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Molecular detection of bovine tuberculosis in El Rank area, North Upper Nile State, Sudan

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Abstract

Bovine tuberculosis (TB) is an infectious disease of cattle and one of the biggest challenges in the cattle industry. It is caused mainly by the bacterium *Mycobacterium bovis (M. bovis)*, which can also infect and cause TB in other mammals. In this study, we aimed to investigate the presence of the bovine tuberculosis in the El Rank area, north upper Nile State, Sudan. The semi-structured questionnaire, conventional methods (Ziehl's-Neelsen staining, culture in Lowenstein-Jensen medium) and polymerase chain reaction (PCR) were used to detect the bovine tuberculosis in the El Rank area. The results of the semi-structured questionnaire indicated that the Kenana cattle were at a high risk of infection (50%), especially the females around the age of 3 years. For the conventional methods and PCR, a total of forty milk samples and thirty aspirated lymph node samples were investigated for the presence of the bacterium. Only 1/30 (3.3%) of lymph node samples showed the characteristic appearance of the acid fast bacilli of a serpentine cord of *M. bovis*. This sample also showed a growth in the Lowenstein-Jensen medium containing pyruvate. The PCR results to amplify the *rpoB* gene (235bp) of *M. bovis* showed a high sensitivity to detect the bacterium. It confirmed the presence of *M. bovis* in 26 (65%) of milk samples and 12 (40%) of lymph node samples. Our results confirmed the presence of *M. bovis* in El Rank area, north upper Nile State, Sudan and it also indicated the sensitivity of the PCR as a rapid tool to detect the infection of *M. bovis.*

Key words: Bovine tuberculosis, Detection, Ziehl's-Neelsen stain, Lowenstein-Jensen medium, PCR.

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Introduction

Bovine tuberculosis (TB) is a chronic disease of animals caused by bacteria called *Mycobacterium bovis*, (*M. bovis*) which is closely related to the bacterium that causes human and avian tuberculosis. Although the cattle are considered to be the true hosts of the *M. bovis*, the disease has been reported in many other domesticated and non-domesticated animals. Today, the bovine TB remains an important disease of cattle, wild animals, and is a significant zoonosis. The disease is prevalent in Africa, parts of Asia and America [1]. Animal TB causes loss of animals and its production and it also considered as a risk to human health [1]. In some African countries, the co-infection of human immunodeficiency virus (HIV) with bovine TB was also reported [2]. The disease is contagious; it can spread by contact with the infected animals. The usual route of infection is by inhaling infected droplets which are expelled from the lungs by coughing. Calves and humans can also become infected by ingesting raw milk from infected cows [3].

The usual clinical signs of bovine TB include: weakness, loss of appetite, weight-loss, fluctuating fever, intermittent hacking cough, diarrhea, large prominent lymph nodes. However, a sub-clinical form of the disease was also observed. The bovine TB could be diagnosed by conventional methods like smear technique and culture method, especially Lowenstein-Jensen medium [4]. In addition, the molecular biology techniques such as PCR were also used to detect the bacteria. Novel genes were targeted for PCR diagnosis of Mycobacterium [5] such as *hsp65* (165 bp), *dnaJ* (365 bp), and *rpoB* (235 bp) [6]. The standard measure applied to control the TB infection is the policy of test and slaughter. Pasteurization of milk of has prevented the spread of disease in humans. Treatment of the infected animals is rarely attempted because of the high cost. Vaccination is widely used in animals and a number of new candidate vaccines are currently being tested [7-10].

No previous data are available about the epidemiology of the disease in the El Rank area, north upper Nile State, Sudan as well as no systemic diagnosis of the disease was followed. Therefore, the accurate representation of the disease in the El Rank area has to be addressed. The results of the study could provide a preliminary data about the disease prevalence which might help to design the control measures in the future.

Materials and methods

Survey based on semi-structured questionnaire

In addition to the information obtained from veterinary health authority, local butchers/meat inspectors and public health authority in the El Rank area, a semi-structured questionnaire was also used to collect preliminary information about the disease. Herein, the data of age, sex and breed were recorded (supplementary materials).

Collection of the sample

The lymph nodes (inflamed or caseoustic) were collected randomly from the slaughtered cattle and carefully aspirated with sterile 10 ml syringes. The samples were added to sterile screw-top plastic falcon tube. The milk samples (5 ml) were collected from El Rank's market using systematic, random methods. In both samples, an equal volume of (0.6% cetyl pyridinium bromide and 2% NaCl) was added to decontaminate the samples [11].

