Original Research Article

A Comparative Study Between Some Diagnostic Techniques in Diagnosis of Bovine Tuberculosis

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Abstract

Early detection of bovine tuberculosis in infected cattle is important to eliminate the infected animals from a herd, and to reduce the risk post by animals and animal products to human. Methods for the detection of mycobacteria are continuously being developed. A total of 1050 cows were tested by tuberculin test and the lymph nodes and internal organs of tuberculin positive cows were examined by culture method and confirmed by RT- PCR. The whole blood samples that collected as soon as the reading of the tuberculin test from both the tuberculin positive and tuberculin negative cows were used for detection of gamma interferon release (bovigam, prionics). The obtained results revealed that 57 cows were positive tuberculin test (5.4%), 37 out of them were harbored the acid-fast bacilli of mycobacteria (65 %), which confirmed using RT -PCR, while there are 49 out of 57 tuberculin positive cows were tuberculous by gamma interferon assay (86 %). There are 3 cases out of 57 tuberculin negative cows were tuberculous by gamma interferon assay. The use of gamma interferon assay has the superiority for diagnosis of bovine tuberculosis over the conventional culture method and tuberculin test due to the obstacles of the decontamination process as well as the field problems of tuberculin test.

Keywords: Bovine, Gamma interferon, Tuberculosis, Tuberculin test.

Introduction

Bovine tuberculosis is (bTB) a transboundary disease of high economic and public-health burden worldwide (Mohamed Borham et al ,2021). Bovine tuberculosis is a chronic contagious disease caused predominantly by M. Bovis and infrequently by other pathogenic Mycobacteria (Djemal et al., 2018; Romha et al., 2018). The disease has serious implications on animal health and severe socio-economic impacts due to impaired milk and meat productivity, test, and slaughter regime costs, culling of infected animals, carcass condemnation, movement controls and international trade restrictions (Vordermeier et al., 2016).In humans, TB is one of the top 10 causes of death (WHO, 2017), and M. Bovis is responsible for 10-15% of human TB cases in developing countries (Algammal et al., 2019).The detection of tuberculosis in cattle is based mainly on the measurement of delayed hypersensitivity following the intradermal injection of M. Bovis antigens, which are usually purified protein derivatives PPD. (Whelan et al., 2010). Postmortem diagnosis depends tentatively on adequate visual observation, palpation, and incision of suspected gross tuberculous lesions. However, some pathogens such as Nontuberculous Mycobacteria (NTMs) causing tuberculous-like lesions. Hence, subsequent PM diagnostic procedures are crucial to reach the final diagnosis. Bacterial isolation and PCR techniques are essential methods to confirm suspected bTB cases (Kuria, 2019). Several diagnostic tools are available for M. Bovis infection detection in cattle. These tools can be broadly categorized into direct (targeting the bacterium or its debris) and indirect (measuring the host's immune response to infection) tests. The single intradermal tuberculin test is the most used indirect method for early detection of cattle infected with M. Bovis (Elsohaby et al,2020). The effectiveness of diagnostic approaches based on the detection of the cellular immune response to M. Bovis antigens, skin tests must be conducted in vivo, which is difficult in large-scale epidemiological surveys and

retrospective epidemiological analyses (Liu et al., 2007). Moreover, a few chronically infected animals may go undetected even after skin test which places herds at risk for the bovine-to-bovine spread of infection. Unfortunately, sporadic cases of bovine TB are expected to occur during the final stages of an eradication program in target area due to the extremely long incubation period for this disease and the presence of latent undetectable infection in some animals. The diagnosis of bovine tuberculosis in live animals mainly depends on clinical manifestations of the disease and skin testing. It is known that the skin test lacks sufficient sensitivity and specificity in many cases. Identification of the mycobacterium species is based on the traditional culture method and Ziehl-Neelsen acid-fast stain. Pigmentation, growth rate, colony morphology of cultures of the isolated suspected organism as well as Biochemical tests such as niacin, catalase, nitrate reduction, and urease which used to identify different species (Bennett and Cooke, 2006). Ziehl-Neelsen stain is rapid but lacks specificity and cannot be used to differentiate between the various members of the family Mycobacteriaceae, while the culture procedures usually require 4 to 8 weeks to obtain good growth (Wobeser, 2009). The introduction of PCR and nucleic acid hybridization has greatly reduced identification time and the use of PCR has improved the level of detection in clinical specimens. by amplifying species-specific DNA sequences, and hybridizing the amplified sequence with a labeled probe, 5 fg of mycobacterial DNA (represent one mycobacterium) can be detected in clinical samples. (Lilebaum et al., 2001). The T-cell based interferon gamma release assays, (IGRAs) are anticipated to replace the tuberculin test for detecting the latent tuberculosis as well as the suspected tuberculous animals. IGRAs overcome many drawbacks of TST including application and reading obstacles and the field problem (Gormley et al., 2004). The aim of this work was directed mainly to evaluate the possible application of the gamma interferon assay in farm animals in parallel to TST and evaluate the results with traditional conventional techniques and the use of RT- PCR in diagnosis of bovine tuberculosis in Egypt.

