

## Assessment of Various Classical and Novel Techniques for Precise Diagnosis of *Mycobacterium bovis*

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**Abstract:** *Mycobacterium bovis* is a vigorous virulent microorganism that causes bovine tuberculosis a global infectious disease of cattle result in great economic losses. The high public health significance of the organism is attributed to its zoonotic importance as it can infect human population. The rapid and accurate diagnosis of *M. bovis* is essential to early control and avoiding the enormous bad consequences of the disease. The study was conducted to assess the effectiveness of novel techniques as Gamma interferon assay and polymerase chain reaction compared with ELISA and conventional bacteriological culture in diagnosis of *M. bovis*. A number of 780 cross-breed dairy cattle in Kafr EL-Shiekh governorate were examined by comparative intradermal tuberculin test during 2018. Milk samples were collected from positive reactors for conduction of bacteriological examination and polymerase chain reaction. Serum samples were collected for ELISA using bovine purified protein derivative as well as whole blood samples for performance of Gamma interferon assay. The prevalence of positive reactors by tuberculin test among tested herds was 18 / 780 (2.3%). The bacteriological examination using Middle Brooke 7H9 agar exhibited the isolation of the organism in 10/18 (55.5%), while higher sensitivity was obtained in case of ELISA using bPPD antigen (72.2%). The highest sensitivity was shown in Gamma interferon assay and PCR (83.3%). The results demonstrated that both of Gamma interferon assay and PCR were motivated assays used for early, rapid and quite screening and diagnosis of bovine tuberculosis in the milk and blood samples obtained from live animals.

**Key words:** ELISA, Gamma interferon assay, *Mycobacterium bovis*, PCR.

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### I. Introduction

*Mycobacterium bovis* (*M. bovis*) is a hazardous bacterium that cause bovine tuberculosis which is a widespread disease resulting in grave problems in dairy cows. *M. bovis* is belonging to the *Mycobacterium tuberculosis* complex (MTC), a group of closely linked acid fast bacteria [1]. Cattle are the conserved host of *M. bovis* and the disease causes remarkable economic problems all over the world involving reducing 10-20% of milk yield and weight, condemnation of carcass, mortalities and infertility [2].

In spite of *M. tuberculosis* is the most prevalent reason of human TB cases; a significant portion '0.5 to 7.2%' in developed countries and '10 to 15%' of developing ones takes place due to *M. bovis* infection and is indicated as public health importance; zoonotic TB [3]. Zoonotic bovine TB is recorded as the most recurrent cause of zoonotic tuberculosis in human, foremost acquired via the consumption of unboiled or unpasteurized milk and dairy products. Lower incidence through eating of raw or improperly cooked infected meat or via aerosols inhaled from infected animals during direct human-livestock contact [4]. Unpasteurized raw milk is preferably consumed than boiled milk as a common practice in developing countries particularly, Africa and Middle East; mostly due to its accessibility, suitability, palatability and lower cost. The public health threat of bovine TB is mainly correlated with consumption of unpasteurized dairy products having *M. bovis* [5].

The comparative intradermal tuberculin test is used mostly for screening and detection of infected animals, but the true impact of *M. bovis* is unclear; many of the reactive antigens used in tuberculin test are also exist in non-pathogenic environmental mycobacterial species and this cross reactivity lead to reduce specificity of the test, resulting in non-specific reactors [6].

Although the conventional bacteriological examination (culture isolation methods and biochemical reactions) is the gold standard method used for diagnosis of bovine tuberculosis, but it needs lengthened period of time (may be up to two months) as *M. bovis* is a slowly grower microorganism [7].

Arising from the need of rapid, accurate, and just promising tests is requested; enzyme-linked immunosorbent assay (ELISA), and the more advance gamma interferon assay as well as the molecular

diagnostic methods. These novel techniques can be applied on live animal's lymph node aspirate, blood and milk [8]. The ELISA seems to be the ultimate convenient serological test used for serodiagnosis of bovine tuberculosis. Gamma interferon assay; the release of bovine gamma interferon (IFN- $\gamma$ ) cytokine from sensitized lymphocytes within 20 hours of incubation with specific antigen is measured [9,10]. PCR is the most dependable method used for detection of *M. bovis* in clinical samples. It is specific; targeting a certain sequence of DNA, and quick needs only 48 hours for detection of *M. bovis* [11].

This study was planned to assess the efficiency of recent techniques used for quite diagnosis of *M. bovis* including ELISA, Gamma Interferon Assay and PCR in comparison to conventional methods (tuberculin test and culture techniques).

## II. Materials And Methods

### 1. Sampling:

A total of 780 cross-breed dairy cattle in Kafr EL-Shiekh governorate were tested by comparative intradermal tuberculin test during 2018. Milk and whole blood samples were collected from positive reactors.

