Prevalence of bovine tuberculosis in a dairy cattle farm and a research farm in Ghana

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How to cite this article:
Asante-Poku, A., Aning, K.G., Boi-Kikimoto, B. & Yeboah-Manu, D., 2014, 'Prevalence of bovine tuberculosis in a dairy cattle farm and a research farm in Ghana', *Onderstepoort Journal of Veterinary Research* 81(2) Art. #716, 6 pages. http://dx.doi.org/10.4102/ojvr.v81i2.716

Note:
Proceedings of the 2nd One Health Conference in Africa. Jointly organised by the Southern African Centre for Infectious Disease Surveillance and the Tanzania National Institute for Medical Research, held at the Snow Crest Hotel in Arusha, Tanzania from 16th to 19th April 2013: http://www.sacids.org/kms/frontend/index.php?m=119.

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The aim of the study was to estimate the prevalence of bovine tuberculosis (BTB) and to identify the mycobacterial species causing BTB in a dairy farm and research farm. Six hundred and eighty-five cattle were screened for BTB by using the Comparative intradermal tuberculin test (CTT). Positive reactors were slaughtered and carcasses were taken for isolation of mycobacterial species. This was followed by speciation of isolates using both standard conventional and molecular assays. Seventeen of the cattle were positive by CTT, giving a crude BTB prevalence of 2.48% among cattle from the two farms. Six of the 17 samples (35.30%) yielded positive acid-fast bacilli cultures and three of the isolates were identified as *Mycobacterium tuberculosis* complex (MTBC), which were sub-divided into two *Mycobacterium tuberculosis* sensu scrito (Mtb) and one *Mycobacterium africanum*; the remaining three were *Mycobacterium* other than tuberculoisis (MOTT). Spoligotyping further characterised the two Mtb isolates as Ghana (spoligotype Data Base 4 number 53) and Latin American Mediterranean (LAM), whilst spoligotyping and Single Nucleotide Polymorphism (SNP) analysis typed the *M. africanum* as West African 1. Microseq 500 analysis identified two of the MOTT as *Mycobacterium flavescens* and *Mycobacterium Moriokaense* respectively, whilst the remaining one could not be identified. This study observed the prevalence of bovine TB among cattle from two farms in Ghana as 2.48% and confirms the public health importance of *M. africanum* as a pathogen in Ghana.

Introduction

Bovine tuberculosis (BTB) is a major public health problem that has been neglected over the years, especially in Sub Saharan Africa. It is considered to be among the seven highly neglected zoonotic diseases of the world and has a major impact on international trade of animal products (Sahraoui *et al*. 2009). Despite its importance, very little has been done regarding the creation of awareness of the disease. Bovine Tuberculosis has been controlled in the developed countries due to the successful implementation of the test and slaughter (TS) policy of all infected cattle and compensation of affected farmers by governments. Sub Saharan Africa, which is home to more than half of the world's cattle population, has been the hardest hit for various reasons. Firstly, in most African countries cattle are used to show economic status in the society and secondly they serve as the main source of income for many farmers (Otte & Chilonda 2002). Moreover, countries in Africa are yet to fully implement the TS policy; this is mainly because of the lack of financial commitment on the part of governments to compensate farmers with infected animals.

Tuberculosis is caused by a group of gram-positive bacterial species that are genetically close; they are referred to as *Mycobacterium tuberculosis* complex (MTBC). Even though they are genetically similar, they appear to differ in host specificity. Whilst *Mycobacterium tuberculosis* (*M. tuberculosis*) and *Mycobacterium africanum* (*M. africanum*) are said to be the main pathogens of human TB, *Mycobacterium bovis* (*M. bovis*) is thought to be the main causative agent of BTB. However, some findings from recent studies seem to have challenged this notion. Whilst some studies implicated *M. bovis* in humans (Grange & Yates 1996; de Kantor & Ritacco 1994), others showed the involvement of *M. tuberculosis* and *M. africanum* in cattle (Romero *et al*. 2011; Cadmus *et al*. 2010).