Materials and Methods

In this study a total of 1050 tuberculin tested cows were examined for tuberculosis by single intradermal tuberculin skin test and the lymph nodes of tuberculin positive cows were examined by cultural method as well as by PCR, in addition to the use of gamma interferon assay on whole blood samples of the tuberculin positive cows and in contact tuberculin negative cows at EL-Menofia, El-Sharkia, and El-Behaira governorates.

Mycobacterial culture according to the procedure descried by (OIE, 2009)

Processing: After sterilized, trimming of fat and fibrous tissues in a biohazard cabinet, approximately 5 gm of each tissue sample was sectioned into tiny pieces in a sterile mortar using a sterile scalpel then crushed, grinded and homogenized using a pestle with adding 5ml of distilled water (DW).

Decontamination: The homogenates were decontaminated by adding equal amount of 4% H2SO4 then incubated at

37°C for 30 min. The obtained mixture was diluted with 16 ml of sterile DW, centrifuged at 3000 rpm/20 min. The supernatant was discarded into 5% phenol whilst the sediment was re-suspended in 0.5 ml sterile DW.

Inoculation of media: Inoculation of the deposit onto Lowenstein-Jensen slants and incubation at 37°C under aerobic conditions then examined daily during the first week and then weekly for 2-3 months. The grown mycobacterial colonies were stained with Ziehl-Nelsen stain for confirming the acid-fast bacilli.

Real time PCR for detection of Mycobacterium tuberculosis complex:

The infected samples of tuberculin positive cows were examined using Mycobacterium tuberculosis complex RT-PCR kit (biovision):

Preparation of the samples for DNA extraction:

Each piece of infected tissue was homogenized in PBS (0.14M NaCl, 4mM KCl, 8mµ Na_2HPO_4, 2mM KH_2PO_4, pH 6.5 buffer) according to Wards et al. (1995).

Isolation of mycobacterial DNA from infected tissues:

The extraction was carried out according to instruction of extraction kit (Sigma) as follow:

Lysis and digestion: Twenty mg of grinded tissue + 180 ul digestion sol. + 20 ul proteinase K were mixed and incubated at 56°C for 3hr.

Fixation: The lysate was transferred to purification column, centrifuged for 1 min./ 8000 rpm, then the

collection tube was discarded, and the column was placed into a new collection tube.

Washing: A 500 ul wash buffer 1 was added, centrifuged for 1 min./ 10000 rpm then discarded flow- through, 500 ul wash buffer 11 was added + centrifuged for 4 min./ 14000 rpm, and the collection tube was discarded.

Elution: The column was transferred to a new microfuge tube, the elution buffer was added + incubated 2 min., then centrifuged for 1 min./ 10000 rpm.

Detection of M. tuberculosis complex:

Real time PCR was performed according to the kit obtained from biovision® Real-time PCR was performed according to   Michel, et al. (2011) using MTplex dtec-RT-qPCR Test (Edifici-Quórum3, Spain) that comprises a series of species - specific targeted reagents designed for detection of all species contained in the Mycobacterium tuberculosis complex.

Genomic marker	IS6110
Holding species	M. tuberculosis complex
PCR-product size	254
Sequence 5´ 3´	INS1: CGTGAGGGCATCGAGGTGGC INS2: GCGTAGGCGTCGGTGACAAA

(Van Soolingen, 1991)

Extracted DNA from the suspected samples was subjected to RT- PCR. The primers and TaqMan probe target a sequence conserved for all strains belonging to Mycobacterium tuberculosis complex. The reaction of 20 µl final volume consisted of 10 µl Hot Start-Mix qPCR 2x, 1 µl MTplex dtec-qPCR-mix, 4 µl DNase/RNase free water and 5 µl DNA sample., the reaction conditions consisted of one cycle of 95˚C for 5 min followed by 45 cycles of 95˚C for 30 sec and 60˚C for 60 sec for hybridization, extension, and data collection. The reaction was run in Applied Biosystem StepOne™ Real Time PCR System and FAM fluorogenic signal was collected and the cycle threshold of the reactions was detected by StepOne™ software version 2.2.2 (Life Technology).

