CHARACTERIZATION OF *MYCOBACTERIUM AFRICANUM* SUBTYPE I AMONG COWS IN A DAIRY FARM IN BANGLADESH USING SPOLIGOTYPING

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Abstract. Acid-fast bacilli (AFB) were detected in the autopsy lung tissue homogenate samples of four cows (variety Frisian cross) in a dairy farm in Bangladesh. Histopathological examination of the lung tissue demonstrated prominent granulomas, caseating necrosis and calcification indicative of tuberculosis (TB) infection. Mycobacteria could not be cultured from the tissue homogenate samples by Lowenstein-Jensen based conventional culture method though AFB were evident by Ziehl-Neelsen (ZN) staining of the smears of tissue homogenate and in paraffin embedded tissue slices. Spoligotyping performed on DNA extracts of paraffin embedded lung tissue samples confirmed the AFB as a member of the *M. tuberculosis* complex (MTBC) with a pattern assigned to *M. africanum* subtype I. This characterization by spoligotyping was confirmed by subjecting *M. africanum* subtype I isolates from other parts of the world to an alternative identification method based on DNA polymorphism in the *gyrB* gene (Hain Life Science, GmbH, Nehren, Germany). Since *M. africanum* is believed to be a human pathogen, general infection in cattle may be a public health threat. The presence of these bacteria in the animal reservoir most likely originated from a caretaker.

INTRODUCTION

Tuberculosis (TB) is a chronic respiratory disease in animals as in humans. *Mycobacterium bovis* is the causative agent of TB in cattle and sometimes zoonotic transmission from TB infected animals to humans occurs (Collins and Grange, 1987). In many African countries *M. bovis* infections have been identified as a major zoonotic problem in human beings and animals (WHO, 1994). However, in many developing countries, there is a lack of available information regarding zoonotic TB caused by *M. bovis* (Cosivi *et al*, 1998). This organism has a broad-spectrum host range, such as cows and buffaloes (Niaz and Siddiqi, 1979; Hein and Tomasovic, 1981; Keet *et al*, 1996), and white tailed deer (O'Brien *et al*, 2002), becoming a reservoir of infection for humans and cattle. The potential role of wildlife in the maintenance and spread of *M. bovis* infection in

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domestic livestock is of particular importance in countries where eradication programs have substantially reduced the incidence of bovine tuberculosis, however sporadic outbreaks still occur (Aranaz *et al*, 2004).

Being highly infectious in nature, TB can be horizontally transmitted among healthy animals from a common source through aerosol or ingestion (O'Reilly and Daborn, 1995). Therefore, it is important to identify TB infected animals, which should be removed immediately to stop further transmission of TB among cattle of the same herd. Human beings acquire infection through close contact with TB infected animals as well as by consuming raw or un-pasteurized milk from TB infected cows. Thus, TB infected animals are dangerous for both livestock and human beings.

M. africanum was first identified and reported as a separate sub-species of *M. tuberculosis* complex (MTBC) in 1968 (Castet *et al*, 1968; Wayne *et al,* 1986). It has been isolated from sputum samples of TB patients in Europe (Grosset *et al*, 1971; Grange and Yates, 1989), the United States (Desmond *et al*, 2004), and Africa (Kallenius *et al,* 1999; Bonard *et al,* 2000). *M. africanum* has traditionally been identified by phenotypic criteria, occupying a position between *M. bovis* and *M. tuberculosis* according to biochemical characteristics. Based on biochemical criteria, strains of *M. africanum* have been divided into *M. africanum* subtype I and *M. africanum* subtype II (David *et al*, 1978). *M. africanum* subtype II has been identified as a major cause of human tuberculosis in Uganda (Niemann *et al*, 2002). The organisms in *M. tuberculosis* complex are genetically related and very difficult to differentiate from each other by biochemical characteristics. Recently, discrimination by spoligotyping (Viana-Niero *et al*, 2001), PCR based regions of difference (RD) (Brosch *et al*, 2002; Parsons *et al*, 2002; Sola *et al*, 2003) and *gyrB* polymorphism (Richter *et al*, 2003) have been proposed for adequate and accurate differentiation of the members of *M. tuberculosis* complex.

