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RESEARCH ARTICLE

Conventional and molecular detection of Mycobacterium bovis in milk of cows and its public health hazard

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Abstract

Bovine tuberculosis is highly infectious zoonotic disease of economic and public health importance. The disease can transmit to human by direct contact with infected animals or by drinking unpasteurized milk and milk products. The study was performed considering the public health hazards related to elimination of Mycobacterium bovis (M.bovis) through milk of cows. 420 cows reared in private dairy farms at kalyoubia province, Egypt were examined by single intra- dermal tuberculin test and the results revealed that 1.9% (8/420) of tested animals were positive reactors. In addition to milk samples collected from tuberculin positive cows and examined by conventional methods and Polymerase Chain Reaction (PCR) to detect M. bovis .The results showed that Acid fast bacilli by direct smear were detected in 2 (25%) of milk samples and by culturing and identification, only 1 (12.5%) of milk samples was positive for M. bovis. PCR results revealed that only one sample (12.5%) was positive. Moreover, 25 farm workers were tested by tuberculin test and the results revealed that 8% (2/25) were positive reactors, while their sputum were subjected to Acid–Fast Staining of which 1(4%) was positive and by culturing, all the examined sputum samples were negative. Our results concluded that PCR results were in concordance to that of conventional culturing but PCR is rapid and screening test for detection of M. bovis in milk. Moreover, the presence of M. bovis in milk represented a major health hazard to the herdsmen and consumers.

Introduction

Bovine tuberculosis is a major zoonotic disease with worldwide distribution especially in developing countries where disease is endemic, as an important for both economic and public health reasons **(Pandey e*t al.*, 2013).**

Bovine tuberculosis is becoming increasingly important due to the susceptibility of humans to the disease caused by M. bovis and there is evidence that M. bovis infections may be more significant than generally considered **(Santhil *et al.*, 2014).** The proportion of human cases in developing countries due to M. bovis is accounted 3.1% for all forms of tuberculosis **(Leite et al., 2003)**. Moreover, importation of infected animals, incomplete removal of infected cases and movement of tuberculosis exposed animals between herds are the main causes of increasing incidence of tuberculosis in cattle make exposure of human population to M. bovis more likely. **(Moussa *et al.*, 2005).**

In developing countries, particularly in low income group, Bovine tuberculosis is still prevalent and is responsible for significant economic loss in animal production through reduced milk yields and low reproductive performance. **(Boland et al., 2010)** .Bovine tuberculosis is generally transmitted to human from animals in three main ways, inhalation of infected droplet nuclei containing M.bovis; ingestion of contaminated materials as milk and its products and directly among workers who are in direct contact with infected animals. **(Moussa *et al.*, 2005)**.

M.bovis infection is certainly an occupational hazard to agricultural workers who may acquire it by inhaling cough spray from infected cattle **(Kleeberg *et al.*, 1984).** While M. bovis is a major cause of pulmonary tuberculosis in cattle; it is also the primary cause of extra- pulmonary tuberculosis in humans, where cow milk is usually consumed fresh and unpasteurized **(Ofukwu *et al.*, 2008)**. Most human tuberculosis cases due to M. bovis occur in young individuals and result from drinking or handling contaminated milk. As a result, cervical lymphadenopathy, intestinal lesions, chronic skin tuberculosis (Lupus vulgaris), and other non pulmonary forms are particularly common. **(Thoen et al., 2006).**

Early diagnosis of Mycobacterial infections play a vital role in control of tuberculosis. Diagnosis of bovine tuberculosis is under taken by the widely spread tuberculin tests. Tuberculin tests have traditionally been used for diagnosis of tuberculosis in cattle and humans. Tuberculin test is easy to perform on a large scale on livestock, but it has the inconvenience of having a broad range of specificity and sensitivity due to presence of common antigens in all mycobacteria and most of these antigens are skin reactive **(Figueiredo *et al.*, 2010)**. Confirmation of the diagnosis is achieved by Acid Fast Bacilli (AFB) microscopy and conventional lowenstein Jensen (L.J) culture which remain the corner stone of diagnosis of tuberculosis **(Nagi *et al.*, 2005)**. Despite the fact that microbiological culture is highly specific, a positive result takes a long time to be obtained (6-8 weeks) and species identification procedures extend the reporting time even further. As the accurate diagnosis plays an important role in the control of tuberculosis, it is necessary to develop new diagnostic methods for bovine tuberculosis which could identify M. bovis directly in biological samples as milk or blood, without having to culture them and which would also improve the predictive value of tuberculin test. Polymerase Chain Reaction (PCR) has been described as an important tool of the diagnosis of bovine tuberculosis , since it is rapid, more accurate, sensitive and most efficient method **.(Ishag *et al.*, 2014)** .The aim of the present study was to determine the prevalence of bovine tuberculosis in dairy cattle in private farms at kalyoubia province , Egypt using the single intra- dermal tuberculin test and to detect Mycobacterium bovis in the milk obtained from tuberculin positive cattle by conventional and molecular method. Moreover to highlight the risk of consuming raw milk and public health hazards among farm workers.

