et al.* 2006). *Blastomyces dermatitidis* primarily affects humans and dogs, causing a chronic suppurative or granulomatous respiratory infection that can disseminate to other tissues and organs, especially the skin (Chapman *et al.* 1997). However, infections have also been reported in cats and horses (Schmiedt *et al.* 2006). This report documents *P. brasiliensis* and *B. dermatitidis* infections in beef cattle in Kenya in association with granulomas grossly indistinguishable from those caused by *M. bovis*.

Materials and methods

Beef cattle carcasses were inspected for tuberculosis lesions in two abattoirs in Nairobi, Kenya, between July 2009 and November 2009. The animals slaughtered originated mainly from the nomadic pastoral communities in the arid and semi-arid northern areas of the country. Carcasses

were randomly selected and inspected according to procedures established by legislation. The lungs, the pleura, abdominal organs and the lymph nodes of the head region, thoracic cavity and the mesentery were palpated, incised and examined. Samples of affected tissues were collected individually into sterile plastic bags, transported to the laboratory and preserved at -20 °C . The samples were then processed to be examined for acid-fast bacilli (AFB) and for *Mycobacterium* cultures according to standard procedures (OIE 2009; World Health Organization [WHO] 1998a, 1998b). Tissue samples were homogenised using Griffith tubes, decontaminated with 4% sodium hydroxide and neutralised with phosphate buffered solution. Smears were then prepared from the homogenates, stained according to the Ziehl-Neelsen (ZN) method and examined microscopically. Samples of the homogenates were cultured in Löwenstein-Jensen (LJ) medium and BBL™ MGIT™ medium (*Mycobacterium* growth indicator tubes, Becton, Dickinson and Co, USA). Smears were prepared from the BBL™ MGIT™ culture tubes and stained with the ZN stain. Mycobacteria isolated in LJ medium were identified by molecular, analysis using the genotype MTBC assay kit (HAIN Lifescience, Nehren, Germany), as described by Gathogo, Kuria and Ombui (2012). Briefly, a loopful of bacteria was collected from each AFB-positive LJ slant, suspended in 300 µL of purified water in a 1.5-mL microcentrifuge tube and incubated in boiling water for 20 min to inactivate the mycobacteria. This was followed by incubation in an ultrasonic bath to break the mycobacterial cell walls. DNA was extracted by centrifugation and amplified in a thermocycler. The amplification conditions consisted of 5 min of initial denaturation at 95 °C, 10 cycles of 30 s each at 95 °C and 2 min each at 58 °C, 20 cycles of 25 s each at 95 °C, 40 s at 53 °C and 40 s at 70 °C, and a final extension at 70 °C for 8 min. Hybridisation and detection were carried out using a semi-automated washing-and-shaking device (TwinCubator®, HAIN Lifescience, Nehren, Germany). Labelled hybridisation membrane strips were added into wells of the plastic reaction tray containing the amplicons and hybridisation reagents, after which plates were incubated. Colourimetric detection of hybridised amplicons was performed by addition of streptavidin-conjugated alkaline phosphatase and the appropriate substrate. After the final washing step, the strips were air dried, fixed on a data sheet and examined visually.

Subsequent to detection of fungi in the ZN preparations, histological examination of the lesions was carried out to confirm the presence of fungi. Formalin-fixed samples of the lesions were routinely processed for histopathology and embedded in paraffin wax. They were sectioned at 5 µm thickness, stained with the periodic acid-Schiff reagent and examined microscopically.