### 2. Preparation of milk samples:

The milk samples were aseptically obtained from the positive reactors (n= 18), brought to laboratory on ice box, the whole procedures were carried out according to OIE (2013) [12] in a bio-safety cabinet. Milk samples were portioned for the culture isolation and direct PCR analysis. The milk samples were decontaminated by adding a double volume of sterile 4% NaOH solution then centrifuged at  $400 \times g$  for 10 min before the skim milk and the fat layer was removed. Afterwards, sulfuric acid 8% is added to stabilize pH, the sediment was washed and stored at  $-80^{\circ}\text{C}$  until assaying.

### 3. Microbiological culture:

A part of sediment was smeared for direct Ziehl-Neelsen (ZN) staining, another part was inoculated in Middle Brooke 7H9 agar (Difco) labeled tubes, incubated aerobically at  $37^{\circ}\text{C}$ , seven days then examined daily till 15 days. The type and rate of growth of the colonies was recorded and considered negative when no colonies were seen. Other smears were made from isolated colonies, fixed by gentle heating, stained by ZN method and examined microscopically for acid-fast organisms [13].

### 4- ELISA assay:

The procedure used for determining antibody responses in the ELISA as described by the manufacturer (UNIBIOTES). Briefly, serum samples were diluted 1:1 in the sample diluent supplied with the kit. Then 100  $\mu\text{l}$ /well of the diluted sample was added to a 96-well microtiter plate pre-coated with mycobacterial bovine Purified Protein Derivative (bPPD) antigen and incubated at room temperature for 60 min. After washing, 100  $\mu\text{l}$  of a monoclonal anti-bovine IgG-horseradish peroxidase conjugate was added to each well, and the plates were re-incubated for 30 min, followed by another washing step. The substrate (100  $\mu\text{l}$ /well) was added to each well, and the plates were incubated for 15 min at room temperature, and the plates were read at 450 nm (in an ELISA microtiter reader). A positive result was defined by the manufacturer as an S/P ratio of  $\geq 0.3$ .

### 5. DNA extraction and detection in milk:

Milk sample (2 ml) was centrifuged at 10000 rpm for 5 min and whey was discarded. The pellet which constituted both milk fat and sediment was suspended in 400  $\mu\text{l}$  Tris-EDTA buffer and 1.2% Triton and incubated for 2 hours at  $37^{\circ}\text{C}$  prior to proteinase K treatment, GeneJET Genomic DNA Purification Kit as recommended by the manufacturer (Thermo Fisher Scientific). The reaction mix (final volume 50  $\mu\text{L}$ ) contained 5  $\mu\text{l}$  of  $10 \times$  PCR buffer, 200  $\mu\text{M}$  of each dNTP, 2.5 U of recombinant *Taq* polymerase (Thermo Fisher), 2.0 mM  $\text{MgCl}_2$ , and 5  $\mu\text{L}$  (50 ng) of purified DNA template with 0.2  $\mu\text{M}$  of primers employed were as JB21 (5'TCGTCCGCTGATGCAAGTGC-3') and JB22 (5'CGTCCGCTGACCTCAAGAAAG-3') as amplification of *RvDIRv2* operon specific for *M bovis* [14].

*M. bovis* complex was included as positive control in the PCR amplifications while *E. coli* strain ATCC35150 used as a negative control. Amplification was performed in a T3 Biometra thermal cycler with the following parameters: 5 min at  $94^{\circ}\text{C}$ , followed by 37 cycles of 1 min at  $94^{\circ}\text{C}$ , 1 min at  $68^{\circ}\text{C}$ , and 1 min at  $72^{\circ}\text{C}$ , and a final elongation step at  $72^{\circ}\text{C}$  for 7 min. The annealing time was doubled and the elongation step was included, in order to increase amplification. PCR products (500 bp) were resolved in 2% agarose gel electrophoresis and stained with ethidium bromide (10  $\mu\text{g}/\text{mL}$ ).

### 6- Gamma interferon assay:

The test was applied on the whole blood samples of the tuberculin positive cows by using gamma interferon assay kit and sensitizing antigen (BOVIGAM®, Prionics- Germany). Firstly, freshly whole blood samples were collected into heparinized collecting tube and transported to the laboratory within 3-7 hours, then were incubated at 37 °C for 16-24 hours after stimulation by specific antigen (bPPD) in order to stimulate the production of IFN- $\gamma$  by sensitized T lymphocytes, and IFN $\gamma$  production was measured in duplicate samples by sandwich ELISA [15].

### III. Results And Discussion

Within the last decades, promoting of novel adapted techniques for early and accurate diagnosis of bovine tuberculosis is in great need. The disease is considered a public health issue threatens human communities through not only direct contact but also via consumption of non-heat treated milk obtained from infected cows [16].

Relying on the comparative intradermal tuberculin test; the positive reactors examined in Kafr EL-Shiekh governorate farms, were 18 with the prevalence of bovine tuberculosis was 2.3%. The low average incidence is come from the efforts of authority to perform the regular testing of animals unless the reemergence due to the importation of live animals from countries where bTB is common [17].