MATERIALS AND METHODS

1- Animal samples

A total of 420 cows reared in private dairy farms at kalyoubia province, Egypt were examined by single intra-dermal tuberculin test as screening for tuberculous infection by cooperation of Regional Veterinary Service Department in kalyoubia province. In addition to milk samples were collected from tuberculin positive animals (10 ml of milk obtained from the udder while milking into sterile McCartney bottles after cleaning, washing and disinfecting the udder to avoid contamination from environment).

2- Human samples

Twenty-five persons who had occupationally contact with these animals (farm workers) were examined by tuberculin test. Moreover, sputum samples were collected from them on three consecutive days early in the morning before breakfast in wide necked plastic containers.

All milk and sputum samples were transferred to the laboratory of Tuberculosis department in Veterinary Sera and Vaccine Research Institute, Abbasia, Egypt in a cooler box with ice and kept refrigerated at 4°C until analysis with in 24 hrs. The samples were examined for isolation and identification of Mycobacterium bovis by using conventional methods (Direct smear- Culturing –Biochemical) and molecular method by Polymerase Chain Reaction (PCR).

-Tuberculin testing in animals (According to Ovdiennkop *et al.*, 1987)

A narrow zone (at the middle third of neck of the tested animals) was marked by clipping the hair. The skin thickness was pre measured using caliper. The 0.1 ml of PPD (purified protein derivative) tuberculin obtained from (Veterinary Sera and Vaccine Research Institute, Abbasia, Egypt) was injected intra-dermal . The results were recorded after 72 hrs post injection, skin thickness was measured and the difference between the 1st and 2nd reading of skin thickness was recorded according to General Organization of Veterinary Services **(GOVS, 1992).**

-Tuberculin testing in farm workers (According to Sinder, 1982)

5 tuberculin units were injected in the skin of the dorsum of the hand , the skin test was read after 48 to 72 hrs. It is considered positive if the injection was followed by in duration of 10 mm or more in diameter.

3- Processing and conventional examination of samples

Milk and sputum samples were processed for isolation of mycobacteria, following standard procedure for homogenization, suspension, centrifugation and decontamination **(Petroff; 1915 and Vestal *et al.*, 1977)**. The samples were centrifuged at 3000 rpm for15 minutes and the supernatant was discarded into 10% formalin. The sediments were suspended in 2 ml of sterilized physiological saline solution. To the suspension, equal volume of sterilized 6% hydrochloric acid for decontamination and incubated at 37°C for 30 minutes. The mixture was centrifuged at 3000 rpm for 30 minutes. The sediment was neutralized with 4% sterile sodium hydroxide solution and one drop of 0.05% phenol red indicator. (The change in color from purple to pink indicates correct neutralization) then, re centrifuged and the sediment was ready for microscopic and culturing examination.

3-a. Microscopic examination of the sediment

The sediment of previously prepared samples was spread on clean slides and then dried, slightly fixed over flame and stained with Acid Fast Stain (Ziehl –Neelsen stain).The stained smears were examined under oil immersion lens of the microscope for demonstration of Acid Fast Bacilli (AFB) which stain as bright rose/ red rods with a blue back ground **(WHO 1998)**.

3-b.Culture of the sediment and species differentiation

The obtained sediment was thoroughly mixed and spread on the surface of each of the slants of glycerinated and pyruvated modified Lowenstein Jensen media in McCartney bottles **(Ernst, 1990).** The bottles were incubated at 37°and were examined daily for 7 days and periodically once week for 6-8 weeks. Suspected colonies were identified morphologically and microscopically **(Cruickshank *et al.*,1975)**. Biochemical tests and confirmation of species were applied according to **Brasil (1994)**.