Results and discussion

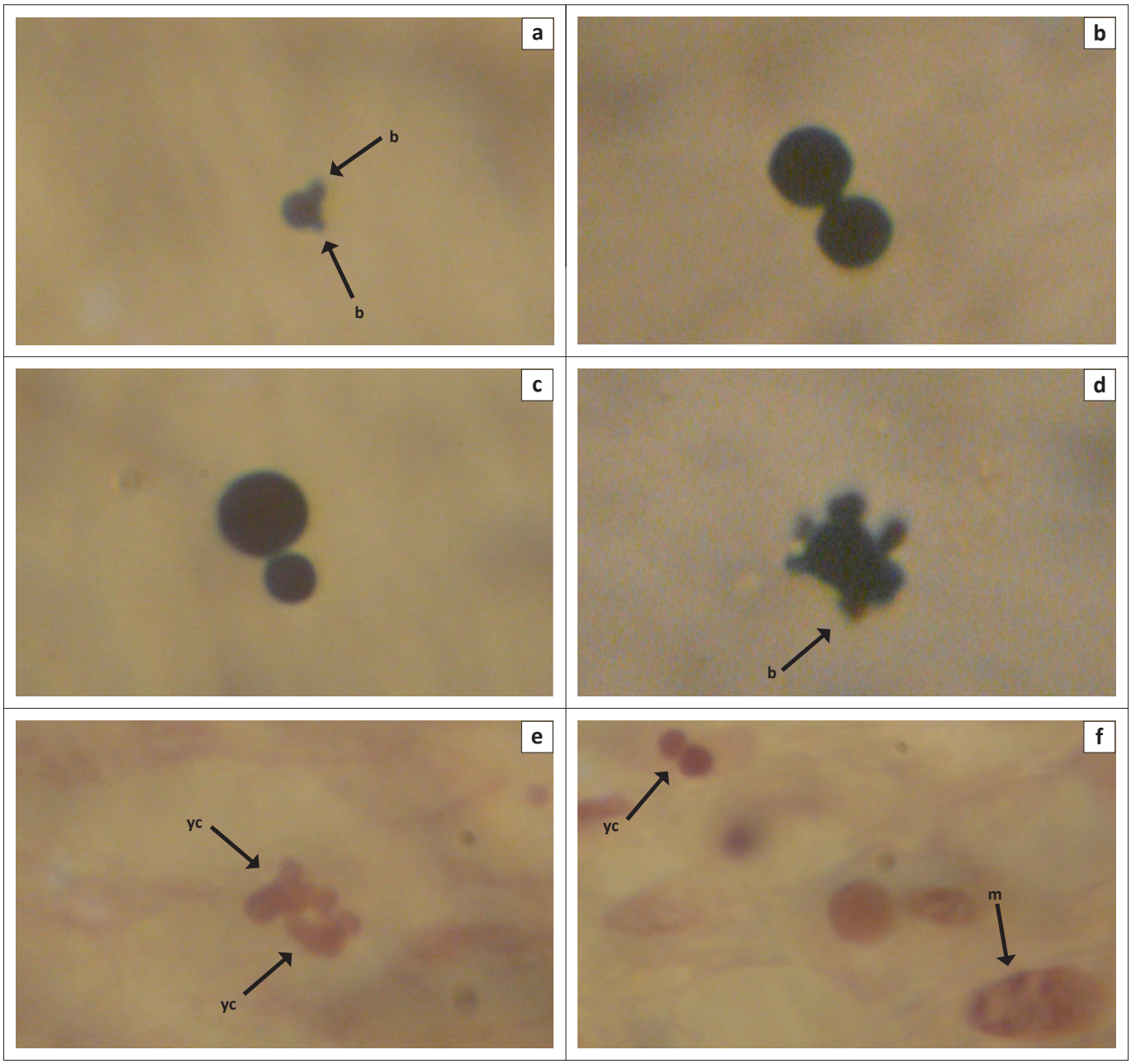
Of the 929 carcasses examined in total, 176 (19%) presented with tuberculous lesions. The majority of the lesions were localised (173/176; 98.29%) and were observed in bronchial lymph nodes (94), posterior mediastinal lymph nodes (94), lung parenchyma (14), liver parenchyma or portal lymph nodes (3), and in other lymph tissues (57). Fungi were detected as acid-fast negative cells in smears of 58 cases (32.9%), either alone (29; 16.4%) or concurrent with AFB (29; 16.4%). *Mycobacterium* spp. were detected from only 35 cases (19.8%). Of the 58 cases showing the presence of fungi, four presented with lesions in the lungs, whilst lesions in lymph nodes and other tissues and organs were seen in the remaining cases (Table 1). The fungi comprised two types: blastospores with multiple buds attached to the mother cell by a narrow neck, resembling a mariner's wheel and typical of *P. brasiliensis*, or large thick-walled cells with a single broad-based bud, identified as *B. dermatitidis* (Figure 1a and 1b). These fungi were also detected in some BBL™ MGIT cultures (Figure 1c and 1d) and *B. dermatitidis* cells were detected in some tissue sections (Figure 1e and 1f). *Mycobacterium* spp. were isolated from all 64 lesions that were AFB positive, with 19 isolates being identified as *M. bovis*.

