Global Veterinaria 18 (3): 151-157, 2017
ISSN 1992-6197

DOI: 10.5829/idosi.gv.2017.151.157

Uses of Staphylococcal Protein A "SPA" and Streptococcal Protein G "SPG" for Diagnosis of Bovine Tuberculosis

[1]F.H. Omnia and [2]M.A.M. Saad

[1]Animal Health Research Institute,
Bacteriology Department -TB unit, Dokky, Giza, Egypt
[2]Animal Reproduction Research Institute, Haram, Giza, Egypt

Abstract: Bovine tuberculosis (BTB) is a major infectious and zoonotic disease; mainly of developing countries. Several methods for diagnosis were presently in use. Serological tests are still relatively, simple, rapid and of low cost. In the present study the diagnosis of BTB in cows was depending on two immune responses either cellular or humoral based on PPD antigen. A slide-based co-agglutination (SCAT) test was developed using the non-species specific diagnostic tools staphylococcal protein A (SPA)or streptococcal protein G (SPG); either separately or in combination. The degree of positivity for agglutination was rated from positive (+) to very strong (++++) and was measured over a range of reaction times. Agglutination time ranged from two to eight minutes. Eighteen serum samples taken from infected cows; positive by Tuberculin skin test (TST), indirect ELISA and gamma interferon release assay "Bovigam" were tested. Eleven (61%) were positive using SPA while 15 (83%) were positive with both SPG alone and with the two reagents used in combination. SPA/G conjugated with fluorescein (FITC) was also used as a confirmatory test (SPA/G-FAT).A second group of six samples, TST positive and Bovigam negative, two of the six samples were SCAT positive by SPG. One of these two was also positive by SPG-FAT. Using these two immunodiagnostic proteins, SPA and SPG, inparallel with other diagnostic methods "TST, iELISA and IFN-γ assay gave good diagnostic specificity and sensitivity for distinguishing infected from uninfected cows. Further studies are needed to investigate and evaluate these findings to improve diagnosis of BTB. A simple, low-cost, rapid, specific and sensitive field test is needed to achieve effective disease control. This slide based test could easily be used in a field setting and would be a practical, low cost, high-throughput assay that avoids the need for species-specific reagents.

Key word: Bovine tuberculosis · Serological tests · Tuberculin skin test · Immunodiagnostic proteins

INTRODUCTION

Tuberculosis (TB) affects one-third of the world's population and is a serious health problem worldwide. *Mycobacterium tuberculosis* (MTB) infects about 8.8 million people annually. In Egypt, MTB has been estimated to infect18 per 100,000 of the population and the mortality rate is 0.82 per 100 [1].

BTB is a common zoonotic disease, caused by *Mycobacterium bovis* (*M. bovis*) and it is responsible for significant economic losses worldwide. Diagnosis is based on the detection of cell mediated immunity in response to tuberculin; a purified protein derived from *M. bovis* (PPD)[2].

*M. bovis*isa member of the mycobacterium tuberculosis (TB) complex; a range of mycobacteria that causes TB in different animal species. Cattle are the primary host for *M.bovis* infection [3].

Bacterial culture is considered the gold standard for TB diagnosis; having close to 98% specificity. However, mycobacteria are slow-growing, with 6–8 weeks being required for culture growth and trained personnel and appropriate culture facilities are needed. The need for technical expertise can be particularly problematic in developing nations [4].

Serological diagnosis of TB is based on detection of circulating antibodies (Humoral immune response). The major advantages of antibody-detection tests are that

Corresponding Author: Omnia Fekry, Animal Health Research Institute –
Bacteriology Department -TB unit, Dokky, Giza, Egypt.

they are inexpensive and relatively easy to perform. However, low sensitivity of the antibody-detection tests remains a concern. Several attempts have been made to develop an ELISA for detection of antibody response against *M. bovis* infection. Purified protein derivative (PPD) was used as an antigen to measure antibody responses in animals with experimental BTB infection [5, 6] but the cross reactivity of PPDB with closely related mycobacterial species has always been a concern [7].