The threshold cycle (CT) was defined as 10 times the standard deviation of the mean baseline fluorescence emission calculated for PCR cycles 3–15. For a sample to be considered positive, the corresponding amplification curve had to exhibit three distinct phases (geometric, linear, and plateau) that characterize the progression of the PCR reaction.

The gamma interferon assay:

BY Using gamma interferon assay kit and Sensitizing antigens (Sensitizing PPD-B and Sensitizing PPD-A-Prionics- Germany). The test was applied on the whole blood samples of tuberculin positive cows and the same number of tuberculin negative in contact cows. The test was standardized and vitrificated as the instruction of the kit.

Stage one: whole blood culture method:

Blood samples collection: Five ml of each blood sample was collected into heparinized blood collecting tubes. Gently the blood was mixed and inverted it for several times, then the samples were transported to the laboratory within 12 hours. Aliquot of heparinized blood (1.5 ml) from each sample was dispensed into 24 well tissue culture tray, then 100 µl of PBS was added (nil antigen control), avian PPD and bovine PPD using aseptic technique to the appropriate 3 wells containing the tested blood, the plate was shacked ten times clockwise or anticlockwise on a flat smooth surface and avoid frothing the blood. The tissue culture trays were incubated, containing blood and antigens for 16-24 hours at 37°C in a humidified atmosphere and of the 24-wells trays were centrifuged at 500 g for 10 min., carefully, approximately 500 µl plasma were removed from above the sedimented red cells. Each sample was expressed in duplicate.

Stage two: Bovine IFN-γ EIA

Freeze dried components of the used reagents were reconstituted and 50 µl of green diluents was added to the required wells, then 50 µl of test and control samples was added to the appropriate wells containing green diluents.

Control samples were added to each plate, then the plates were mixed, and each plate was covered with a lid and incubated at room temperature on a plate shaker at a setting of 600 rpm for 60 min. The plates were washed with wash buffer and 100 µl of freshly prepared conjugate reagents was added to wells.

Freshly prepared enzyme substrate solution (100 µl) was added, the plates, mixed and covered each plate with a lid and incubate at room temperature on a plate shaker at a setting of 600 rpm for 60 min. and 50 µl of enzyme stopping solution was added to each well, and then mixed by gentile agitation. Read the absorbance of each well was carried out within 5 min. by using a 450 nm filter (with a 620-650 nm reference filter). The

absorbance values were then used to calculate the results.

Interpretation of the results. The interpretation of the obtained results of Gamma interferon assay for the detection of bovine tuberculosis was carried out according to the following equation:

Positive= OD bovine PPD - nil antigen ≥ 0.1; and OD bovine PPD- avian ≥ 0.1

Negative = OD bovine PPD - nil antigen ≤ 0.1; and OD bovine PPD- avian≤ 0.1

Results

Results of tuberculin test showed that: Fifty-seven cows out of 1050 tested cases were tuberculin positive (reactors) with a percentage of 5.4%.

The conventional culture technique was carried out on the infected tissues of tuberculin positive cows and the results revealed that 31 out of 57 cows were harbored the acid-fast bacilli with a percentage of 54.4 % (table, 1).

The results of RT-PCR showed that 49 out of 57 tuberculin positive cows were positive and the amplification blot represent the results is shown in figure 1.

Table 1. Bacteriological findings of different samples of tuberculin positive and tuberculin negative cows by different diagnostic techniques.

Animal status		No. of samples	Bacteriological finding			
Samples			Microscopic examination	Culture method	PCR	IGRAs
Tuberculin (+)	Whole blood	57	--	--	--	49
	Lymph nodes	57	27	31	49	--
Tuberculin (-)	Whole blood	57	--	--	--	3

As regards to the use of gamma interferon assay on the 57 whole blood of tuberculin positive cows and 57 contact tuberculin negative cows, results showed that there were 49 infected cows with a percentage of 86%.