Bovine TB prevails in many developing countries but is mostly under reported (Cosivi *et al*, 1998). There are scattered reports of bovine TB from India (Lal, 1969) and Pakistan (Niaz and Siddiqi, 1979). Two separate studies have also reported the incidence of bovine TB in cows from two geographical locations (3% from the northwestern part and 3.4% from the northern part) of Bangladesh in the late 1990s (Pharo *et al*, 1981; Samad and Rahman, 1986). However, there are no reports on the isolation of Mycobacteria from cattle in Bangladesh. In the present study both conventional and molecular techniques were applied in an attempt to detect and directly or indirectly identify *M. tuberculosis* complex in the infected lung tissue samples of four cows of a dairy farm in Dhaka, Bangladesh. These cows died of unknown disease.

MATERIALS AND METHODS

Collection of tissue samples

The bovine lung tissue samples were collected from the Central Disease Investigation Laboratory (CDIL) at Dhaka, Bangladesh. This laboratory receives organs of dead animals from different farms of Bangladesh for subsequent diagnosis to investigate the cause of mortality.

Processing of autopsy lung tissue of cows

Each tissue sample was divided into two pieces immediately upon receiving it at the tuberculosis laboratory. After homogenization, one piece was processed for staining and culture, the other piece was processed for histopathological examination.

Preparation of tissue homogenate

Tissue for homogenization was cut into small pieces using a sterile surgical blade in a class II biological safety cabinet and homogenized in an equal volume of sterilized distilled water. The tissue homogenate was kept in-

side the hood for 3-5 minutes to allow the sedimentation of the bigger tissue particles. The supernatant portion of the homogenate was decanted into a 50 ml falcon tube for Ziehl-Neelsen (ZN) staining and culture.

Histopathology

Lung tissue samples were immersed in buffered formalin and embedded in paraffin. Paraffin embedded tissue sections of 3 µm were cut and mounted on glass slides (Superfrost/plus, Menzel-Glaser, Germany), dried overnight at 37ºC and kept at room temperature (Raquib *et al,* 1995). Paraffin sections were deparaffinized and stained with hematoxylin and eosin, then examined by light microscopy.

ZN staining of tissue homogenate and tissue sections

Two loops full (5 mm diameter) of tissue homogenate were smeared on a glass slide. The smear was ZN stained and examined for acid-fast bacilli (AFB). To detect AFB in the paraffin embedded lung tissue, three tissue sections 3 µm thick were stained with modified ZN staining (Prophet *et al,* 1992).

Culture

The homogenized tissue sample was decontaminated following standard procedures (Petroff, 1915). Briefly, 5 ml tissue homogenate was mixed with 10 ml autoclaved (121ºC at 15 pounds/inch^2 for 15 minutes) sodium hydroxide (4%), vortexed vigorously and constantly in a horizontal shaker at room temperature for 15 minutes. The homogenized tissue extract was centrifuged at 3,000*g* at room temperature for 15 minutes. The sediment was washed in 20 ml autoclaved distilled water and centrifuged again at 3,000*g* for 15 minutes at room temperature. The supernatant was discarded and two loop-fulls of sample from the sediment were inoculated on two Löwenstein-Jensen (L-J) slants without glycerol. The inoculated L-J slants were incubated at 37ºC and examined weekly for two months. L-J slants showing no growth of Mycobacteria were incubated further for an additional 4 weeks.

DNA extraction from paraffin embedded lung tissue

Three 14 µm sections were cut from the paraffin embedded lung tissue of 4 cows for extraction of DNA following the procedure described by van der Zanden *et al* (1998). Briefly, from each sample, three paraffin embedded tissue sections (14 µm each) were cut and mixed with 150 µl of Chelex solution in a 1.5 ml eppendorf tube. After thoroughly mixing and incubating at 100ºC for 30 minutes with occasional vortexing, the sample was centrifuged at 13,000*g* for 10 minutes. The interface between the paraffin layer and the Chelex particles was transferred to a fresh eppendorf tube and used for PCR.

Spoligotyping

Amplification of the spacers was performed using DNA extract from paraffin embedded tissue and primers (DRa and DRb) corresponding to the direct repeat (DR) region of the genome of *Mycobacterium tuberculosis* following the procedure described by van der Zanden *et al* (2002, 2003). Briefly, 10 µl of DNA extract from paraffin embedded tissue was mixed with 40 µl of PCR mix containing 5 µl 10 x PCR buffer, 1.5 µl magnesium chloride (final concentration 1.5 mM), 1 µl dNTP mix (final concentration 0.2 mM dNTP each), 5 µl primer DRa, 5 µl primer DRb, 0.25 µl Taq polymerase and 22.25 µl water. The tubes were placed in a DNA engine for amplification for 3 minutes at 96ºC for DNA denaturation, 1 minute at 55ºC for primer annealing, 30 seconds at 72ºC for primer extension and finally 5 minutes for final primer extension. The cycle was repeated 39 times. PCR products were kept at -20ºC until use.