The Tuberculin Skin Test (TST) is by far the most effective test used in the eradication of BTB in developing countries. The test is performed by injecting a small volume of PPDB under the skin of the animal and measuring a change in the thickness of the skin at the site of injection after 48–72 hours. However, false-positive reactions are possible owin gto exposure to environmental mycobacteria such as *M.avium* (MA) and MA subspecies *paratuberculosis* (MAP). TST can also cause false-negative reactions due to immunosuppression, desensitization towards tuberculin, sub potent use of tuberculin and lengthy exposure to a field strain [8].

Purified protein derivative (PPD) is currently the only available skin test reagent used worldwide for the diagnosis of tuberculosis (TB) [9].

Staphylococcal protein– A (SPA) is a protein bound to the cell wall of the pathogenic bacterium *Staphylococcus aureus* and it binds to the Fc portion of most mammalian immunoglobulin G (IgG). Therefore, this protein has a unique potential for making "Universal" immunoassays [10]. Some strains of *S. aureus* synthesize protein - A, a group-specific ligand that binds the Fc region of IgG from many species. It binds to human, mouse, rabbit, cat, cow, dog, goat, guinea pig, horse and sheep IgG but binds only very weakly to hamster IgG and not at all to chicken [11]. SPA is increasing importance as a tool in both quantitative and qualitative immunological techniques. SPA remains markedly stable on exposure to high temperatures, low pH and denaturing agents [12, 13].

Streptococcal Protein-G SPG is an immunoglobulin-binding protein expressed in group C and G streptococcal bacteria; much like protein A but with differing specificity. The native molecule also binds albumin, however, because serum albumin is a major contaminant of antibody sources-,the albumin binding site has been removed from recombinant forms of protein G [5].

Fluorescent antibody dyes are commonly employed as labels in primary binding tests; one of the most important being fluorescein isothiocyanate (FITC). FITC is a yellow-coloured chemical that can be bound to antibodies without affecting their reactivity. When irradiated with invisible ultraviolet light, FITC emits violet green light, so FITC- labeled reagents are used in both direct and indirect fluorescent antibody tests [14]. Fluorescent microcopy is more sensitive, but its application is limited by high cost and by issues relating to the use of mercury vapour lamps in conventional fluorescent microscopes [15].

The purpose of this study was to develop alternative serodiagnostic assays for BTB and to investigate the effectivity of SPA and or SPG as immunoglobulin binding proteins.

MATERIALS AND METHODS

Serum Samples: Table (1): BTB infection in cattle remains a major cause of zoonotic and economic problems. BTB may be diagnosed using culture of suspect tissues or through a range of *in vitro* assays designed to detect either antibody or cell-mediated immune responses. Antibody responsesare useful indicators of BTB.

In this study, the aim was to compare the diagnostic efficacy of two serological tests for BTB infection with that of TST, Bovigam and iELISA. The SCAT, using SPA and/or SPG, as non-species specific universal immune assaysand a fluorescent antibody technique (FAT), were used to test serum samples taken from animals that had already tested positive for BTB by these established techniques.

Twenty six bovine serum samples were kindly provided by the Animal Health Research Institute (AHRI) Department of Bacteriology, (Tuberculosis Unit). Sera were classified into threegroups:

Group 1: Eighteen serum samples from animals known to be naturally infected and identified as positive by TST, I ELISA and Bovigam.

Group 2: Six serum samples were taken from animals positive by TST but Bovigam negative and IELISA negative.

Group 3: Two negative control samples obtained from cows negative by TST, IELISA and Bovigam.

Tuberculin skin test: (TST): All 26 animals used for this study had been skin tested at AHRI (Animal Health Research Institute), using the reference test. PPDB was used for the bovine tuberculin skin test, resulting in 24 cows TST positive while the two healthy controls were TST negative.

Table 1: Tested serum samples for TST, INF- γ and iELISA(n=26):

		Diagnostic Tests		
Groups	Animals No	TST	INF- γ	iELISA
1	18	18 +ve	18 +Ve	18 +Ve
2	6	6 +ve	6 - Ve	2 - Ve
3	2	2 - Ve	2 - Ve	6 - Ve
Total	26	24	18	18

TST: Tuberculin skin test.