Bovine tuberculosis is diagnosed in the field by the detection of a delayed hypersensitive response to Purified Protein Derivative (PPD) antigen in live cattle; this is perceived to be the first point of care diagnostic tool. However, this test lacks specificity due to antigenic similarity among various members of the *Mycobacterium tuberculosis* complex (MTBC) and also with other mycobacterial species. Microscopic detection of acid-fast bacilli in impression smears is simple and performed in the laboratory, but lacks specificity and sensitivity. Microbiological isolation and specie identification are the final proof methods for diagnosis of BTB (Proaño-Pérez *et al*. 2011). Yet, microscopy is still widely used in sub Saharan African because it is very cheap.

Ghana is one of the countries where it is believed that BTB is still an epizootic disease (Veterinary Services Division 2008), yet data about the disease is very scanty. In Ghana, veterinary officers

usually diagnose BTB based on post mortem detection of TB lesions in animal carcasses during inspection of slaughtered animals at various abattoirs. However, macroscopic occurrence of lesions usually indicates the advanced stages of bovine TB (Corner 1994; Shitaye *et al*. 2006). The present study was designed to estimate the prevalence of BTB among cattle from a dairy and research farm using the comparative intradermal tuberculin test, followed by laboratory analysis for isolation and speciation of the infecting bacteria.