The suspected lymph nodes and suspected organs of all these animals were subjected to P/M examination and the isolation of mycobacterial species on the specific culture media and use of RT -PCR for confirmation. Results showed that 3 cows proved to be tuberculous out of 57 tuberculin negative cows and confirmed by P/M examination and isolation of mycobacterium species as well as using RT -PCR.

Table 2. Sensitivity results of the used diagnostic techniques of the tuberculin positive cows (57 cases).

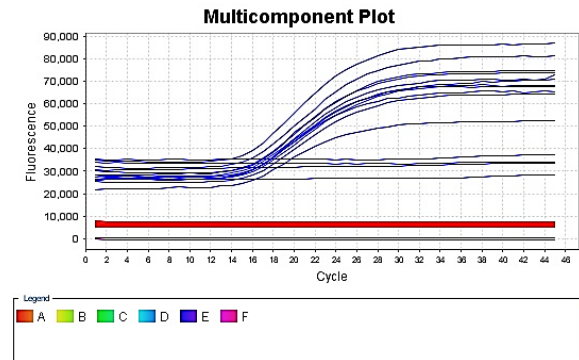| Method of diagnosis	Bacteriological findings		Sensitivity
	Negative	Positive	
Culture method	26	31	54.4 %
Microscopical ex.	30	27	47.4 %
PCR	8	49	86 %
Igras	8	49	86 %

Fig. 1. The amplification blot of RT -PCR positive samples.

Analysis for the amplification blot in its linear form:

1- The figure represents positive samples at cycle 12 and one control positive sample.
2- Three negative samples and one control negative sample.
3- The used reference dye is (FAM).
4- The run is for 45 cycles.

Discussion

Early detection of bovine tuberculosis in infected cattle is important to eliminate the infected animals from a herd, adequate measure to localize the outbreak of infection and consequently to reduce the risk that post by animals and animal products which might be source of infection for human must be considered (Anaelon et al. ,2011) The diagnosis of bovine tuberculosis is based on routine testing with the intradermal tuberculin test. Skin tests require containment of the animals on two occasions with a 72-h interval for measuring the thickness of the skin fold (Brasil,2004) Management is hampered in herds raised extensively or on farms without adequate facilities, which is a common situation in developing countries. This difficulty is probably one of the main reasons for why epidemiological surveys of tuberculosis planned in the eradication program are in

a less advanced stage than those for brucellosis. The results of tuberculin skin test showed low sensitivity and specificity as it revealed 57 positive cows out of 1050 tested animals, meanwhile the animals that give positive skin test and CMI positive test should be considered highly suspect of being infected. Such subclinical infected animals may account for the significant number of reactor cattle appearing at slaughter without lesions. Furthermore, these subclinical reactor cattle could, over time, develop disease, contributing to a significant number of herd breakdowns which are difficult to attribute to a precise source. Meanwhile, McIlroy et al. (1986) reported the sensitivity as 92.7%, which may compare to other similar studies, obtained a figure of 87%. and a sensitivity of 89.3%. Reports indicate the very low percentage of positive reactors detected during the national control program during the study conducted by (Borham et al.,2021). Elsohaby et al (2020) assessed the accuracy of diagnostic tests for the diagnosis of bTB and used Many diagnostic tests, including culture, histopathology, PCR, and SCT. The conventional culture technique was carried out on the infected tissues of tuberculin positive cows and the results revealed that 31 out of 57 cows were harbored the acid-fast bacilli with a percentage of 54.4%. The sensitivity of the cultural method was discussed by Taylor et al. (2001) who mentioned that the isolation of mycobacteria and their identification based on phenotypical characters. Particularly, culture methods have been constantly used as a "golden standard" but because these methods are much more time consuming, their use is on the decline (Thoen et al., 2006) and they have been replaced by other diagnostic methods. As regards to isolation of mycobacterium species by cultural method, the viability of M. Bovis in the organs samples is essential. The viability could be reduced by either freezing/unfreezing practices or by treatment with the selected decontaminant reagent, which is not completely innocuous for M. Bovis. One of the main factors influencing the success of primary isolation of M. Bovis from clinical specimens is the type of the culture media used. The times at which the M. Bovis colonies were detected in the tubes were considerably longer than those earlier reported (Bennett and Cooke, 2006). The low frequency at which the slants were observed (60 d) could justify a certain delay for the colony detection. A factor that influences the successful mycobacterial isolation is the decontamination procedure. No ideal reagent has