INF- γ:Gamma Interferon release test (Bovigam).

iELISA: Home Indirect ELISA.

Discordant: T.S.T positive and INF- γ negative.

+Ve: Positive, -Ve: Negative.

Home Indirect ELISA (iELISA): The ELISA plate was coated with PPDB and used to screen serum samples and to detect those positive for BTB [16]

Gamma Interferon Release Assay (IFN-γ): Whole blood samples previously collected from twenty six cows at AHRI were set up for overnight incubation *in vitro* with PPDB and cell supernatants were assayed for IFN-γ release using the commercially produced Bovigam kit.

Antigen: PPDB was used as an antigen in the two different serological techniques; rapid slide co-agglutination test with SPA and SPG and FAT and also in the TST, iELISA and Bovigam IFN-γ, release assays.

Immunodiagnostic Agents: SPA and SPG for the SCAT had previously been prepared by the Bacteriology Department (TB unit) at AHRI and the Animal Reproduction Research Institute (ARRI) according to the methods described by Kessler[17] and Sting *et al.*[18]. The degree of agglutination was evaluated in terms of a "plus" scale and was read after two – eight minutes.

Fluorescent Antibody Technique FAT: SPA and SPG conjugated to fluorescein isothiocyanate, SPA-FA and SPG-FA, previously prepared by ARRI according to Kessler [17] and Sting *et al.*[18]were used as an immunodiagnostic laboratory test, following the method of Hudson and Hay [19].

RESULTS

Results Are Summarized in Table 1: The results of SPA and SPG SCAT carried out for all three experimental groups are summarized in Table 2

SPA SCAT: Of the 18 animals in group 1, 11 (61%) were seropositive for PPDB binding by SCAT. The degree of positivity was classified by the degree of agglutination from + (Slightly positive) to 4+ (Very strongly positive). A sandy appearance was classified as ± and

Table 2: The reactivity of SPA and SPG in serodiagnosis of bovine tuberculosis

	SPA		SPG		SPA/SPG
Animals	Slide agglutination test	SPA-FAT	Slide agglutination test	SPG-FAT	Slide agglutination test
1	+	-	-	-	-
2	±	-	-	-	-
3	+++	+	++	+	+++
4	++	-	±	-	+
5	+	+	+++	+	+
6	±	-	+	-	+
7	+	+	++	+	+
8	-	-	++	+	+
9	-	+	+	+	++
10	+	-	+	+	++
11	++	+	++++	+	+++
12	+++	+	+++	+	+++
13	++++	+	+++	+	++++
14	++	+	++++	+	++
15	±	-	+	+	+
16	+	+	+++	+	+
17	-	-	+	-	-
18	-	-	+	+	+
Total +Ve	11	9	15	13	15

Reactions: (+) Positive,(++) Moderate,(+++) Strong, (++++) Very strong,(±) Suspected, Undetectable, (-) Negative (Sandy like appearance).

considered suspect but counted as seronegative. Seven animals (39%) were seronegative by SCAT, four of these being ±. Of the 11 seropositive animals, five were +, three 2+, two 3+ and one 4+.

The six TST positive, Bovigam negative animals in group 2 and the two negative control animals were all seronegative by SPA SCAT.

SPG SCAT: Fifteen out of 18(83%) animals in group 1 were seropositive by SPG SCAT. Six were +, three scored 2+, four 3+ and two 4+. The SPG test detected responses to PPDB in four more animals than SPA. Of these, three were + and one 2+. The two animals seronegative by SPG and the one classified as suspect were all seronegative by SPA. However, one of the two animals seronegative by SPG and the one ± were both seropositive by SPA.Of the six animals in group 2, TST positive but Bovigam negative, two were seropositive by SPG SCAT.

Mixed SPA/G-SCAT: A similar percentage of seropositive animals were found when SCAT using a combination of SPA and SPG was carried out. Of the three seronegative animals, one had tested positive by SPA and one positive by SPG. Eight animals were seropositive +, three scored 2+, three + and one 4+.

Fluorescent Antibody Technique FAT: The results of this assay are summarized in Table 2.