Materials and methods

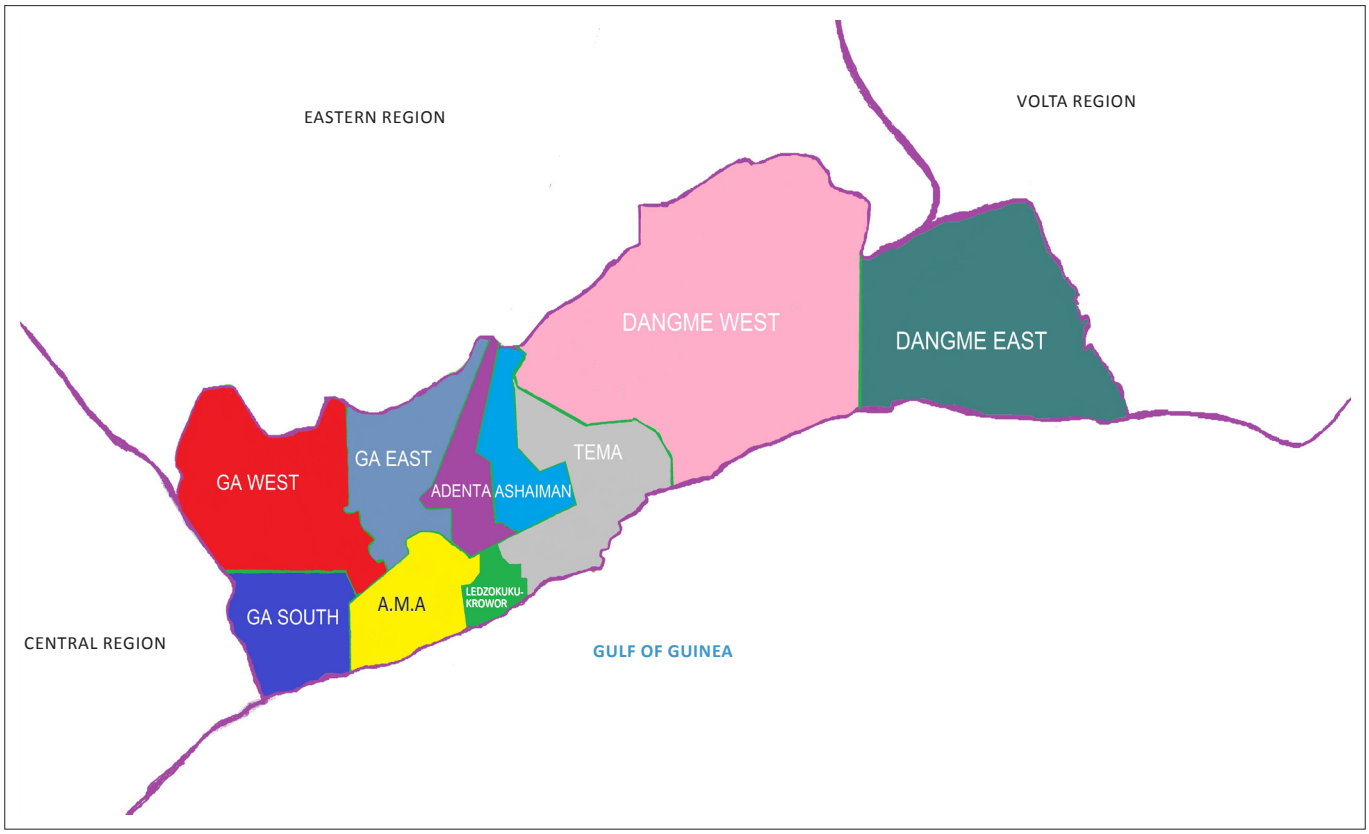
Study area and cattle population

The study was conducted in two government-owned farms (Farm A and Farm B) located 16.09 km apart in the Greater Accra region of Ghana (Figure 1). These two farms were selected based on the availability of demographic data on the cattle. Farm A, is situated on hilly land with easy access to fodder. The farm stocks mainly cattle, a few horses and goats, which are all for breeding purposes. It has a herd size of 400 (28 bulls, two breeding bulls and 370 cows or heifers) made up of Sanga cattle from Burkina Faso and Friesan-Sanga. Grazing is within the confines of the farm and the cattle are kept close to human housing. It is the main source of pasteurised milk and other milk products, such as yoghurt, within the community and surrounding areas. Farm B, which was set up mainly for research and teaching, is situated on low-lying land with rich fodder. The farm stocks various animals including sheep, goats, cattle, ducks, chickens and donkeys. It has a herd size of 285 comprising Sanga and white Fulani breeds. Although its main purpose is research and teaching, the farm undertakes some commercial activities to complement the research activities. Ante-mortem examination (including sex, breed and body condition) of all the cattle was performed by two Veterinary personnel and trained staff.

Comparative Tuberculin skin test

Comparative tuberculin skin test was performed according to the previously outlined protocol (Ó Reilly & Daborn 1995). Briefly: two injection sites were located at the middle side of the neck, one above the other, separated by at least 12 cm. The hair was shaved around the sites to a radius of about 2 cm. The skin folds at both sites were measured with a calliper and the measurements were recorded. An aliquot of 0.1 mL bovine Purified Protein Derative (PPD-B) was subsequently injected intradermally into the lower injection site, and similarly, an avian Purified Protein Derative (PPD-A) was injected at the upper site.

After 72 h, the thickness of the skin folds at both sites was measured and recorded. The tuberculin test results were interpreted based on the World Organisation for Animal Health (OIE) recommended cut-off of greater than 4 mm skin-fold thickness (World organisation for Animal Diagnostic Manual 2004). A bovine reactor was defined as an animal in which the relative increase in skin thickness at the injection site for PPD-B was at least 4 mm greater than the increase



Source: Wikipedia, n.d., *Districts of Ghana*, viewed 09 December 2013, from http://en.wikipedia.org/wiki/Districts_of_Ghana

FIGURE 1: Map of Greater-Accra Region of Ghana indicating the Adenta district where the present study's Farms were located.

in skin thickness at the injection site for PPD-A. A negative reactor was identified when there was no reaction to the bovine antigen or when the difference of the skin thickness at the injection sites did not exceed 2 mm. An inconclusive reaction was recorded if reaction to both PPD-B and PPD-A exceeded 2 mm, but the difference between the bovine and avian reaction was < 4 mm.

Slaughter and sample collection

Detailed veterinary inspection was carried out on all *M. bovis* tuberculin-positive cattle. The lungs, liver, spleen, kidney and mammary gland were palpated carefully and inspected both externally and internally. Mandibular, retropharyngeal, tracheobronchial, mediastinal, hepatic, mesenteric and supramammary lymph nodes were sliced into thin sections and inspected *in situ* for detection of visible lesions. About 2 cm^3 of the suspected tissue specimens were taken from infected organs using a sterile knife, the sample was then kept in Phosphate Buffered Saline (PBS) pH 7.2 in sterile 50 mL centrifuge tubes and transported on ice to the Noguchi Memorial Institute for Medical Research for microbiological analysis.

