Journal of Infectious Diseases and Therapy
ISSN: 2332-0877

Malama et al., J Infect Dis Ther 2013, 1:3
DOI: 10.4172/2332-0877.1000114

Research Article Open Access

Isolation of *Mycobacterium Bovis* from Human Sputum in Zambia: Public Health and Diagnostic Significance

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Abstract

The WHO reported in 1998 that 3.1% of tuberculosis cases in humans worldwide are attributable to *M. bovis* and that in 0.4-10% of sputum isolates from patients in African countries, *M. bovis* is isolated. Sputum samples were collected from a total of 917 smear-positive TB patients enrolled in a national drug resistance survey in the nine provinces of Zambia and another 100 patients enrolled in a separate TB survey conducted in the pastoral area of Namwala district of Southern province of Zambia between 2008 and 2011. Based on Spoligotyping, eight of the isolates from both surveys were confirmed as *M. bovis* belonging to the SB 0120 Spoligotype. The two surveys provided an opportunity to document isolation of *M. bovis* from sputum samples from patients diagnosed with TB from both urban and pastoral areas of Zambia. This study therefore, highlights the public health significance of *M. bovis* in Zambia and the importance of screening for *M. bovis* as part of routine diagnosis procedures. Hence, a targeted treatment for those human patients suffering from zoonotic tuberculosis is recommended to address important differences in pathology and treatment response between different mycobateria.

Keywords: *Mycobacterium bovis*; Spoligotyping; Public health; Zambia

Introduction

Zambia, with a human population of 13 million people, is ranked among the world's top ten countries with a high burden of human tuberculosis (TB) caused by *Mycobacterium tuberculosis*. The World Health Organization (WHO) estimates the incidence of human TB in Zambia to be 707/100 000 [1,2]. Human TB, although an ancient disease, has re-emerged with devastating impact upon global public health and currently one of the most widespread infectious diseases and a leading cause of death due to a single infectious agent among human adults in the world [3]. TB is caused by members of the *Mycobacterium tuberculosis* complex (MTC), which include *Mycobacterium tuberculosis, Mycobacterium bovis, Mycobacterium africanum, Mycobacterium caprae, Mycobacterium microti, Mycobacterium pinnipedii and Mycobacterium canettii [3]. M. bovis* shows a high degree of virulence for both humans and animals [4]. The WHO reported in 1998 that 3.1% of tuberculosis cases in humans worldwide are attributable to *M. bovis*, however, depending on the country 0.4-10% of sputum isolates from patients in African countries were identified as *M. bovis* [5]. This is despite the fact that *M. bovis* is associated with extra-pulmonary disease in humans [5]. Data on the prevalence of human disease due to *M. bovis* in Zambia and other developing countries is limited, owing to the technical problems posed by isolation and identification of this species during routine diagnosis [6-8]. The other additional factor that these developing countries are now facing is the HIV/AIDS pandemic, which favours human-to-human transmission of *M. bovis* leading rapidly to disease [4]. In a study conducted in Uganda by Oloya et al. [9], a prevalence of 7% *M. bovis* infections was reported in humans suffering from cervical lymphadenitis in a pastoral community in the Karamoja region [9]. Another study conducted in Tanzania on human patients with cases of lymphadenitis by Kazwala et al. [7] found 16% of cases due to *M. bovis* [7]. In Zaire (now Democratic republic of Congo), *M. bovis* was isolated from gastric secretions in two of five patients with pulmonary TB [10]. Although *M. bovis* is associated with extra – pulmonary TB, it has however, been isolated from sputum samples of patients with pulmonary TB from Nigeria [11]. In addition, TB due to *M. bovis* infection could be an economic and public health threat in developing countries [12].

We report the isolation, diagnostic significance and possible public health implication of *M. bovis* isolation from human TB patients in Zambia.