considered the sort of samples handled and the way they were obtained. No many studies exist in the literature about this matter, including the same culture media, similar samples, and the same decontamination product (Dimitri et al., 1996). However, the successful results of culture might be related rather to a proper sample collection and preservation (especially the integrity of lymph node connective capsules and refrigeration of samples quickly after collection) than only to decontamination procedure. However, the results of RT-PCR showed that 49 out of 57 tuberculin positive cows were tuberculous in a rapid and more accurate assay (Soliman et al.,2017, Lilebaum et al.,2001, Taylor et al., 2001 and Tortoli et al., 2001). According to the obtained results of this study, the PCR technique is more sensitive than culture and direct smear. The high sensitivity in addition to the potential for rapid detection of M. tuberculosis complex, makes this test a useful tool for detection of M. tuberculosis complex. As regard to the sensitivity percentage of each used techniques, the gamma interferon assay and PCR assay revealed the highest sensitivity percentage reached 86% ,followed by culture method and microscopically examination as 54.4% and 47.4% respectively .Interferon gamma assay is OIE listed as an alternative test for international trade (Anon, 2008) and approved as a complementary bTB test as described in USDA, APHIS, Bovine Tuberculosis Eradication, Uniform Methods and Rules, and by the European Union (Anon, 2002). This in vitro assay is a laboratory-based test detecting specific cell-mediated immune responses by circulating lymphocytes. Briefly, the assay consists of two stages. Firstly, the heparinized whole blood is incubated with antigens (i.e., PPDs, specific antigens) for 18–24 h. Antigenic stimulation induces production and release of IFN-c by predominantly T lymphocytes. Secondly, the IFN-c present in the plasma supernatants is quantified in a sandwich ELISA. With PPDs, a differential optical density (OD) value is determined by subtracting the OD value achieved with PPD-A stimulation from that of PPD-B. The IFN-c assay (Bovigam, Prionics,) is being incorporated into bTB eradication programs in many countries (De la Rua-Domenech et al., 2006) either in a serial testing regime as confirmatory test after CFT to enhance specificity or in a parallel testing regime to enhance sensitivity of TSTs. In this study ,49 cows out of 57 tuberculin positive cows were tuberculous by IFN assay. Advantages of the IFN-c

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assay are its increased sensitivity, the possibility of more rapid repeat testing, no need for a second visit to the farm and more objective test procedures and interpretation in comparison to the TST. IGRAs offer the possibility of identifying BTB positive herds using sera collected for other epidemiological studies, such as for brucellosis, prior to tuberculin skin tests, thereby increasing the diagnostic coverage (Vordermeier et al., 2008 and Buddle et al., 2001). The results of IGRAs revealed the positivity of 3 tuberculin negative cows. This is another useful aspect in the detection of cattle in advanced stages of tuberculosis with false-negative results on the skin test. These findings agree with that reported by Whelan et al.,2004 and Gormley et al., 2004).

Conclusion

The results presented in this study indicate that the IGRAs method improves tuberculosis diagnosis, with the advantage that the method is rapid and more sensitive than other methods. IGRAs could be applied and used as a routine diagnostic test.

References

Al-Gaabary, Magdy. (2021). Abattoir survey of bovine tuberculosis in Tanta, center of the Nile Delta, with in silico analysis of gene mutations and protein-protein interactions of the involved Mycobacteria. Transboundary and Emerging Dis. 2021:00:1-17

Algammal, A. M., Wahdan, A., & Elhaig, M. M. (2019). Potential efficiency of conventional and advanced approaches used to detect Mycobacterium bovis in cattle. Microbialpathogenesis,134, 103574.

Anaelon, Nwanta, IKechukwuJ. Onunkwo, Sundy W. Ezemaand Nnaemekac. Umeononigwe (2011). Zoonotic tuberculosis: A review of epidemiology, clinical presentation, prevention, and control. Journal of public Health and Epidemiology.2(6), pp.118-124.

Anon (2002). Commission decision of 8 juli in Amending Annex B to council directive 64 /432.E.E.C

Anon, (2008). Bovine Tuberculosis. Diagnostic techniques. OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals. 2.4.7., 686–689.