SPA-FAT: A total of 9 of the 18 serum samples (50%) were positive for BTB by SPA-FAT, compared with 11 seropositive by SPA SCAT. One animal positive by SPA-FAT had been seronegative by SPA SCAT. However, all those animals that had been 4+ or 3+ by SPA SCAT and all but one of those 2+ were also seropositive by SPA-FAT.

SPG-FAT: Thirteen of the fifteen animals from group 1 that were seropositive by SPG SCAT were also positive using SPG-FAT. The two additional seronegative animals were both scored as + by SPG SCAT.

DISCUSSION

M.bovis is the primary causative organism of BTB. While this organism is specifically adapted to infect cattle, it is also infectious to humans and is of significant zoonotic importance.Most animals infected with TB show no clinical signs of the disease [20, 21].

Cows were chosen for this study as they are the primary host of BTB infection. The animals tested had already been diagnosed positive for BTB by three *ante mortem* tests, TST, iELISA and Bovigamas summarized in Table 1.

TST is used worldwide for the diagnosis of BTB and is based on the *in vivo* intradermal inoculation of purified protein derivative from *M.bovis*(PPDB) [22]. It is the most effective tool used in the eradication of BTB in developing countries [7].

The standard diagnostic test for *M. bovis* is the intradermal tuberculin tests still in wide spread use. This test is both sensitive and specific [23 24] and remains in widespread use. However, the frequency of false reactions has been considered to limit the value of the test somewhat [23, 25].

Antibody ELISAs have been widely used over the years for diagnosis of BTB. It has been suggested that they could be used as a complement to tests based on cellular immunity [24, 26,27].Many antigens have been employed in ELISA. They include complex antigens such as purified protein derivative (PPDB). However, ELISA using PPDB as the antigen although sensitive and able to detect antibodies to mycobacteria, lacks specificity [6, 28].

A home iELISA using a plate coated with PPDB has been validated as a sensitive and specific serological method for useas a laboratory test to confirm the results obtained by TST [29].

The Bovigam test quantifies *in vitro* release of IFN-γ following whole blood culture under PPD-B stimulation[22].However, this assay is costly and requires well-trained personnel to carry out the test [4]. The IFN-γ test, as most commonly used, is generally accepted to have a higher sensitivity than single comparative intradermal skin test. However, its use as general screening tool is not advocated because of issues with specificity [21]. The specificity of the PPDB based gamma interferon test is not as high as single intradermal tuberculin test 31]. (In our study, out of the 26 animals tested, 24 were BTB positive by TST but six of these were Bovigam negative and may be described as discordant.

While the initial IFN-γ assays were based on PPD antigen [25] epitope- defined IFN-γ assay kits have now become established for the specific detection of infection with tubercle bacilli in both humans and cattle [35),

The test kits need to be refrigerated during storage and transportation, This may difficult for veterinary clinics in remote areas. Additionally, after the blood samples collected, they require specific handling, such as shaking the tubes and incubating the blood samples within specific time period.

Immunoglobulin binding proteins have been used in a large number of indirect primary assays, especially in veterinary diagnostics because of their applicability to multiple species. Thes erodiagnostic tests used in this study were SPAand SPG SCAT and FAT (SPA-FAT and SPG-FAT) and were designed to detect humoral antibodies directed against [23].

SPA and SPG SCAT were read by eye and the results evaluated according to the degree of agglutination. This could mean that some of the discrepancies detected between the parallel tests were attributable to operator error.

In this study, we have used a simple, rapid, low cost, sensitive, specific, stable and visually detectable SPA and SPG SCAT test that is readable after two to eight minutes. SPA and SPG are proteins that will bind to the Fc portion of most mammalian class G IgG. They therefore have a unique potential for use inuniversal immunoassays involving both quantitative and qualitative immunological techniques [32]. We have also conjugated SPA and SPG to FITC and used these reagents in a FAT assay for antibodies to BTB. We have assessed these assays against the established measures of immune responses to BTB, TST and the *invitro* IFN- γ assay.