The results of the investigation indicated that granulomatous lesions in the beef cattle carcasses examined were caused either separately by *Mycobacterium* spp. or dimorphic fungi, or concurrently by both bacterial and fungal infections. Bovine tuberculosis is endemic in many African countries (Ayele *et al.* 2004). In tropical and subtropical countries the disease is of particular importance as a large proportion of the population depends on livestock for their livelihood. Lack of control or eradication of the disease affects trade in animals and animal products (Biet *et al.* 2005). The zoonotic nature of bovine tuberculosis further puts the human populations at risk. Accurate diagnosis is therefore important in design and implementation of control programmes. In the present report dimorphic fungi were identified in tuberculous-like lesions as acid-fast negative cells. *P. brasiliensis* and *B. dermatitidis* are known to cause granulomatous lesions grossly similar to those caused by tuberculosis (Baily *et al.* 1991; Borges-Walmsley *et al.* 2002; Frean, Blumberg & Maureen 1993). In the presumed endemic region, paracoccidioidomycosis has also been found to

TABLE 1: Cause of granulomatous lesions recovered from slaughter cattle in Nairobi, Kenya, as detected according to direct acid-fast staining, culture and DNA typing.

Lesion location	ZN staining			Culture and DNA analysis	
	AFB	AFB and fungi	Fungi	*Mycobacterium* spp.	*Mycobacterium bovis*
Bronchial lymph nodes	8	12	14	20	4
Mediastinal lymph nodes	7	11	11	18	11
Lung	5	0	4	5	3
Liver and portal lymph nodes	1	0	0	1	0
Other lymph nodes and organs	14	6	0	20	1
Total (of 176)	**35**	**29**	**29**	**64**	**19**

AFB, acid-fast bacilli; ZN, Ziehl-Neelsen



b, multiple buds on *P. brasiliensis* cell; m, macrophage; yc, yeast cells; ZN, Ziehl–Neelsen; PAS, periodic acid-Schiff

FIGURE 1: Photomicrographs of dimorphic fungi observed in tuberculous lesions in beef cattle in Kenya, (a) *Paracoccidioides brasiliensis* and (b) *Blastomyces dermatitidis* fungal cells in lymph node lesion smears, (ZN × 1000); (c) *P. brasiliensis* and (d) *B. dermatitidis* cells in BBL cultures, (ZN × 1000). Note the multiple buds, shown at b, on the flattened *P. brasiliensis* cell; (e) *P. brasiliensis* and (f) *B. dermatitidis* yeast cells in lymph node sections, (PAS x 1000).

occur concurrently with tuberculosis in 10% of human cases (Borges-Walmsley *et al.* 2002). In the present report, the fungi occurred concurrently with tuberculosis in 16.4% of the cases and separately in 17% of the cases. Clinically, dimorphic fungi mimic tuberculosis (Borges-Walmsley *et al.* 2002; Parvin *et al.* 2010) and chronic systemic mycoses caused by dimorphic fungi may therefore be confused with tuberculosis during ante-mortem examination of animals. Examination of acid-fast stained smears of post-mortem specimens can, however, provide a preliminary diagnosis of mycobacterial disease (OIE 2009).

Previous reports indicate that beyond the presumed endemic geographical region, paracoccidioidomycosis occurs only in patients who have previously resided in South or Central America (Miyaji & Kamei 2003; Shelbume & Hamil 2002). Detection of *P. brasiliensis* in cattle in the present report was an indication that this fungal infection does, however, occur outside the endemic area. It is noteworthy that climatic conditions similar to those in the presumed endemic regions of South and Central America occur in northern Kenya. Although *B. dermatitidis* infections are most common in dogs and humans in North America, some isolated cases have been reported in Africa, Israel, India and Bangladesh (Carman *et al.* 1989; Parvin *et al.* 2010; Rouhou *et al.* 2008). In Africa, and especially South Africa, infections have previously been reported only in humans.

Conclusion

The present report documents native *P. brasiliensis* infections outside the reported endemic region and *B. dermatitidis* infection in a livestock animal. Dimorphic fungi should therefore be considered as an important differential diagnosis for tuberculosis during meat inspection in the region. In the present investigation, 16.4% of the cases were found to be caused exclusively by the fungi. Bovine tuberculosis is an important zoonosis and its diagnosis at slaughter requires condemnation of parts of or the whole carcass. Dimorphic fungi are not zoonotic and a diagnosis would therefore avert condemnation. However, observation of infection in cattle in the present report may also indicate infection in other animals, including humans, in the region.

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Competing interests

The authors declare that they have no financial or personal relationship(s) that may have inappropriately influenced them in writing this article.

Authors' contributions

J.N.K. (University of Nairobi) designed the project, S.M.G. (University of Nairobi) collected the samples and both authors contributed to sample analyses. J.N.K. wrote the manuscript.

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