Identification of the bacteria by smearing methods

A direct smear was prepared in the field from each sample of lymph node and milk, dried and fixed with heat by passing it through benzene flame 2-3 times. Later, the samples were stained with Ziehl's-Neelsen stain according to the previous methods [12]. The slides were observed under the microscope using 100χ oil immersion lens to detect the red or pink color against the blue counterstained (the characteristic feature of the acid fast bacilli) and the results were reported, according to the International Union Against Tuberculosis and Lung Diseases (IUATLD) as follows: No acid fast bacilli (AFB) in at least 100 fields (0/negative), 1-9 AFB in 100 fields (actual AFB counts), 10-99 AFB in 100 fields (+) and 1-10 AFB per field in at least 50 fields (++).

Isolation of the bacteria by culturing on Lowenstein-Jensen medium

The samples were washed three times with 35-40 ml of PBS and the pellet was suspended in 0.1 ml of PBS. About 0.5 ml of specimen was inoculated into Lowenstein-Jensen medium and cultured at 37°C. The culture was examined daily to detect the growth of the bacteria up to 8 weeks [13]. The characteristic features of the *M. bovis* (poor, slow growth and dysgonic colonies) were observed. All the cultures with a characteristic feature of the bacteria were further analyzed by smearing methods to confirm the appearance of the acid fast bacilli.

Confirmation of the bacteria by polymerase chain reaction (PCR)

DNA extraction and quantification

One ml of milk and lymph node specimens were centrifuged at 12,000 rpm for 10 minutes. The pellet was collected and 100 µl of 5% (w/v) Chelex-100 resins (Bio-Rad) was added and heated at 100ºC for 5 minutes in a Thermal Cycler machine. The samples were vortexed for 30 seconds. The heating and vortexing process was repeated at least 3 times. The samples were centrifuged at 12,000 rpm for 1.5 minutes and the supernatant was collected as a template DNA. The extracted DNA was quantified by Nanodrop-1000 apparatus and either used immediately or sorted at -20 ºC for subsequent use.

Polymerase chain reaction (PCR)

Our study describes a PCR assay using *M. bovis* specific DNA markers (*rpoB* gene, 235 bp) according to the previous methods with minor modifications [14]. The target gene *rpoB* was amplified using following primers. Tb1-F CGTACGGTCGGCGAGCTGATCCAA and Tbc-R CCGACAGTCGGCGCTTGTGGGTCAA. The PCR mixture (total 25 µl) was as follows: 0.7 µl distilled water, 2.5 µl of 5 mM dNTPS mix, 2.5 µl of 10X reaction buffer solution, 2 µl of 25 mM $MgCl_2$, 1 µl of primers (10 pmol), 5 µl of DNA and 0.3 µl of Taq polymerase. The mixture was transferred to a Thermal Cycler (Perkin Elmer 480-USA) and subjected to the following condition: 94ºC/5 min, 30 cycles of 94ºC/30 sec, 74ºC/30sec, 74ºC/30 sec and final extension at 74ºC/5min. The PCR product was visualized in 2% agar along with the DNA molecular marker 50 bp (S7025, USA) and was documented with a Gel-Doc System (Bio-Rad and AlphaImager).

Data analysis

Data obtained from different experiments were recorded as the mean ± standard deviation (SD) and subjected to one-way analysis of variance (ANOVA) using the SPSS data analysis software (SPSS Inc., Chicago, IL, USA). Differences between means were considered significant at $p \leq 0.05$.

Results

Epidemiological data

The epidemiological data were obtained from the analysis of the questionnaire. The analysis of the questionnaire revealed that the disease affects the Kenana, Baggara and Fellata cattle by 50%, 30% and 20% respectively. It also showed that the cattle with age of 3 is highly susceptible to the infection (45%) followed by the age of 2 (40%) and age of 4 (15%). The females (55%) were infected more than the males.

Microbiological analysis

Smear microscopy

The smear prepared from the milk samples were negative for the acid fast bacilli, while only 1/30 (3.3%) of the lymph node samples showed a characteristic feature of the acid fast bacilli of the serpentine cord (Fig. 1).

Culture on Lowenstein-Jensen medium

No growth was observed in cultured milk samples. Only one lymph node sample 1/30 (3.3%) showed a growth in Lowenstein-Jensen medium

containing pyruvate (Fig. 2A). It was the same sample that was positive during the initial screening by the smearing methods. This sample was further confirmed by the smearing methods and presented a feature of the acid fast bacilli of the serpentine cord (Fig. 1B).

