



RESEARCH ARTICLE

Evaluation of a fluorescence polarization assay using MAP whole-protein antigen for the detection of anti-*Mycobacterium avium* subsp. *paratuberculosis* antibodies in cattle and buffaloes

Slathia, P.¹, Narang, D.^{1*}, Chandra, M.¹, Kaur, P.¹

¹Department of Veterinary Microbiology, College of Veterinary Science, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana-141004, Punjab, India

*Corresponding author: deeptivet@rediffmail.com

ARTICLE HISTORY

Received: 24 April 2026
Revised: 13 July 2026
Accepted: 17 July 2026
Published: 30 September 2026

ABSTRACT

Johne's disease, caused by *Mycobacterium avium* subsp. *paratuberculosis* (MAP), is a chronic granulomatous enteritis of ruminants that causes substantial economic losses worldwide due to reduced productivity. Rapid and reliable serological methods are important for disease surveillance and control. The present study evaluated the relative diagnostic performance of a fluorescence polarization assay (FPA) using fluorescein-labelled MAP whole-protein antigen for the detection of anti-MAP antibodies in cattle and buffalo sera. A total of 218 serum samples were collected from cattle and buffaloes with a history of chronic intermittent diarrhea from organized dairy farms in Punjab, India. Whole protein extracted from MAP strain K10 was used as the tracer antigen in the FPA. Receiver operating characteristic (ROC) curve analysis was performed, and the results were compared with those obtained using a commercial enzyme-linked immunosorbent assay (ELISA). ELISA identified 20 seropositive samples, whereas FPA identified 35 seropositive samples. ROC analysis established an optimal cutoff value of >39.15 mP (operational threshold >39 mP), corresponding to a relative sensitivity of 85% and a relative specificity of 71.1%, with an area under the ROC curve (AUC) of 0.830 ($P < 0.001$), indicating good discrimination between ELISA-positive and ELISA-negative samples. These findings suggest that FPA using MAP whole-protein antigen is a simple and rapid serological assay that may serve as a useful complementary method to ELISA for the detection of anti-MAP antibodies in ruminants.

Keywords: *Mycobacterium avium* subsp. *paratuberculosis*; Fluorescence polarization assay (FPA); Receiver operating characteristic (ROC); Enzyme-linked Immunosorbent Assay (ELISA).

INTRODUCTION

Johne's disease (JD), also known as paratuberculosis, is a chronic granulomatous enteritis of ruminants caused by *Mycobacterium avium* subsp. *paratuberculosis* (MAP). It represents a major constraint to livestock productivity worldwide because of reduced milk yield, weight loss, and premature culling (Garcia & Shalloo, 2015; Singh *et al.*, 2014). The disease is persistent, contagious, and frequently fatal, with clinically affected animals exhibiting progressive weight loss and emaciation (Roller *et al.*, 2020). MAP is transmitted predominantly via the fecal-oral route. During the advanced stages of infection, additional transmission pathways, including transplacental transmission and excretion of MAP in milk and colostrum, may occur as a result of hematogenous dissemination of the organism. Concurrently, infected animals shed large numbers of MAP organisms in their feces, contaminating feed, water, pasture, and teats, thereby increasing the risk of within-herd transmission (Whittington & Sergeant, 2001).

Effective control of paratuberculosis relies heavily on early and accurate diagnosis. Conventional diagnostic methods include fecal microscopy, bacterial isolation, and post-mortem examination, with

bacterial culture considered the gold standard (Collins, 2011). These methods are often applied in combination or used to confirm results from immunological assays (Pfyffer, 2015). However, the slow growth rate of MAP and susceptibility to contamination make culture-based diagnosis labor-intensive and time-consuming, requiring approximately 5–16 weeks for confirmation (Silva *et al.*, 2018). These limitations create a significant diagnostic gap, particularly for early or subclinical infections where rapid identification is essential for effective disease control.