Bennett RM and Cooke RJ. (2006). Costs to farmers of a tuberculosis breakdown. Vet. Rec, 158:429–432. biology, 141:2131–2138.

Borham M., A. Orabiey, A. El-Gedawy, Y. Hegazy, M. AL-Gaabary (2021). Tuberculin test errors and its effect on detection of bovine tuberculosis. J hellenic vet med soc 2021, 72(4) πεκε 2021, 72(4).

Brasil: Secretaria de Defesa Agropecuária, Ministério da Agricultura, Pecuária e Abastecimento. Instrução Normativa Nº 6 de 8 de janeiro de (2004). Approve o Regulamento Técnico do Programa Nacional de Controle e Erradicação da Brucelose e Tuberculose Animal. Diário Oficial da União, Brasília; 2004:6–10.

Buddle BM, Ryan TJ, Pollock JM, Andersen P, de Lisle GW (2001). Use of ESAT-6 in the interferon-gamma test for diagnosis of bovine tuberculosis following skin testing. Vet Microbiol. 3;80(1):37-46.

De la Rua-Domenech, R., A.T. Goodchild, H.M. Vordermeier, R.G. Hewinson, K.H. Christiansen, and R.S. Clifton-Hadley, (2006). Ante mortem diagnosis of tuberculosis in cattle: a review of the tuberculin tests, gamma-interferon assay, and other ancillary diagnostic techniques. Res. Vet. Science 81,190–210

Dimitri, R.A.; Husseinen, H.S.,;Mikhail , D.C. and Abbas , A.M.(1996). Detection of tuberculosis in slaughtered animals by PCR. J. Egypt. Vet. Med. Assoc., 56: (4).

Djemal, S.E., Camperio, C., Armas, F. et al. (2018). Detection of a streptomycin-resistant Mycobacterium bovis strain through antitubercular drug susceptibility testing of Tunisian Mycobacterium tuberculosis complex isolates from cattle. BMC Vet Res 14, 296.

Gormley, E., M.B. Doyle, K. McGill, E. Costello, M. Good, and J.D. Collins, (2004). The effect of the tuberculin test and the consequences of a delay in blood culture on the sensitivity of a gamma-interferon assay for the detection of Mycobacterium bovis infection in cattle. Vet. Immunol. Immunopathol. 102, 413–420.

Ibrahim Elsohabya, Yasser S. Mahmmoda, Marshal M. Mweud, Heba A. Ahmede, Mohamed M. El-Diastyf, Attia A. Elgedawyg, Eman Mahrousg, Fatma I. El Hofy (2020). Accuracy of PCR, mycobacterial culture and interferon-γ assays for detection of Mycobacterium bovis in blood and milk samples from Egyptian dairy cows using Bayesian modelling. Preventive Veterinary Medicine 181. (2020), 105054.

Ibrahim Elsohaby, Jawher I. Alahadeb, Yasser S. Mahmmod, Marshal M. Mweu, Heba A. Ahmed, Mohamed M. El-Diasty, Attia A. Elgedawy, Eman Mahrous and Fatma I. El Hofy (2021). Bayesian Estimation of Diagnostic Accuracy of Three Diagnostic Tests for Bovine Tuberculosis in Egyptian Dairy Cattle Using Latent Class Models. Vet. Sci. 2021, 8, 246.

Kuria, J. K. (2019). Diseases Caused by Bacteria in Cattle: Tuberculosis. In: Kaoud, H. A. h. E., editor. Bacterial Cattle Diseases [Internet]. London: IntechOpen;

Lilebaum W, Pessolani MCV, Fonseca LSF. (2001). The use of Ag85 complex as antigen in elisa for the diagnosis of bovine tuberculosis in dairy cows in Brazil. Journal of Veterinary Medicine., 48:161–166.

Liu S, Guo S, Wang C, Shao M, Zhang X, Guo Y, Gong Q. (2007). A novel fusion protein-based indirect enzyme-linked immunosorbent assay for the detection of bovine tuberculosis. Tuberculosis, 87:212–217.

McIlroy, S.G., S.D. Neill and R.M. McCracken, (1986). Pulmonary lesions and Mycobacterium bovis Identification of Mycobacterium bovis in bovine excretion from the respiratory tract of tuberculin clinical samples by PCR species-specific primers. Vet Record, 118: 718-721.