Materials and Methods

Study area and sample collection

Sputum samples were collected from a total of 917 smear-positive TB patients enrolled in a national drug resistance survey in the nine provinces of Zambia and another 100 patients were enrolled in a separate TB survey conducted in the pastoral area of Namwala district of Southern province of Zambia between 2008 and 2011.Trained and well qualified staffs were involved in patient recruitment. Only consecutive newly diagnosed patients were recruited. Patients were requested to submit a spot and an early morning sputum sample in a 50ml screw-cap centrifuge tube (Falcon, Becton Dickinson, USA) before they started TB treatment.

Sputum samples that were not transported on the same day to Chest Diseases Laboratory (CDL) in Lusaka were stored in the fridge at 4°C until the day of departure. The samples were then wrapped in absorbent material and packed in internationally recommended containers for transporting infectious substances. Each sample was accompanied by a transportation form and a clinical questionnaire.

Sample processing

Upon receipt of specimen at the Chest Diseases Laboratory (CDL),

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Received August 29, 2013; **Accepted** September 24, 2013; **Published** October 01, 2013

Citation: Malama S, Muma JB, Olea- Popelka F, Mbulo G (2013) Isolation of *Mycobacterium Bovis* from Human Sputum in Zambia: Public Health and Diagnostic Significance. J Infect Dis Ther 1: 114. doi:10.4172/2332-0877.1000114

the name on the transportation form and that on the specimen container were verified to make sure that they belonged to the same person. The two sputum samples were then logged into the register book and given a laboratory number.

Decontamination

The sample was liquefied and decontaminated for 15 minutes using the NaOH NALC method giving a 1.5% final concentration of sodium concentration (BBL™ MycoPrep™ KIT, Becton Dickinson). Samples were then suspended in sterile phosphate buffer (PBS, pH 6.8) after decontamination and centrifuged at 3000Xg for 15 minutes. After centrifugation, the tubes were left for 5 minutes to allow the aerosols to settle before opening and decanting the supernatant in a biosafety cabinet. The sample was then re-suspended in 2-3 ml of phosphate buffer. Smears were also made from the concentrated sediments to confirm that the sample received was truly positive. Results were graded using the International Union against Tuberculosis and Lung Diseases (IUATLD) recommendations. Only one sample was opened at any given time to avoid cross contamination and the phosphate buffer was also aliquoted into small volumes.

Inoculation

The processed specimens from the national survey were inoculated on both MGIT broth (BBL™ MGIT™ 960 [BD]) and solid media Lowenstein -Jensen (L-J) which was locally made containing Glycerol, while those from the Namwala survey were inoculated on solid media LJ containing Glycerol and pyruvate and incubated at 37°C. Left over of the decontaminated sputum was heat killed at 96°C for 20 min and stored in a 2 ml screw cap tube at -20°C. The L-J slopes were incubated at 37°C in an incubator and cultures were checked daily for the first 3 days and after that once a week for 8 weeks. If no growth was observed after 8 weeks, the culture was recorded as 'No growth' and the tube was discarded. The time to detection was calculated as the day from when the sample was inoculated to the day when colonies were visible. Samples were also inoculated onto MGIT according to recommended standard operating procedures [13]. The results were entered both into the computer and on the worksheet.

Identification tests

Cultures from both surveys were identified as *M. tuberculosis* complex (MTC) using the Capilia TB test (Capilia TB, TAUNS Laboratories INC, Shizuoka, Japan) according to the manufacturer instructions. The Capilia test is a laminar flow assay that detects the MPB64 antigen which is secreted during growth into the media and is specific for *M. tuberculosis* complex.

DNA extraction and spoligotyping analysis

The extraction of DNA was done using a quick method. The quick method was performed as follows: 500 µl of MGIT broth from the bottom of a positive culture was aliquoted into a 2ml micro centrifuge tube. The cells were killed by heating at 96°C for 20 min. If the DNA was from an L-J culture, then a loopful of cells was mixed with 250 µl of 1X TE buffer. The cells were equally killed by heating at 96°C for 20 min. The DNA was then stored at -20°C until needed. Spoligotyping was done following the protocol developed by Kamerbeek et al. [14].