To overcome these constraints, immunological assays have gained prominence as practical tools for large-scale screening. Enzyme-linked immunosorbent assays (ELISA) are widely used but exhibit variable sensitivity depending on the stage of infection (Aitken *et al.*, 2024; Martinez *et al.*, 2012). The fluorescence polarization assay (FPA) is a homogeneous immunoassay that enables real-time detection of antigen-antibody interactions in solution, eliminating washing steps and reducing assay time and technical variability (Jameson & Ross, 2010). FPA has been successfully applied in the diagnosis of various bacterial and viral diseases (Jolley & Nasir, 2003); however, its application for the detection of MAP-specific antibodies remains limited.

Therefore, the present study aimed to compare the relative diagnostic performance of the fluorescence polarization assay (FPA) using fluorescein-labeled MAP whole-protein antigen with that of a commercially available ELISA for the serological detection of anti-*Mycobacterium avium* subsp. *paratuberculosis* antibodies in cattle and buffaloes.

MATERIALS AND METHODS

Study design and sample collection

This prospective study was conducted between January 2022 and December 2022. A total of 218 samples of serum were collected from cattle as well as buffaloes with a history of chronic intermittent diarrhea from organized dairy farms in Punjab, India. Fresh blood samples were collected aseptically by jugular venipuncture, allowed to clot, and centrifuged to separate serum. The sera were stored at -20°C until further serological analysis. Samples were collected consecutively from eligible animals during the study period to minimize selection bias.

Approval of nimal Ethics Committee

The study was conducted following approval from the Institutional Animal Ethics Committee (IAEC) (497/GO/Re/SL/01/CPCSEA).

Enzyme-linked immunosorbent assay (ELISA)

All serum samples were tested using the commercially available PARACHEK® 2 ELISA kit (Prionics) according to the manufacturer's instructions. Test results were interpreted according to the manufacturer's recommended criteria. Results of bovine serum samples which were above or equal to the cut-off 15 Percent Positivity (%P), were considered as positive. No borderline category was used in the present study. ELISA served as the reference (comparator) assay for evaluating the relative diagnostic performance of the fluorescence polarization assay (FPA).

Antigen preparation

Standard culture of MAP (ATCC K10, IMTECH, Chandigarh) was grown on Middlebrook 7H10 media then the colonies from the Middlebrook 7H10 media were scraped and inoculated in Middlebrook 7H10 broth (HiMedia). The suspension was then autoclaved for 15 mins at 121°C at 15lbs pressure and centrifuged for 10 mins at $10,000 \times g$ at 40°C to eliminate the cell debris. After centrifugation, 1 milliliter of the supernatant was extracted and collected in a separate eppendorf. In a concentrator (Eppendorf 5301), the supernatant was concentrated for 1 hr. to a volume of 500 μL . The remaining supernatant was utilized as the final component/antigen (whole protein) for further use after the procedure. Additionally, the protein's OD was examined in NanoDrop.

FITC conjugation and purification

The antigen was conjugated with fluorescein isothiocyanate (FITC) and purified using a Sephadex G-25 column under light-protected conditions (Surujballi et al., 2002). Through the sephadexG-25 column, the conjugated antigen was passed slowly. In collecting tubes, 60 fractions in total were obtained. A Tecan Infinite F200 Pro fluorescence polarization analyzer was used to measure the fluorescence intensity, while a NanoDrop spectrophotometer was used to determine the protein concentration of each fraction. Fractions exhibiting the highest fluorescence intensity and protein concentration were selected, pooled, and used as the tracer. The pooled tracer was stored at 4°C until further use.