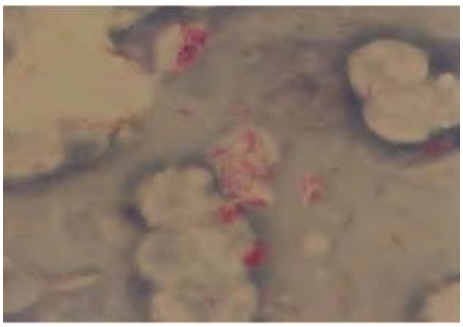

Fig. 1: Smear showing acid fast bacilli of *M. bovis* under 100χ oil immersion object. Smear from lymph node samples (Cattle). Note the characteristic appearance of the acid fast bacilli of a serpentine cord.

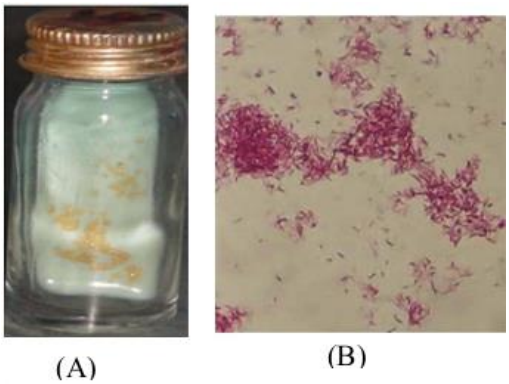

Fig. 2: The growth of *M. bovis* from lymph node sample. (A) Mycobacterium colonies in Lowenstein-Jensen medium containing pyruvate. (B) Smear from lymph node growing colonies to confirm the acid fast bacilli that were detected in Fig. 2A.

Molecular detection by PCR

The *rpoB* gene was detected in 26/40 (65%) of milk samples and 12/30 (40%) of lymph node samples. The band showed the typical size of *rpoB* gene (235 bp) for the *M. bovis* (Fig.3).

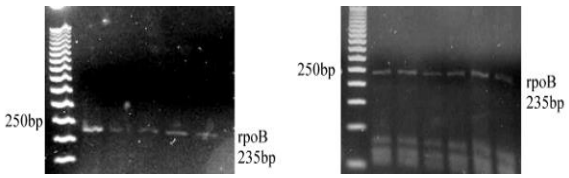

Fig. 3: Amplification of *M.bovis rpoB* gene. (A) Lymph node samples and (B) Milk samples. DNA marker 50 bp (S7025, USA) was used.

Discussion

Although bovine tuberculosis could affect the animal production and increase the risk to the public health, still there is a less attention from the veterinary authorities to establish a routine surveillance system for the diagnosis of the disease. The ultimate goal of this study was to detect bovine tuberculosis in cattle and to raise the awareness of the concerned authorities. The findings of this study indicated that tuberculosis in cattle is an existing problem in the El Rank area. We did not observe a severe contamination in the culture of Lowenstein-Jensen medium except two samples (7%). This indicates the efficiency of the 0.6% cetyl pyridinium bromide + 2% NaCl as a transport medium to detect *M. bovis* [15].

The data showed that three breeds were common in the investigated area: Kenana, Baggara and Fellata breeds. The accurate data about their population size was not available. However, this study indicated that Kenana breed was highly affected among other breeds. This might indicate the breed effect in the disease spreading. These results were similar to the observation in Chad that local breeds have different susceptibility to the disease (Mbororo zebu and the Arab zebu cattle) [16] and was further supported by the same observation in Cameroon [17]. The difference in breed susceptibility to the disease could suggest the genetic contribution in the development of the disease in addition to the environmental effect.

The present study reflects the early onset of the disease since the age around 2-4 was infected. This represents a health threatening situation where the disease gets worse with age progression. The results from this study could also address a serious health problem since drinking un-pasteurized milk and eating raw meat still abundant in some societies. Smear results showed no acid fast bacilli except one lymph node sample, this could be attributed to low Mycobacterium load in the collected samples [18]. The Mycobacterium load is often low in bovine specimens and they can only be visualized if the bacterial quantity is at least 5×10^4 mycobacteria/ml [19-20].

In conclusion, this study revealed the presence of the bovine tuberculosis in the El Rank area, although there were no clinical signs of the disease observed in cattle herd. It also proved that PCR using *rpoB* gene (235 bp) is a rapid, accurate, sensitive and specific test to diagnose the bovine tuberculosis. However, culturing methods are still essential to monitor the response of the disease to the therapeutics and to test the antimicrobial susceptibility as well. Finally, early detection of tuberculosis is crucial to initiate the treatment and interrupt the train of the transmission of the disease.

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