Compared with some other serological tests, these assays may be useful in improving the control of BTB through rapid, sensitive &specific and low cost immunodiagnostic tests. However, SPA/G SCAT would appear to detect more infected animals than FAT.

There is currently no single test that will fulfill all the criteria necessary to identify all infected animals [21]. Serological tests have several advantages over other methods including short turn- around time, relativity simple procedures and low cost. However, the existing serodiagnostic methods for BTB are of low sensitivity and specificity [25]. The serological assays used in this study could be used as ancillary methods for the detection of infected animals [33].

The two serological techniques used here were aiming to improve the detection of humoral immune response -to BTB. Two bacterial cell wall proteins known to bind to IgG were used in agglutination (SPA and/or SPG SCAT) and fluorescence (SPA or SPG FAT) assays. SPA/SPG SCAT and FAT both use inexpensive reagents, do not require the production of specific antisera and would therefore be cheaper to use than other serodiagnostic techniques. The SCAT tests were the most sensitive, with SPG being the superior reagent, detecting 83% of infected animals [34]. The superior sensitivity of SPG is probably owing to its reacting with both IgG1 and IgG2 in sera. Native SPG will also bind to albumin but the albumin binding site is removed from the recombinant protein.

Fluorescent labeling is a useful technique owing to its high sensitivity and to the ease of integrating a label onto the marker [35]. Protein A is capable of binding to the Fc portion of immunoglobulins, especially IgGs, from a large number of species.

Fluorescent dyes are commonly employed as labels in primary binding tests. One of the most commonly used is FIT which emits violet green light on stimulation with UV. FITC-labeled anti bodies are used in both direct and indirect fluorescent antibody testing [36].Most serodiagnostic tests such as FAT use specific antibodies. However, we have shown that SPA,SPG or a combination of the two can be used effectively as non-species specific immunodiagnostic tools.

Ourattempts to improve diagnostic testing indicate that the multiple antigens and recombinant PPDB as well as uses of recombinant streptococcal protein G may improve the accuracy of rapid field serodiagnosis of BTB.

Co-infection with parasites, most notably liver fluke and other mycobacterial diseases such as John's disease influence the diagnostic sensitivity of both the comparative skin and the interferon gamma test [37]. PPDB was used as an antigen to measure antibody responses in animals with BTB infection but the cross reactivity of PPDB with closely related mycobacterial species has always been of concern [6].

CONCLUSIONS

This study has attempted to develop a rapid, accurate and low cost diagnostic tool for detection of BTB infection in the field that would improve on existing field tests. Our findings suggest that the SCAT assay, using PPDB and a combination of SPA and SPG could be of value as a primary screen in the diagnosis of BTB in cattle.

REFERENCES

1. WHO, 2010. Global tuberculosis control Global tuberculosis control.Preparation and Conjugation of Staphylococcal proteinA and streptococcal protein G with fluorescein isothiocyanate as non-species specific diagnostic kits.