Michel AL, Cooper D, Jooste J, de Klerk LM, Jolles (2011). Approaches towards optimizing the gamma interferon assay for diagnosing Mycobacterium bovis infection in African buffalo (Syncerus caffer). Prev Vet Med, 98:142–151

Borham M, Oreiby A, El-Gedawy A, Hegazy Y, Hemedan A, Al-Gaabary M. (2021). Abattoir survey of bovine tuberculosis in tanta, centre of the Nile delta, with in silico analysis of gene mutations and protein-protein interactions of the involved mycobacteria. Transbound Emerg Dis. 2021 Jan 23.

OIE- world organization for animal health. Bovine tuberculosis. (2009). In: Manual of diagnostics tests and vaccines for terrestrial animals. V. 1, pt. 2, sec. 2.4, chap. 2.4.7.

Ratledge C. Nutrition, growth, and metabolism. En: Ratledge C, Stanford J, editors. The biology of mycobacteria. London: United Kingdom Academic Press; 1982. p. 185-271.

Riad, E.M. Fareed, A.F., Tanio, A.I. (2014). standardization of the most accurate methods of DNA extraction from tissues of tuberculous animals. conference of scientific assoc. of animal health institute 2-6-2-2014.111-116.

Romha, G., Gebru, G., Asefa, A., & Mamo, G. (2018). Epidemiology of Mycobacterium bovis and Mycobacterium tuberculosis in animals: Transmission dynamics and control challenges of zoonotic TB in Ethiopia. Preventive veterinary medicine, 158,1-17.

Soliman, Y. A., El-Hamshary, M., & Rasmy, M. (2017). Molecular characterization of mycobacterial isolated from human and animals. Research Journal of Applied Biotechnology, 3(1), 28-35.

Taylor M.J., Hughes M.S., Skuce R.A., Neill S.D. (2001). Detection of Mycobacterium bovis in bovine clinical specimens using real-time fluorescence and fluorescence resonance energy transfer probe rapid-cycle PCR. Journal of Clinical Microbiology, 39, 1272–1278.

Thoen.c.o., steele j.h., Gilsdorf m.j. (2006). Mycobacterium bovis infection in animal and human .2nd ed. Blackwell publishing professional, Anes, Iowa, usa.317pp.

Tortoli E., Nanetti A., Piersimoni C., Cichero P., Farina C., Mucignat G., Scarparo C., Bartolini L., Valentini R., Nista D., Gesu G., Tosi C.P., Crovatto M., Brusarosco G. (2001). Performance assessment of new multiplex probe assay for identification of mycobacteria. Journal of Clinical Microbiology, 39, 1079–1084. 1, Academic Press, London, New York, Pairs, San Diego.

Van Soolingen, D., P.W.M. Hermans, P.E. de Haas, D.R. Soll and J.D.A. van Embden, (1991). Occurrence and stability of insertion sequences in Mycobacterium tuberculosis complex strains: evaluation of an insertion sequence-dependent DNA polymorphism as a tool in the epidemiology of tuberculosis. Clin. Microbiol. J., 29: 2578-2586.

Vordermeier, H. M., Jones, G. J., Buddle, B. M., Hewinson, R. G., & Villarreal-Ramos, B. (2016). Bovine tuberculosis in cattle: vaccines, DIVA tests, and host biomarker discovery. Annual review of animal biosciences, 4,87-109.

Vordermeier HM, Whelan AO, Hewinson RG. (2008). The scientific case for the gamma-interferon Bovigam assay. Gov Vet J. 19:38–43.

Vordermeier, H.M., A. Whelan, K. Ewer, T. Goodchild, R. Clifton-Hadley, J. Williams, and R.G. Hewinson, (2006). The BOVIGAM assay as ancillary test to the tuberculin skin test.Gov. Vet J. 16, 72–80.

Wards B.J., Collins D.M., De Lisle G.W. (1995). Detection of Mycobacterium bovis in tissues by polymerase chain reaction. Veterinary Microbiology, 43, 227–240.

Whelan, A.O., M. Coad, Z.A. Peck, D. Clifford, R.G. Hewinson, and H.M. Vordermeier, (2010). Influence of skin testing and overnight sample storage on blood-based diagnosis of bovine tuberculosis. Vet. Rec. 155, 204–206.

WHO (2017). Global tuberculosis report. WHO, 2017.

Wobeser C (2009). Bovine tuberculosis in Canadian wildlife: An updated history. Can J Vet Res, 50:1169–1176.