No ethical clearance was required for this study because it was performed on slaughtered animals and organ confiscation is part of routine monitoring in Ghana.

Cultivation of Mycobacterial species

Sample processing was carried out using aseptic techniques in a biosafety cabinet to avoid cross-contamination between samples. Specimens were processed according to standard methods (Thoen & Steele 1995). In brief: 1 cm^3 of suspected lesion was manually homogenised in a sterile mortar containing 2 mL Phosphate buffered saline. The resulting suspension was transferred into a sterile screw cap tube and decontaminated using the Petroff method. After neutralisation, the homogenate was concentrated by centrifuging for 20 min at 3000 rpm and left to stand for 5 min before opening; this was to allow the generated aerosols to settle. Decontaminated specimens were inoculated on four Lowenstein-Jensen slopes; two were supplemented with 0.4% sodium pyruvate to enhance the isolation of *M. bovis* and *M. africanum*, the remaining two were supplemented with glycerol for isolation of *M. tuberculosis.* The cultures were incubated at 37 °C and were read weekly for macroscopic growth until 12 weeks.

Ziehl-Neelsen microscopy

Smears prepared from decontaminated specimen and bacterial isolates were stained by Ziehl-Neelsen and graded according to the International Union against Tuberculosis and Lung Diseases (IUATLD) guidelines.

Biochemical assay

Isolates that were confirmed as acid-fast bacilli (AFB) were further characterised by susceptibility to p-nitro benzoic acid (PNB), pyrazinamidase activity (PZA), nitrate reduction and niacin production using standard procedures (World Health Organization 1998).

DNA extraction

DNA of confirmed AFB-positive isolates was extracted using a previously described protocol (Kaser *et al*. 2009). About 5 µL loop full of harvested bacteria was heat killed in 300 µL of extraction buffer (50 mM Tris–HCl, 25 mM Ethylenediaminetetraacetic acid (EDTA) and 5% monosodium glutamate). After cooling, 100 µL of a 50 mg/mL lysozyme solution was added and incubated with shaking for two hours at 37 °C. Sixty micro litres of 20 mg/mL Proteinase K solution in a 10 x buffer [100 mM Tris–HCl, 50 mM EDTA, 5% sodium dodecyl sulphate (pH 7.8)] were then added and incubated at 45 °C overnight. The bacterial cell wall was fully disrupted by adding 200 µL of 0.1 mm-diameter zirconia beads (BioSpec Products) to each sample and was vortexed at full speed for four minutes. Beads and undigested tissue fragments were removed by centrifugation at 14 000 rpm for three minutes and the supernatants were transferred to fresh tubes for phenol-chloroform (Fluka) extraction. The DNA contained in the upper phase was precipitated with ethanol and re-suspended in 100 µL of water.

Spoligotyping

Commercially prepared amino-linked spacer oligonucleotides were diluted to the indicated concentration with 0.5 mM $NaHCO_3$. Membranes were then prepared following previously published procedures (Goyal *et al*. 1997). In brief: the membrane (Pall Biosupport, Michigan) was activated by incubation in 16% (weight or volume) 1-ethyl-2-(3-dimethylaminopropyl)carbodiimide (Sigma Chemical, St. Louis, Missouri) for ten minutes at 25 °C. Following a brief wash with deionised water, 150 µL of each diluted oligonucleotide (spacer) was applied in a line by using a miniblotter system (MN45; Immunetics, Cambridge, Massachusetts). After incubation at room temperature for five minutes, excess non-bound oligonucleotide solutions were removed from the membrane by aspiration. The membrane was inactivated by incubation in 100 mM NaOH for nine minutes at room temperature, followed by a brief wash with 2 × SSPE (0.36 M NaCl, 20 mM NaH_2PO_4, 2 mM EDTA, pH 7.7) (Gibco-BRL, Grand Island, NewYork) and a five-minute incubation in 2 × SSPE–0.1% sodium dodecyl sulphate (SDS) at 58 °C. The membrane was incubated in 20 mM EDTA for 20 min at room temperature and stored at 4 °C until use.