Test protocol for fluorescence polarization assay

The FPA was conducted using a 96-well black Nunc microplate (Thermo Fisher Scientific). Each well received 180 μL of PBSALDS buffer (pH/ 7.2 phosphate-buffered saline [HiMedia] comprising 0.05% lithium dodecyl sulfate and 0.1% sodium azide [MP

Biomedicals]), followed by 10 μL of serum sample. After an initial 2-minute incubation at room temperature, baseline fluorescence polarization was recorded using a manual gain setting with 25 flashes and default emission filters. Next, 10 μL of fluorescein-labeled MAP whole-protein tracer was added to every well, mixed gently, incubated for additional 2-minutes in dark. Final fluorescence polarization measurements were taken under optimized conditions: gain optimum, 25 flashes, 100 s settle time, with excitation at 485nm as well as emission at 533nm (Surujballi et al., 2002). Values were expressed in millipolarization (mP) units, which quantitatively reflect the level of anti-MAP antibodies present in the serum samples.

Data analysis

The data were analyzed using receiver operating characteristic (ROC) curve analysis with MedCalc Statistical Software (MedCalc Software Ltd., Ostend, Belgium). Initially, fluorescence polarization assay (FPA) results, expressed in millipolarization (mP) units, were plotted to visualize the distribution of positive and negative samples. ROC curve analysis was then performed to evaluate the relative diagnostic performance of the FPA by plotting sensitivity (true positive rate) against specificity (false positive rate) across a range of cutoff values. The optimal cutoff value was determined using Youden's index, which maximizes the sum of sensitivity and specificity. The overall discriminatory ability of the assay was assessed by calculating the area under the ROC curve (AUC). For comparative evaluation, the Parachek® 2 ELISA results were used as the reference (comparator) assay. As bacteriological culture, the definitive gold standard for MAP diagnosis, was not performed in the present study, the ROC analysis and the derived estimates of sensitivity and specificity represent the relative diagnostic performance of the FPA with respect to the ELISA comparator assay rather than its true diagnostic accuracy for MAP infection.

RESULTS

A total of 218 serum samples were initially screened for anti-MAP antibodies using the *Mycobacterium paratuberculosis* Test Kit for Cattle (Parachek® 2, Prionics). Of these, 20 samples tested positive by ELISA and were considered as the reference standard for subsequent evaluation of the fluorescence polarization assay (FPA). In the FPA, MAP whole-cell protein labeled with fluorescein isothiocyanate (FITC) was used as the tracer antigen for the detection of specific antibodies. The results obtained from FPA were directly compared with ELISA outcomes to assess diagnostic concordance between the two assays. Receiver operating characteristic (ROC) curve analysis was performed using MedCalc software to determine the optimal cutoff value for FPA. A dot plot was generated to visualize the distribution of positive and negative samples. The ROC analysis identified the cutoff value that provided the best balance between sensitivity and specificity, enabling effective discrimination between infected and non-infected animals.

Using MAP whole-protein as the antigen, 35 out of 218 serum samples were identified as positive by FPA (Table 1). Comparison with ELISA results established an optimal cutoff value of 39.15 mP (operational cutoff: >39 mP), which corresponded to a relative sensitivity of 85% and a relative specificity of 71.1% (Figure 1). The area under the ROC curve (AUC) was 0.830 ($P < 0.001$) (Figure 2), indicating good diagnostic performance. The AUC value reflects

Table 1. Contingency table comparing FPA with the ELISA comparator assay

FPA Result	ELISA-positive	ELISA-negative	Total
Positive	True Positive (TP)	False Positive (FP)	35
Negative	False Negative (FN)	True Negative (TN)	183
Total	20	198	218