The PCR product was hybridized with the Biodyne C membrane (Pall Biosupport, Portsmouth, UK) having nucleotides of a known sequence of *Mycobacterium tuberculosis* attached to it by reverse line blotting. Then, the hybridized membrane was exposed to X-ray

film for the detection of the hybridization signal. The x-ray film (Hyperfilm™ ECL, Amersham Bioscience UK) was manually read for a complete pattern of spacers between the DRs harbored by a particular strain. Finally the spoligo patterns were matched with the Spoligo Database located at the National Mycobacteria Reference Laboratory, National Institute of Public Health and the Environment (RIVM), Bilthoven, the Netherlands.

Genotyping

The Genotype MTBC assay (Hain lifescience GmbH, Nehren, Germany) was performed for the indirect identification of Mycobacteria, which infected the four cows. Three mycobacterial isolates with a similar spoligo pattern but isolated from different sources were retrieved from the National Mycobacteria Reference Laboratory, Bilthoven, the Netherlands. One of these strains was earlier determined as *M. tuberculosis* and was isolated from a human being from the Netherlands. The remaining two cultures were isolated from Oryx (strain # 24 from the Netherlands and # 69 from Saudi Arabia) (Brosch *et al*, 2002). DNA from these strains were amplified for the *gyrB* gene and the PCR product was hybridized on the paper strip following the procedure of the manufacturer (Hain Life Science, GmbH, Nehren, Germany).

RESULTS

Characteristic AFB cells were detected in all the ZN stained smears of tissue homogenate from the four cows.

Lung tissue samples from all the cattle showed the presence of granulomas (different sizes), caseating necrosis, fibrosis and calcification, indicating TB infection (figures not shown).

By 8 weeks of incubation, no characteristic mycobacterial colonies were observed on the L-J slant. Incubation for an additional four weeks did not result in growth of *Mycobacterium* in either sample.

The spoligo patterns for the three cows (Z-1, Z-2 and Z-3) were identical but the spoligo pattern for the fourth cow (Z-4) was slightly different. The spoligo pattern of the latter strain was considered truncated compared to the other three cows (Z-1, Z-2 and Z-3) but belonged to the same cluster. The spacers 8, 9 and 39 were absent and resembled the strains with the spoligotyping signature of *M. africanum* subtype I (Viana-Niero *et al*, 2001). This indicates that a single strain of *M. africanum* subtype I infected these four cows. The spoligo patterns in these cows were compared to the spoligo database at the National Mycobacteria Reference Laboratory (National Institute of Public Health and the Environment RIVM), Bilthoven, the Netherlands.

Ten identical spoligo patterns were found in the data base. Information was only available for three strains. Two *M. bovis* strains, 24 and 69, were used in earlier studies (Kremer

Fig 1–Spoligo patterns of *M. africanum* subtype I detected in paraffin embedded lung tissue of cattle. The solid boxes indicate the presence of a spacer as detected by hybridization while the empty boxes indicate the absence of spacers as there was no hybridization.

et al, 1999; Brosch *et al*, 2002); one *M. tuberculosis* strain was of human origin. The DNA from these three strains was tested with the GenoType MTBC kit resulting in bands 1-7 and 10, and was typed as *M. africanum* subtype I.

DISCUSSION

Classical TB in human beings is usually caused by *M. tuberculosis* and bovine TB is caused by *M. bovis* (Dankner *et al*, 1993). However, there are reports of the isolation of *M. tuberculosis* infection in elephants in North America (Mikota *et al*, 2000, 2001) and in 8 different species of animals from zoological porks in South Africa (Michael *et al*, 2003). Occasional isolation of *M. tuberculosis* infection in cattle has been reported in several countries, such as 13 of 14,439 cattle (0.09%) in England over a period of 16 years (Lesslie and Birn, 1970), in USA in 1 of 7,007 cows over a 5 year period (Thoen *et al*, 1981), in 2 of 1,135 cattle (0.18%) from Germany over a 10 year period (Schliesser *et al*, 1976), and in 2 studies from Slovakia, 1 in 553 cows (0.18%) over a one year period (Popluhar *et al*, 1970) and in 2 of 6,684 cows (0.03%) in 1972 (Popluhar *et al*, 1974). There is only one published report of *M. africanum* infection in cattle from Germany (Erler *et al*, 2003), probably because of the difficulty of differentiating *M. africanum* from the other members of *M. tuberculosis* complex.