4- Polymerase Chain Reaction (PCR) analysis

All kits and reagents used in PCR analysis were obtained from (QIAGEN GmbH; Hilden, Germany).

4-a. DNA extraction from milk samples

DNA was extracted from milk samples collected from tuberculin positive cows by a modification done by **Carvalho *et al.*, (2014)** of QIAmp DNA Mini kit (Qiagen).One milliliter of milk was centrifuged at 1000rpm for 5 minutes; the pellet was suspended in 180 ml of 20 mg/ml lysozyme in 20 mM Ttris. Hcl,(P.H 8); 2 mM EDTA; and 1.2% Triton, and incubated for 2 hours at 37°C prior to proteinase K treatment, as recommended by the manufactures' protocol.

4-b. DNA amplification by PCR

The extracted DNA from all samples of bovine milk were subjected to PCR by using primers containing forward JB21(5'-TCGTCCGCTGATGCAAGTGC-3')and Reverse JB22(5'-CGTCCGCTGACCTCAAGAAG-3') amplifying a 500 bp genomic fragment of the RvD1- Rv2031c gene specific for M. bovis as also described by **Rodriguez *et al.*, (1995)** and **Rodriguez *et al.*, (1999)**. The primers were obtained from Life Technologies (Applied Biosystems). The reaction was performed in a final volume of 50 µl containing 1x reaction buffer, 2.5 U of Taq polymerase, 0.2m M each deoxy nucleoside triphosphate , 1.5 m M magnesium chloride; 2 µg of each DNA and 20 pmol of each primer . Target DNA was denatured by initial incubation for 5 min at 94°c followed by 30 cycles of denaturation at 94°c for 1 min, annealing at 68°c for 1 min and extension at 72°C for 1 min and final extension at 72°c for 7 min. (Amplified PCR products generated in samples were compared with standard M.bovis positive control, obtained from Veterinary Sera and Vaccine Research Institute, Abbasia, Egypt. After amplification, PCR products were electrophoreses on 1% agarose gel containing ethidium bromide staining (0.5ug/ ml) and visualized under U V light.

RESULTS

-Tuberculin test in cows:-

A total of 420 cows were tested by single intra-dermal tuberculin test, only 8(1.9%) of these cows were positive (Table 1).

- Microbiological analysis (Conventional analysis):-

Acid Fast Bacilli were detected in 2 (25%) out of the examined milk smears obtained from 8 tuberculin positive cows, while only 1 (12.5%) of M. bovis was isolated from the examined milk samples by culturing on L. J media & biochemical identification (Table 2)

-Molecular detection by PCR.

PCR was carried out on 8 milk samples of tuberculin positive cows by using primers JB21 and JB22 which amplify a 500- bp fragment of M. bovis .The positive PCR was present in one milk sample (12.5%) as shown in table (2) &Fig (1).

-Tuberculin test in farm workers and bacteriological examination of sputum samples:

A total of 25 farm workers were tested by tuberculin test, only 2 (8%) were positive. Acid Fast Bacilli was detected in only one (4%) sputum smear samples out of examined workers, while all examined sputum samples were negative by bacteriological examination (Table 3).

Table (1) Results of tuberculin test of examined cows

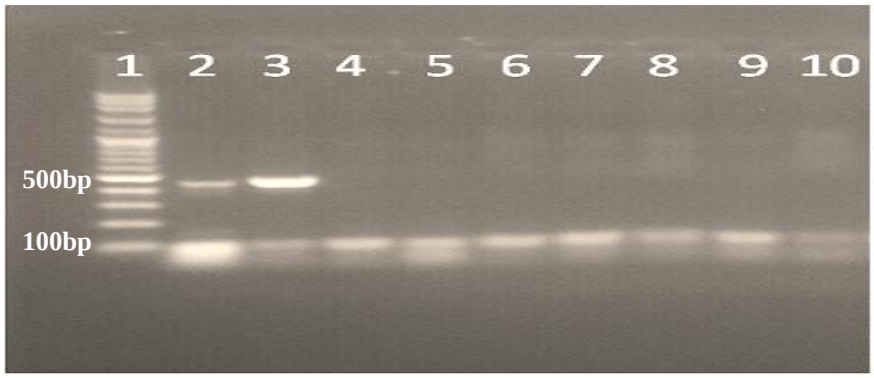
Number of tested cows	Positive tuberculin test		Negative tuberculin test	
	No	%	No	%
420	8	1.9	412	98.1

Table (2) Correlation between Conventional and PCR method of processing milk samples of tuberculin positive cows.