Spoligotyping assay was then carried out as previously outlined (Kamerbeek *et al*. 1997).

16SrRNA gene sequencing

Non-tuberculous isolates identified by biochemical analysis were sequenced for specie identification using microseq 500, as previously published. Sequences obtained were used in BLAST searches of databases at NCBI.

TaqMan Single Nucleotide Polymorphim typing assay

TaqMan real time PCR was performed according to standard procedures using probes designed by Stucki *et al*. (2012). Briefly: two microlitres of DNA was added to a 10-µL-reaction mix containing 0.21 uM each reverse (3'GGCCTGTGACCCGTTCAAC 5') and the forward (3'TCCAGCAGGTGACCATCGT 5') primers. In addition, 0.83 uM each of probe A for ancestral allele (VIC-CGTGGACCTCATG-MGBNFQ) and Probe B for mutant allele (6FAM-CGTGGACCTGATGCMGBNFQ) and 5 µL Taqman Universal MasterMix II (Applied Biosystem, Carlsbad, USA) were added to the reaction mix. The reaction was then carried out using Applied Biosystem, Carlsbad, USA thermal cycler under the following conditions: 60 °C for 30 s, 95 °C for 10 min, 95 °C for 15 s and 60 °C for 1 min for 40 cycles, then 60 °C for 30 s. The fluorescence intensity in the VIC and FAM (6-fluorescein amidite) channels was measured at the end of each cycle.

Data analysis

Tuberculin skin test positivity was calculated using OIE interpretations and the crude prevalence rates were calculated by dividing the positives by the total cattle population. The significance of observed difference between the two farms was calculated using the chi square test; a p-value of < 0.05 indicated significance. The obtained spoligotyping pattern was compared with those available in the international spoligotype database. Species, lineages and clades were assigned according to signatures described in the database.

Results

Tuberculin reactivity in cattle

Seventeen of the 685 (2.48%) cattle screened had a positive reaction to Mycobacterium *bovis* PPD. Thirteen of the positive reactors were from Farm B, giving a farm-specific prevalence of 4.56% (13/285), which was higher than that from Farm A ($p = 0.003$), which had four positives, giving a prevalence rate of 1.0% (4/400). The average middle neck size induration before PPD injection was six mm on both farms. The average post injection skin induration recorded for Farm A animals showed a slightly higher skin thickness of 13.25, whilst Farm B recorded 12.77 mm skin thickness (Table 1).

Direct microscopy

Direct microscopy after decontamination had two of the 17 (11.8%) samples yielding positive AFB, and direct microscopy from impression smears had all samples negative by AFB.

Identification and characterisation of *Mycobacterium* species

Six out of the seventeen cultured samples 6/17 (35.29%) yielded growth that was confirmed as acid-fast bacilli. Nine (52.94%) showed no growth after 12 weeks of incubation, whilst two (11.77%) got contaminated with massive fungal growth. Three (50.00%) of the AFB isolates were confirmed by susceptibility to para nitro benzoic acid as belonging to MTBC and the remaining three were classified as mycobacteria other than tuberculosis (MOTT) by showing resistance to Pyrazinamide. Spoligotyping analysis classified two as *M. tuberculosis* sub lineage Latin American Mediterranean (LAM) and Ghana, respectively, the remaining one as *M. africanum* sub lineage West African 1; this was confirmed by SNP analysis.

Comparing our obtained isolate patterns with the SpolD4 database, 1/3 (33.3%) of the MTBC isolates that had previously defined shared spoliogotype number, whilst the remaining two had undefined patterns. Microseq 500 analysis identified two of the MOTT as *Mycobacterium flavescen* and *Mycobacterium Moriokaense* respectively; the remaining one could not be identified (Table 2).