#	Occupation	Sex*	Age (years)	District	Spoligotype
1	Cattle farmer	M	44	Lusaka	SB 0120
2	Cattle farmer	M	65	Lusaka	SB 0120
3	Cattle farmer	M	20	Lundazi	SB 0120
4	Cattle farmer	M	32	Lundazi	SB 0120
5	Cattle farmer	F	31	Lundazi	SB 0120
6	Cattle farmer	F	48	Lundazi	SB 0120
7	Cattle farmer	M	45	Namwala	SB 0120
8	Cattle farmer	M	39	Namwala	SB 0120

* F= Female; M= Male

Table 1: Occupation, Age, sex, district and spoligotype of eight TB patients from whom Mycobacterium bovis was isolated from sputum in Zambia.

Ethical clearance

The study was approved by University of Zambia, Lusaka, Zambia ethical committee (ref.005-07-07#) and the ERES converge IRB ethical review Board, Lusaka, Zambia (Ref: 2012-Mar-001)

Data analysis

Spoligotyping data in binary form were entered and validated in Excel©2007. These were then copied into the spolDB4.0 database (http://www.pasteurguadeloupe.fr:8081/SITVITDemo) to establish the lineage, sub-lineage and spoligotype international types (SIT) designations. To take into account the effect of imperfect tests, proportions were estimated using the @Risk software with Monte Carlo simulation using 1,000 iterations with the following model settings, given the number of positive candidates (r) out of the total number of persons sampled (n): RiskBeta (α1,α2), where α1=r+1 and α2=n−r+1.

Results

Eight isolates from the two surveys were confirmed either as *M. bovis* by spoligotyping. Six of the isolates were from the national survey (n=917) and 2 from survey conducted in Namwala rural district (n=100) in Southern province. Of the six from the national survey, 2 came from Lusaka urban district (n=260) and the other four strains were isolated from patients from Lundazi rural district (n=18), a district in Eastern province of Zambia. These isolates were recovered from adults TB patients (age range: 20 to 65). Based on this estimate, the likelihood of isolating *M. bovis* from human sputum samples from both surveys (n=1017) was estimated at 0.86% (95% CI: 0.37-1.45%) using @Risk. *Mycobacteria bovis* isolation frequency varied by district, with Lundazi (r=4; n=18) showing the highest prevalence of 24.4% (95% CI: 8.5-45.5%) while that for Namwala (r=2; n=100) and Lusaka (r=2; n=260) districts were 3.0% (95%CI: 0.7-6.9) and 1.15% (95%CI: 0.21-2.9), respectively. Majority (6/8) of the persons involved were male and only two were female, indicating a high probability (69.9 %; 95%CI: 38.9-91.6%) of isolating *M. bovis* from males subjects compared to their female counterparts. All the isolates had the same spoligotype, the SB 0120 (SIT 482) (Table 1).

Discussion

The findings in this study are part of a wider national drug sensitivity survey, carried out to ascertain the sensitivity pattern of *M. tuberculosis* against the commonly used anti-TB drugs in Zambia and also the TB survey conducted in Namwala district to isolate and differentiate the different species of MTC. It was however, interesting to observe *M. bovis* among the isolates hence this report. *M. bovis* is mainly responsible for bovine TB in cattle and in many other domestic and wild animals. It's also a known cause of zoonotic tuberculosis in humans, which is indistinguishable with regard to pathogenesis, lesions and clinical findings to that caused by *M. tuberculosis* [4,6]. *M. bovis* shows a high degree of virulence for both humans and animals [4]. In the two surveys, it was observed that approximately in 1.0% of the sputum samples from patients with pulmonary TB, *M. bovis* was isolated. This figure falls within what has been estimated by WHO for