2. Eirin, M.E., A. Macias, G. Magnano, C. Morsella, L. Mendez, F.C. Blanco, M.V. Bianco, W. Severina, A. Alito, M. Pando, L. De, M. Singh, R. Spallek, FA. Paolicchi, F. Bigi and A.A. Cataldi, 2015. Identification and evaluation of new Mycobacterium bovis antigens in the in vitro interferon gamma release assay for bovine tuberculosis diagnosis.Tuberculosis (Edinb),95(6):795-801. doi: 10.1016/j.tube.2015.07.009. Epub 2015 Aug 8.
3. vanIngen, J., Z. Rahim, A. Mulder, M.J. Boeree, R. Simeone, R. Brosch and D. van Soolingen, 2012. Characterization of Mycobacterium orygis as M.tuberculosis complex subspecies. Emer. Infect. Dis., 18; 653-655.doi: 10.3201/eid1804.110888.
4. Buddle, B.M., P.G. Livingstone and G.W. De Lisle, 2009. Advances in ante-mortem diagnosis of tuberculosis in cattle.New Zealand Veterinary Journal, 57(4): 173-180.
5. Thoen, C.A., M.R. Hall, A. Tannis, B.S. Petersburg and R. Harrington, 1983. Detection of mycobacterial antibodies in sera of cattle experimentally exposed to Mycobacterium bovis by use of a modified enzyme linked immunosorbent assay. In The Proceedings of the 26th Annual Meeting of the American Association of Veterinary Laboratory Diagnosticians, Las Vegas, Nov, USA, pp: 25-38 October. 6-Lilenbaum, W., E.R. Ribeiro, G.N. Souza, E.C. Moreira, L.S. Fonseca, M.A. Ferreira and J. Schettini, 1999. Evaluation of an ELISA-PPD for the diagnosis of bovine tuberculosis in field trials in Brazil.Research in Veterinary Science, 66(3): 191-195.
7. Anon, 2008. "Bovine tuberculosis" in:Manual of Diagnostic Tests and Vaccines for Terrestrial Animals, chapter, 2.4.7:686-689, OIE 2008.
8. DeDent, A., M. McAdow and O. Schneewind, 2007. Distribution of protein A on the surface of staphylococcus aureus. Journal of Bacteriology, 189(12): 4473-4484.
9. Stavri, H., N. Bucurenci, I. Ulea, A. Costache, L. Popa and M.I. Popa, 2012. Use of recombinant purified protein derivative (PPD) antigens as specific skin test for tuberculosis. Indian J. Med. Res., 136: 799-807.
10. Aslam, M. and A. Dent, 1998. Bio conjugation protein coupling techniques for the biochemical sciences. Macmillan Ltd.U.K.
11. Alan, J. and Rabinthorpe, 1996. Coupling antibodies to fluorochrome enzymes and biotin preparation of S.aureus adsorbent. Immunochemistry in practice, 3rded. pp: 249-251&277-279.
12. Montassier, H.J., J.P. AraújoJúnior and A.A. Pinto, 1994. Rapid co agglutination test for the detection and typing of foot and mouth disease virus. J, Virol. Meth. Dec, 50(1-3): 29-41.
13. Andrea, C.D., M.A. Molly and S. Olaf, 2007. Distribution of protein A on the surface of staphylococcus aureus. Journal of Bacteriology, 189(12): 4473-4484.
14. Bhaskar, S., J.N. Banavaliker and M. Hanif, 2003. Large- scale validation of a latex agglutination test for diagnosis of tuberculosis. FEMS Immunology and Medical Microbiology, 39: 235-239.
15. Minion, J., A. Zwerling and M. Pai, 2009. Diagnostics for tuberculosis: what new knowledge did we gain through the International Journal of Tuberculosis and Lung Disease in 2008.International Journal of Tuberculosis and Lung Disease, 13(6): 691-697.
16. Aagaard, C., M. Govaerts, V. Meikle, A.J. Vallecillo, J.A. Gutierrez-Pabello, F.S. Giemes, J. McNair, A. Cataldi, C. Espitia, P. Andersen and J.M. Pollock, 2006. Optimizing antigen cocktail for detection of Mycobacterium bovis in herds with deferent prevalence of bovine tuberculosis ESAT – 6 CFP10 mixture show optimal sensitivity and specificity. J. Clin. Microbiol., 44: 4326-4335.
17. Kessler, S.W., 1975. Rapid isolation of antigens from cells with staphylococcal protein A antibody adsorbent parameters of the interaction of antibody antigen complexes with protein. A.J. Immunol., 115(6): 1617-1624.
18. Sting, R., L.L. Lauerman and H. Blobel, 1990. Isolation of protein A and protein G from the bacterial surface. Zbl.Bakt, 273: 306-312.
19. Hudson, L. and F.C. Hay, 1989. Practical Immunology 3rded. Blackwell, Oxford.
20. Michel, A.L., B. Müller and P. D.vanHelden, 2010. Mycobacterium bovis at the animal-human interface: a problem, or not?Vet. Microbiol. 140(3-4): 371-81. doi: 10.1016/j.vetmic.2009.08.029. Epub 2009 Sep 2.
21. Sam Official Veterinarian Practice Guide, 2011. APHAahvla.defra.gov.uk/…/sam/Sam-Official-Veterinarian-Practice-Guide.pdf
22. Pollock, J.M., B.M. Buddle and P. Andersen, 2001. Towards more accurate diagnosis of bovine tuberculosis using defined antigens. Tuberculosis. (Edinb.), 81: 65-69.
23. Rothel, I.S., S.L. Jones, L.A. Corner, J.C. Cox and P.R. Wood,1990.A sandwich enzyme immunoassay for bovine interferon- gamma and use for the detection of tuberculosis in cattle. Aust. Vet. J., 67: 134-137.