Discussion

This study analysed the prevalence of BTB in two dairy farms. The overall crude prevalence rate among cattle was 2.48%. This figure is far lower than the findings of a previous study that analysed prevalence of BTB in the Dangbe-West district (Figure 1) of the same administrative region, which had a prevalence rate of 13.80% (Bonsu, Laing & Akanmori 2000). The risk of BTB is influenced by a number of factors

TABLE 1: Distribution of tuberculin test positive cattle between the two farms.

Cattle identification number	Skin folds thickness of cows before antigen injection (mm)		Skin folds thickness of cows after antigen injection (mm)	
	M. Avium PPD	*M. Bovis* PPD	*M. Avium* PPD	*M. Bovis* PPD
806-Farm B	7	7	8	12
573-Farm B	6	5	6	15
O16-farm B	6	5	8	10
U349-Farm B	3	4	4	14
570-Farm B	7	8	8	16
G03-Farm B	10	11	12	18
U251-Farm B	4	5	5	12
577-Farm B	7	8	9	15
94-Farm B	5	5	7	11
U231-Farm B	7	7	9	10
Heifer 1-Farm B	6	7	8	11
Heifer 2-Farm B	5	4	7	13
527-Farm B	5	4	6	9
188-Farm A	4	4	5	16
139-Farm A	4	4	6	10
1776-farm A	5	5	6	9
270-Farm A	11	10	12	18

M. Avium PPD, *Mycobacterium Avium* purified protein derivative; *M. Bovis* PPD, *Mycobacterium Bovis* purified protein derivative.

TABLE 2a: Identity of Isolates obtained from the study using both biochemical and molecular assays.

Isolate	Biochemical test			
	Niacin	Nitrate	P-nitrobenzoic acid	Pyrazinamide
Bv01	+	+	–	+
Bv02	–	–	+	+
Bv03	+	+	–	–
Bv04	–	–	+	+
Bv05	–	–	+	+
Bv06	+	–	–	+

TABLE 2b: Identity of Isolates obtained from the study using both biochemical and molecular assays.

Isolate	Spoligotyping profile	Single Nucleotide Polymorphism genotyping	Microseq 500 analysis	Species	Sub lineage
Bv01		ND	ND	*M. tuberculosis*	LAM
Bv02	-	ND	Unknown†	-	-
Bv03		ND	ND	*M. tuberculosis*	Ghana
Bv04	-	ND	*M. flavescens*	-	-
Bv05	-	ND	*M. moriokaense*	-	-
Bv06		L5	ND	*M. africanum*	West African I

M., Mycobacterium.
†, species could not be identified by the methods used.

including: age of animals; breed; herd size; farming practices in terms of stock densities, pasture systems and contact between animals. The main difference in the animals used for this study and another study by Bonsu *et al.* (2000) was the pasture systems used: the present study used animals from a dairy farm, the other study used animals from free grazing farms that were dotted in rural communities. Studies conducted in both developed and African countries have shown that dairy cows experience more stress during gathering for milking, which increases the risk of transmission and susceptibility to BTB (Humblet, Boschiroli & Saegerman 2009). Nevertheless, the practices in rural Ghanaian communities, where animals from different farms congregate around the same stream for watering (Bonsu *et al*. 2000), facilitate contact with both humans and animals from different environs. This increases the risk of transmission and susceptibility to BTB and could account for the observed differences. In addition, the cattle populations in the current study are from farms where veterinary officers frequently screen animals for infections (Dr Kikimoto, pers. comm. n.d.). Thus, our observation confirms the importance of a test and slaughter control strategy for BTB.

The distribution of positive animals was not uniform between the farms studied; Farm B, which is situated on low land, had a higher farm-specific prevalence than Farm A, which is on a hill. Local African breeds have been proven over the years to have a high level of resistance to BTB due to the high endemicity in Africa. Exotic breeds, which are mostly from developed counties where the disease has been controlled, are more susceptible to BTB (Müller *et al*. 2011). This could explain the observed difference, as Farm A stocks mostly local breeds (only two exotic bulls), whereas Farm B has few local breeds (10) and mainly exotic animals. In addition, cattle that graze on hilly terrains tend to spread out, which leads to less contact, whilst those that feed on low and wet lands tend to congregate around the areas with rich fodder, which leads to more contact (Hall *et al*. 1978; Ameni *et al*. 2007). Thus, the difference in the landscape of the two farms might have contributed to this difference.