In this study, the Mycobacteria from these four cow could not be cultured on L-J medium possibly because the organism lost viability due to improper storage or transportation from the dairy farm to the Central Disease Investigation Laboratory (CDIL) in Dhaka, Bangladesh, as well as delay in processing. Therefore, the paraffin embedded tissue samples were used for detection and differentiation of *M. tuberculosis* complex by spoligotyping. The presence of the same spoligo pattern for *M. africanum* subtype I in these cows indicates horizontal transmission among these animals. The spoligo pattern was compared with the spoligo database located at the National Mycobacteria Reference Laboratory, the Netherlands and 10 identical patterns were found. From three of the ten strains, information was available regarding the origin of the isolate. One *M. tuberculosis* strain was of human origin and the other two were *M. bovis* strains isolated from oryx. The three strains were identified as *M. africanum* subtype I in the Genotype MTBC assay.

It is difficult to differentiate members of the *M. tuberculosis* complex even with molecular techniques. The two oryx isolates (24 and 69) were investigated in earlier studies and identified as *M. bovis* by biochemical identification (Kremer *et al*, 1999) and were also tested by PCR-based genomic deletion analysis (Brosch *et al,* 2002). The result from this study was RD1, RD2, RD4, RD5a, RD6, RD12, RD13 and RD14 were present, and RD3, RD5b, RD7, RD8, RD9, RD10 and RD11 were lacking. This profile resembles *M. microti* isolates. The *oxyR* and *pncA* sequences showed sequence polymorphisms characteristic for *M. tuberculosis* strains. A single nucleotide polymorphism (SNP) in the *Mycobacterium tuberculosis* deletion 1 (TbD1) region at codon 551 (AAC-AAG) of the *mmpL6* gene was found, and typical for *M. bovis* and *M. microti*. RvD2, RvD3 and RvD4 were lacking, in contrast to *M. bovis*.

To overcome these difficulties in identification of the different species of *M. tuberculosis* complex, an algorithm to identify individual subspecies was proposed by Huard *et al* (2003). *M. africanum* lacking RD7 and containing RD1, RD4 and RD12, lacking RD7, RD8, RD9 and RD10 was proposed by Mostowy *et al* (2004) and lacking RD9, containing RD12 and a specific combination of *gyrB* gene polymorphisms was proposed by Richter *et al* (2003) and Niemann *et al* (2004). The biochemical identification, spoligo pattern, the 17-20 IS*6110* elements, SNP in TbD1

codon 551 (AAC-AAG), the absence RvD regions, the absence and presence of RD regions and *gyrB* gene polymorphisms shows the difficulty in discriminating *M. africanum* from *M. bovis* and *M. tuberculosis*.

M. tuberculosis complex infections in cattle may be contacted most frequently via sputa and less often via urine and feces of TB infected human beings (Thoen and Steele, 1995; Grange, 1996). Moreover, isolation of *M. tuberculosis* in river water infested with raw wastewater indicates that cattle may acquire infection with this pathogen while drinking such water contaminated with *M. tuberculosis* (Laktis *et al*, 1970). It is unclear how the cows in this study acquired infection with *M. africanum* subtype I. It is essential to test sputum samples of the caretakers of these cows to pinpoint the mode of vertical transmission of *M. africanum* from the TB infected individuals to these cows. Moreover, water supply in the farms should also be tested for the presence of *M. africanum.*

Cattle are a possible reservoir of *M. africanum,* since in certain parts of Africa where tuberculosis in humans is common. *M. africanum* is the most important cause of tuberculosis in humans (Richter *et al*, 2003). In Bangladesh the real scenario is unknown. Therefore, the cattle population should be examined to assess the prevalence of *M. africanum*. These infected cattle may be a possible source of infection for both humans and cows. Further study should be performed to evaluate the prevalence of *M. africanum* in cattle.

M. africanum subtype I was detected in four cows of a dairy farm in Bangladesh. Further studies are need to assess the magnitude of this problem and understand the mode of transmission of *M. africanum* subtype I from cows to humans and vice versa. This could help the government of Bangladesh to take appropriate measures to curtail transmission of TB.

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