developing countries [5]. However, we think this is an underestimation of the true burden of *M. bovis* infection among humans in Zambia since the study samples were obtained from sputum samples only; and also due to the fact that the National TB drug resistance survey contained a large proportion of patients living in urban areas. Results from Lundazi district, which is a pastoral area, show that *M. bovis* is a real public health problem in this cattle producing community. However, the proportion of TB due to *M. bovis* in human could have been much higher had we also sampled lymph node biopsies. On this regard, a higher prevalence of *M. bovis* was found among TB patients in studies conducted in Uganda and Tanzania in which lymph node biopsies were sampled instead of sputum [7,9]. Nevertheless, the isolation of *M. bovis* from sputum indicates the importance of this organism on the overall dynamics of TB and represent a great threat to public health as it provide an additional opportunity for airborne human to human transmission as opposed to its most common transmission route via unpasteurized milk and milk products [4,6]. The other additional factor increasing the risk of *M. bovis* infection among humans in Zambia is that currently the country is suffering from HIV/AIDS pandemic, which favours human-to-human transmission of *M. bovis*. The prevalence of HIV in Zambia in adults stands at 17% and TB notifications have increased 5 fold in the last 20 years, mainly due to the HIV pandemic [15].

Majority (80%) of the positive samples for *M. bovis* were from male subjects. This is probably due to the cultural settings, where in most cases, male members of the household are responsible for herding cattle and thus help themselves with raw milk while out in the field. *M. bovis* infection in humans can be acquired both orally and via inhalation of aerosolized particles containing *M. bovis* from infected animals or from person to person contact [16]. However, the most common route of infection of *M. bovis* is through the oral route by consumption of contaminated milk or other dairy products. It should here be mentioned that all the isolates were obtained from cattle farmers. The two isolates from Lusaka urban were from males who were also cattle farmers but travelled to Lusaka city to visit. The isolation of *M. bovis* from cattle farmers could suggest transmission from cattle because people in pastoral areas of Zambia are known to consume unpasteurized milk and share micro environments such shelter and water bodies with cattle. Therefore, transmission could occur through consumption of contaminated water, raw milk and aerosol. Thus, milk pasteurization plays a crucial role in preventing humans from getting infected by consumption of milk contaminated with *M. bovis* [17]. Pasteurization of milk, however, is not generally available or accessible in many rural areas of Zambia. Thus, the results of our study emphasize the importance of isolating and differentiating *Mycobacterium tuberculosis* complex species among TB patients. This is critical in determining the impact of transmission of this re-emerging zoonotic disease.

Zambia is a country with historically high incidence of TB [1]. Patients with acid fast bacilli (AFB)–positive sputum or those with chest radiographic findings suggestive of active TB who do not respond to general antimicrobial drugs are generally presumed to have pulmonary TB caused by M. tuberculosis. These patients are empirically treated for 6 months with a combination of drugs recommended by WHO. However, this inconclusive diagnosis of pulmonary TB, without identifying the causal agent, is leading to inappropriate treatment for other type of mycobacteria, such as *M. bovis*, which is known to be resistant to pyrazinamide. In Zambia, there are communities living in regions that had a long history of a high prevalence of bovine TB. In addition, the wildlife-livestock interface has been suggested as an important risk factor for transmission of *M. bovis* between Kafue lechwe antelopes (*Kobus leche Kafuensis*) and bovines [18]. An eight-fold increase in the prevalence of bovine TB in cattle has been reported inside the livestock-wildlife interface area compared to bovines outside this high-risk area [19]. Lundazi district is located in one of the provinces with the highest cattle populations in Zambia and consumption of raw milk is not uncommon. Similarly, Namwala district is located in the Kafue Basin area, which is one of the few lacustrine wetlands supporting close to 300,000 cattle with a variety of wildlife species whilst the Kafue lechwe antelope form a mega fauna with an estimated population of 44,000 [20]. In this area, anecdotal data suggest that humans are at high risk of zoonotic TB caused by *M. bovis*.