Six AFB-positive isolates were obtained; of these, three were Mycobacteria Other Than Tuberculosis (MOTT). The isolation of MOTT from carcasses of BTB-positive reactor cattle not only signifies the importance of these bacteria in pastoral communities but also gives an indication of the mycobacterial status of the environment and water sources, as well as their potential to cause infection. These bacteria are commonly found in environmental sources such as water and soil and have a high survival rate. The farm sites used in the study have created pools within the farms from which the animals drink. These pools were found to be contaminated with animal waste, which is well known to enhance the growth of environmental mycobacterial species. Two of the most commonly found MOTT (*M. flavescens* and *M. Moriokaense*) were identified in this study. These two, in addition to several others such as *Mycobacterium terrae, Mycobacterium avium, Mycobacterium chelonae, Mycobacterium gordonae, Mycobacterium fortuitum, Mycobacterium flavescens* and *Mycobacterium smegmati*as, have been implicated as possible causes of BTB (Cleaveland *et al*. 2007).

The identification of *M. tuberculosis* and *M. africanum* as causative agents of BTB is not surprising. Members of MTBC are generally known to be genetically similar, but differ in host specificity. However, in regions like Sub Saharan Africa that have a high burden of human TB coupled with close animal-human contact, there are occasional cases where cross-host specie infections occur (Grange & Yates 1996; De Kantor & Ritacco 1994; Romero *et al.* 2011; Cadmus *et al*. 2010). Whilst most studies give the prevalence of *M. tuberculosis* infection in cattle as ≤1.00%, there are a few exceptions, like Algeria and Sudan with 6.20% and 7.40% prevalence, respectively (Ocepak *et al*. 2005; Boulahbal, Benelmouffok & Brahimi 1978; Sulieman 2002). *Mycobacterium tuberculosis* was isolated from two out of 685 (0.22%) cattle tested with the tuberculin skin test, which put the result in the range of < 1.00%. Present knowledge shows that *M. tuberculosis* does not appear to have an indigenous animal host or reservoir and that the animals which become infected represent most probably accidental hosts acquiring it from humans. In this study, though not directly proven, it can be speculated that animal attendants with active pulmonary TB could be the source of *M. tuberculosis* infection, transmitting it via sputum or urine.

The isolation of *M. africanum* from cattle in Ghana is not surprising. *M. africanum* has been identified as an important TB-causing pathogen unique to West Africa. Country-specific prevalence among pulmonary TB patients can be as high as 50%; it causes about 20% of all TB cases in Ghana (Yeboah-Manu *et al*. 2011; Addo *et al*. 2007). A study conducted in Nigeria that analysed 180 mycobacterial isolates from cattle also identified *M. africanum* in cattle. Equally, Coscolla *et al.* (2013) isolated an MTBC from a wild chimpanzee in Côte d'Ivoire that was shown by comparative genomic and

phylogenomic analyses to belong to a new lineage of MTBC that is closer to *M. africanum*. All of these findings confirm the importance of *M. africanum* as a pathogen of public health importance to West Africa. More resources and efforts need to be put in for understanding both the epidemiology and biology of this pathogen for effective TB control.

Acknowlegements

The authors wish to express their profound gratitude to the Technical staff, Veterinary Division, Ministry of Food and Agriculture and to all farms workers of the two government farms included in this study. Their immense cooperation contributed towards the success of this work. Funding was obtained from the UNICEF/UNDP/World Bank/WHO special program for research and training in Tropical Diseases for DYM.

Competing interests

The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Authors' contributions

D.Y.M. (University of Ghana) conceived and designed the project; A.A.P. (University of Ghana) and B.B.K (University of Ghana) were responsible for performing the experiment; A.A.P., D.Y.M. and K.G.A. (University of Ghana) analysed the data; D.Y.M. and B.B.K. contributed reagents and materials; A.A.P. and D.Y.M. wrote the manuscript.

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