24. Sayin, Z. and O. Erganis, 2013. Diagnosis of bovine tuberculosis PPD-ELISA and sonication-ELISA. Israel Journal of Veterinary Medicine., 68(3).
25. Wadhwa, A., G.J. Hickling and S. Eda, 2012. Opportunities for improve serodiagnosis of human tuberculosis, Bovine tuberculosis and paratuberculosis. Veterinary Medicine International. DOI 10.1155/2012/674238
26. OIE Manual of Diagnostic tests: Vaccines for Terrestrial Animals (Terrestrial Manual), 2009. Vol 2. Chapter, 2.4.7: pp: 1-16.
27. Gandhi, B.M., D.K. Bhargava, M. Irshad, T.C. Chawla, A. Dube and B.N. Tandon, 1988. Enzyme linked proteinA:an ELISA for detection of IgG antibodies against mycobacterium in intestinal tuberculosis. Tubercle, 67(3): 219-224.
28. Pereira Arias-Bouda, L.M., S. Kuijper, H. van Deutekom, R. Van Gijlswijk, I. Pekel, H.M. Jansen and A.H. Kolk, 2003. Enzyme- linked immunosorbant assays using immune complexes for the diagnosis of tuberculosis. Journal of Immunological Methods, 1-2: 115-124.
29. Monaghan, M.L., M.L. Doherty, J.D. Collins, J.F. Kazda and P.J. Quinn, 1994. The tuberculin test." Vet. Microbiol., 40: 111-124.
30. JurajIvanyi, J., 2014. Function and potentials of M. tuberculosis epitopes in immunology. Front Immunol. Mar, 24;5:107. doi: 10.3389/fim
31. EFSA panel on animal heath and Wlfare (AHAW):Scientific opinion on the use of a gamma interferon test for the diagnosis of bovine tuberculosis. EFSA Journal, 10(12):2975. (2012).
32. Eltayb, A., 2012. Trends in Immunolabeled and Related Techniques.Department of Internal Medicine, Faculty of Medicine, King Abdul-Aziz University, Jeddah, Saudi Arabia.
33. Casal, C., A. Díez-Guerrier, J. Álvarez, S. Rodriguez-Campos, A. Mateos, R. Linscott, E. Martel, J.C. Lawrence, C. Whelan, J. Clarke, A. O'Brien, L. Domínguez andA. Aranaz,2014. Strategic use of serology for the diagnosis of bovine tuberculosis after intradermal skin testing.Vet Microbiol. Jun, 4;170(3-4):342-51.doi: 10.1016/j.vetmic.2014.02.036. Epub 2014 Mar 2.
34. Vaillant, A.A.J., P.E. Akpaka, N. McFarlane-Anderson and M.P. Smikle, 2013. Use of Staphylococcal Protein-A and Streptococcal Protein-G for Detection of Red Blood Cells (RBC) Antibodies and Comparison with Other Techniques. British Journal of Medicine & Medical Research, 3(4): 1671-1677.
35. Jiang, H., X. Weng and D. Li, 2011. Microfluidic whole –blood immunoassays.Microfluid Nanofluid, Volume 10, Number, 5, 941
36. Vaughan, J.A., C. Lenghaus, D.J. Stewart, M.L. Tizard and W.P. Michalski, 2005. Development of a Johne's disease infection model in laboratory rabbits following oral administration of Mycobacterium avium subspecies paratuberculosis. Vet. Microbiol., 25;105(3-4): 207-13. Epub 2004 Dec 22.
37. 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 test, gamma- interferon assay and other ancillary diagnostic techniques. Res. Vet. Science, 81: 190-201.