Based on spoligotyping, all the 8 isolates belong to the SB 0120 (SIT 432) Spoligotype. This spoligotype pattern is much conserved to Zambia as it has been observed in cattle and Kafue lechwe antelopes of the Kafue basin in Southern province [21,22]. This therefore, suggests the zoonotic potential of these strains, especially for those isolates obtained from Namwala district. This spoligotype has also been observed in humans from Italy and Germany [23,24]. They have also been isolated from cattle in Algeria, France and South Africa [25-27]. The two surveys have also shown that Spoligotyping is a good differentiation tool of *Mycobacterium tuberculosis* complex and can well be used in Zambia.

Routine TB diagnosis in Zambia is mainly through microscopy and few samples are sent to the reference laboratory for culture [28]. In Zambia, like in most developing countries, where there is considerable evidence to support the conclusion that *M. bovis* is likely to be significantly underestimated as causal agents of extra-pulmonary and pulmonary TB diagnoses, the actual burden of this infection, to date, is only poorly understood.

Current methods used to routinely diagnose TB in humans in Zambia (i.e., culture and acid fast bacillus smear examination); are inadequate for identifying and differentiating the mycobacteria causing disease. It is crucial to correctly identify those patients infected with *M. bovis* because it has been established that this agent is resistant to pyrazinamide, one of the drugs recommended by the WHO for treatment of human TB cases in the initial phase [29]. In addition, *M. bovis* infection in humans is associated with extra-pulmonary TB [30], which represents a more complex set of challenges in terms of pathology; hence, effective antimicrobial treatment compared to patients with primarily pulmonary TB is of the utmost importance in order to achieve the best prognosis for the patient. Furthermore, therapies in patients infected with *M. bovis* are longer than patients infected with *M. tuberculosis* [16] and patients infected with *M. bovis* have a higher mortality rate during treatment compared to *M. tuberculosis* infected patients [31]. Finally, the WHO recently classified human TB caused by *M. bovis* as a "neglected zoonosis" in developing areas of the world [5]. In addition, there is an alarming general lack of awareness (risk perception) by the scientific health care professionals and lay communities about the impact of this public health threat. This issue has been presented in a scientific review entitled "Why has zoonotic tuberculosis not received much attention?" in which the authors conclude that "as the treatment of *M. tuberculosis* disease and *M. bovis* disease is "similar", some clinicians think it is not important to differentiate between the organisms" [32]. However, the available literature as result of few studies, suggest otherwise. Thus, the isolation of *M. bovis* in the two surveys supports the need for the adoption of public health measures such as the pasteurization of milk and control of bovine TB in animals [33,34]. Management of TB patients in areas where *M. bovis* is a potential etiological agent in humans should therefore, not be neglected.

Conclusion

This paper has documented the isolation of *M. bovis* from human sputum from pastoral areas of Zambia. It therefore, highlights the zoonotic, public health and diagnostic importance of *M. bovis*. Therefore, the National Tuberculosis Program (NTP) in Zambia should improve on the diagnoses of *M. bovis* as well as encourage collaboration between Public health and veterinary public health. A 'One health' approach can have positive impact towards the design and implementation of appropriate prevention, surveillance, and diagnosis of mycobacterial infection, especially in areas in which different mycobacteria species are known to be infecting human, livestock and wildlife sharing the same environment. A targeted treatment for those human patients suffering from zoonotic tuberculosis is also recommended to address important differences in pathology and treatment response between different mycobateria.

Acknowledgement

The authors are grateful to the Clinicians, field and laboratory technical staff involved in the surveys. Sydney Malama received financial support from the Norwegian Education and loan fund (Quota scholarship) Where as Grace Mbulo, who co-ordinated the national drug resistance survey under Zambart project, was funded by global fund through the Ministry of Health. The authors further wish to thank Dr. Berit Djonne, Dr. Tone Bjordal Johansen and the technical staff at the Norwegian Veterinary Institute, Bacteriology laboratory for their material and technical assistance.

Competing interests

The authors declare that they have no competing interests.

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