No .of milk samples from tuberculin positive cows	Conventional methods				Positive PCR	
	+ve Microscopic Exam.		+ve culture and identification (M. bovis)			
8	No	%	No	%	No	%
	2	25	1	12.5	1	12.5

Table (3) Correlation of tuberculin, microscopic and bacteriological examination of sputum samples of farm workers

No. of examined workers	+ ve tuberculin test		+ ve Microscopic Exam.		+ ve Culture	
25	No	%	No	%	No	%
	2	8	1	4	0	0



Fig(1): PCR amplification results of M.bovis appeared at 500 bp . Lane 1: Marker,100 bp ladder
Lane 2: Mycobacterium bovis positive control, Lane 3: Mycobacterium bovis positive milk sample
Lanes 4-10: Mycobacterium bovis negative milk samples.

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 tuberculosis in cattle and to raise the awareness of people.

Bovine tuberculosis infection in cattle usually diagnose in the live animal on the basis of delayed hyper sensitivity reaction which is the standard method for detection of bovine tuberculosis **(Ereny, 2011)**. In the present study, single intra dermal tuberculin test results showed that 1.9% of the investigated animals were positive. These results are nearly similar to that recorded by **Moussa *et al.*, 2011** (2.46%)**, Hazem et al., 2012** (0.96%) and **Pandy et al., 2013** (2.6%) but lower than that reported by **Mossad *et al.*, 2009** (4.6%) as this study was conducted in small holder private farms with small herds**, Cook *et al.*, (1996)** indicated that breed of cattle , housing and gathering of animals at watering and grazing sites have influenced the prevalence of tuberculosis. Although the intra-dermal tuberculin test has been the widest used diagnostic technique, but it lacks sufficient sensitivity and specificity in many cases. Tuberculin test depends on several factors, including high quality reagents, as well as the immunological status of the animal. Furthermore, negative tuberculin test does not mean that the animal is not infected ; on the other hand, a positive test can only mean a delayed hypersensitivity reaction due to previous exposure **(Rodriguez *et al., 1999)***. The infected animals secret M. bovis in milk that referred to the risk of human infection through unpasteurized , untreated consumption of milk or using raw milk for producing cream, butter or curd among cattle owners and herdsmen **(Qamar and Azhar, 2013)**. So detection of M. bovis in milk samples by conventional (microscopical and bacteriological) and Molecular methods is very important . The results of Acid Fast Bacilli in milk samples examined by direct smear using ziehl- Neelsen stain , table (2) showed that out of 8 milk samples ,only 2 milk samples were positive, while the results of bacteriological examination , only one milk sample out of 8 samples was positive.

The results of AFB in milk samples examined by direct smear were higher than that recorded by culturing of samples from tuberculin positive reactors. These results may be due to the fact that Ziehl- Neelsen method was used for staining of tubercle, other acid fast and saprophytic bacilli and can't differentiate between them **(Cruickshank et al., 1975)** and because the microscopical examination can not differentiate between M. bovis and other mycobacteria. Our results confirm the conclusion of **Wards *et al.*, (1995)** who stated that Ziehl- Neelsen staining of clinical specimens lack sufficient sensitivities and specificities as the specimen is required to give a positive results by AFB staining when it loads more than5.000 to10.000 bacilli /ml **(Liehardt&Cook,2005)**. Mycobacterium bovis could be detected by culturing in 1 (12.5%) milk sample out of 8 milk samples obtained from positive tuberculin .These results are nearly similar to **Ameni *et al.*, 2003** (9.3%) and **Al- Attar, 2010** (10.2%) but higher than recorded by **Benkahla *et al.*, 2011** (4.9%) and **Majeed et al., 2013** (2.3%). The difference in these results may be attributed to that the size of the herd is a determining factor in outbreak of bovine tuberculosis and host genetics also play a role in the susceptibility to M. bovis **(Cleaveland et al., 2007)**. The obtained results indicated that culturing followed by the biochemical tests has been used for detection of mycobacterium bovis .Culturing gives good sensitivity. Overall, the sensitivity of the direct Acid fast smear is lower than that of culture. Moreover, detection of M. bovis in milk samples by bacteriological examination although it has a specificity that approaches 100%, but the slow growth of the organism results in delay in its diagnosis. For these reasons, the PCR described here was evaluated for detection of bovine tubercle bacilli in fresh unprocessed milk from tuberculin positive cows. The results in table (2) indicated that 12.5% of samples were positive for PCR (one out of 8 samples). Compared with previously described PCR assays in milk samples as **Naima *et al.*, 2008** (29%), **Figueirdo *et al.*, 2012** (12%) and **Carvalho *et al.*, 2014** (5%) . The variable PCR results can be explained as bacilli shed from tuberculosis cows, depending on the progression of the disease, and may be associated with cell mediated immunity **(Romero et al., 2006).** Other explanations to variable PCR results may be related to the amplification conditions, extraction method, sequence and polymorphism of targets used. A reliable PCR- based diagnostic assay must have a target DNA sequence that is specific for the microorganism to be detected and that must also be present at most, if not all, isolates of the organism. The 500 bp fragment amplified primers JB21 and JB22 fulfills the requirement, since it is capable of discriminating M.bovis from related strains **(Bauerfeind *et al.*, 1996)**