In contrast the appearance of higher prevalence in other African countries is due to the lack of regular testing like Ethiopia, a number of 701 cattle were tested for bovine tuberculosis (bTB) using comparative intradermal tuberculin (CIDT) test and the apparent prevalence was 9.1% [18]. On the same manner, the prevalence among cattle in the highlands of Cameroon using two population-based tuberculin skin test (TST) screening was 13.14% [19].

Concerning the bacteriological examination, the obtained results of Middle Brooke 7H9 agar culture exhibited positivity in 10 milk samples as prevalence of bovine tuberculosis was (55.5%). These results agreed with that obtained by de Azevedo Issa et al [20], which the isolation ranged between 55 to 62%, while the results higher than those of Yates et al [21], who isolated *M. bovis* through Middle Brooke 7H9 by 38.8 to 43%. It is suggested that the Middle Brooke 7H9 as an agar-based media is useful because of the earlier appearance (2 weeks) of colonies than the egg based medium (8 weeks) [22].

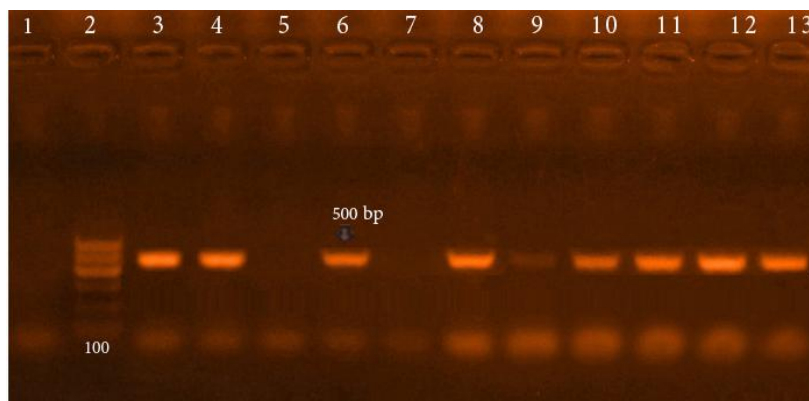
Touching to results of ELISA using bPPD antigen as shown in figure (1), the number of positive cases were 13, supposed sensitivity of ELISA was (72.2%) which higher than the culture methods. There was a higher ELISA result; 87.8% [23]. On the other hand there was a low sensitivity in other studies 50% [24] and 63% [25]. ELISA when used in bulk tank milk samples may be not useful for accurate diagnosis of *M. bovis* due to the dilution occurred by other negative milk in cattle herd [26].



**Figure (1):** ELISA using bPPD antigen performed on the positive reactors

Regarding of Gamma interferon assay, the data implied that the positive blood samples were 15 gave a sensitivity of gamma interferon assay by using bPPD antigen was 83.3% which is higher than ELISA (72.2%). Higher results were frequently recorded as 93 up to 100% [27, 28], this high sensitivity of the technique gives the chance to detect the infected animals as positive and the protocol may render a trustful assay for the antemortem diagnosis of TB from the primary phase of *M. bovis* pathogenesis. From the other sight there are some limitations for using the IFN- $\gamma$  assay on a large scale as a routine field test; the high cost and the tested blood specimens must be handled within 8 hours between collection and lymphocyte culture preparation [16].

In our investigation, PCR was used for direct detection of *M. bovis* from milk samples by amplification of *RvDIRv2* gene specific for *M. bovis*, which responsible for expression of regulatory proteins. A number of 15 samples (83.3%) from the tested ones were positive with specific amplicon size 500 bp; figure (2). PCR revealed more sensitivity and specificity in comparison with ELISA and nearly similar with Gamma interferon assay. These results were harmonized with other similar data; 82 to 100% in bulk tank milk samples [29].



**Figure (2):** PCR amplification of RvD1Rv2 gene specific for *M. bovis* at 500bp, lane 1: negative control, lane 2: ladder 100bp, lane 3: positive control.

Direct detection of bovine TB by molecular-based techniques in dairy animals after preliminary screening was found to be more sensitive and specific compared to the traditional ones [8]. PCR is found to be an easy, low cost, sensitive and specific method for diagnosis of *M. bovis* among infected dairy herds compared to other used techniques and efficient in control measures of bovine tuberculosis.

#### IV. Conclusion

Gamma interferon assay is seemed to be more sensitive than ordinary ELISA and could get over the problems of ELISA results. Recently, it is the most advised assay used for diagnosis of bovine tuberculosis in the field; otherwise the only drawback of utilizing this assay in large scale is being expensive. PCR is the easy, sensitive and specific technique used for direct detection of *M. bovis* in milk samples minimizing the diagnosis time from 4-8 weeks in conventional culture methods to 48 hours. The novel techniques should be disseminated in a large scale for rapid and accurate diagnosis of bovine tuberculosis. This concept can reduce the risk of transmission of zoonotic organism through raw milk consumption which is considered a public health threat.

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