By Comparing the PCR results to the findings with the gold standard method of conventional diagnosis using staining and culture on L.J media. It was found that PCR result matched the culture results in the examined samples. The PCR give high sensitivity and specificity for the mycobacterium and is more accurate and faster than conventional method for diagnosis **(Al- Saquar et al., 2009)**.

Although the number of M. bovis positive samples was low, the habit of pooling milk may still pose a public health danger to milk consumers **(Schelling *et al.*, 2000)**. Furthermore, **kleeberg *et al.*, (1984)** indicated that one cow with tuberculous mastitis can excrete enough viable tubercle bacilli to contaminate the milk of up to 100 cows when milk pooling and bulk transportation is used. Some investigators have pointed out the risk of human infection through using raw milk for producing cream, butter or curd among cattle owners and herdsmen in community **(Bonsu *et al.*, 2001)**. Because most of farmers either sell their milk to local people or pool milk in units for selling milk products without heat treatment, risk of milk contamination with M. bovis is a potential major health hazard to consumers.

Furthermore, the cattle owners are poor understanding of bovine tuberculosis exacerbates the situation **(Hassanain *et al.*, 2009)**

In this study, only 2 (8%) out of 25examined farm workers were positive by tuberculin test, while examination of their sputum by direct smear using Ziehl- Neelsen stain gives only 1 (4%) positive, while all their sputum samples were negative for M. bovis isolation. Positive tuberculin test may be due to vaccination by BCG and direct smear using Z.N method was used for staining of tubercle, other acid- fast and saprophytic bacilli and can not differentiate between them **(Cruickshank *et al.*, 1975)**. The inability to isolate M. bovis from sputum samples in this study does not exclude the importance of zoonotic tuberculosis. As WHO reported in 1998 that 3.1 % of tuberculosis cases in humans worldwide are attributed to M. bovis and that 0.4 – 10% of sputum isolates from patients in African countries could be M. bovis **(Cosivi *et al.*, 1998)**.

The presence of zoonotic M. bovis in milk poses a serious public health hazard to the herdsmen and consumers. This is because in countries, where cow milk is not usually pasteurized before use, tuberculosis due to M. bovis emerged as a major cause of extra- pulmonary tuberculosis in humans. Moreover, the emergency of M. bovis infection is linked to the genetic and antigenic relationship existing between M. bovis and M. tuberculosis, leading to identical clinical disease in humans. **(Bonsu *et al.*, 2001).**

CONCLUSION

It could be concluded that bovine tuberculosis still constitutes a public health hazard and every effort should be made to control such disease in Egypt. Detection of M. bovis in freshly drawn milk from tuberculin positive cows emphasizes the risk of transmission of tuberculosis to human through consumption of contaminated unpasteurized milk and milk products. It also proved that PCR could be effectively used as a diagnostic and rapid screening test for the detection of M. bovis in milk, as conventional diagnostic procedures are often time consuming and have low sensitivity and or specificity. Moreover, PCR used to detect the presence of M. bovis in biological samples as milk and thus become an important tool for the control and eventual eradication of the disease**.** The only way to be protected from the disease is through prevention. It is important to limit the exposure of the herd to other infected cattle. There is a need for enhanced public health education to raise awareness on the consequences of consuming potentially contaminated milk. Also, measures need to be adopted to test and eliminate positive reactor cows from the herd.

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