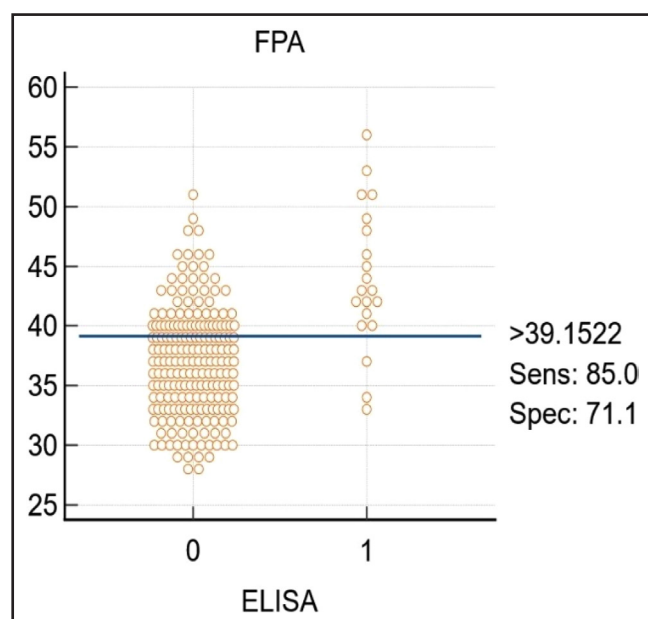


Figure 1. Distribution of fluorescence polarization values. The horizontal line represents the ROC-derived optimal cutoff value of 39.15 mP, which was rounded to >39 mP for relative diagnostic interpretation. (0= ELISA Negative, 1= ELISA Positive).

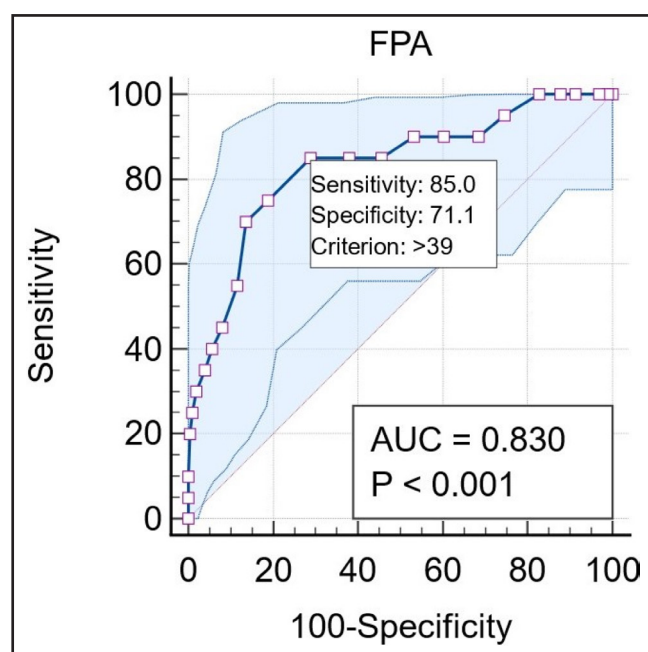


Figure 2. ROC analysis of MAP whole protein FPA with ELISA.

the ability of the FPA to discriminate between seropositive and seronegative serum samples classified by the comparator assay. Overall, these findings demonstrate good relative diagnostic performance of the FPA for the serological detection of anti-*Mycobacterium avium* subsp. *paratuberculosis* antibodies.

DISCUSSION

Accurate diagnosis of mycobacterial infections relies on a combination of complementary diagnostic methods, including bacteriological culture, molecular assays, histopathology, cell-mediated immune assays, and serological tests, depending on the

stage of infection and the purpose of testing. Within serological assays, the use of species-specific or immunodominant antigens has been shown to improve diagnostic specificity and reduce cross-reactivity, thereby enhancing the performance of assays for the detection of tuberculosis and paratuberculosis in animals (Torres-Velez *et al.*, 2019). The incorporation of such antigens into immunoassays, including ELISA and fluorescence polarization assay (FPA), has been shown to enhance both sensitivity and specificity compared to conventional diagnostic approaches (Barkema *et al.*, 2023; Chaubey *et al.*, 2016).

FPA has emerged as a rapid, versatile, and field-adaptable alternative to traditional serological assays. Unlike ELISA, FPA is a homogeneous assay that requires only a single reagent, eliminating washing and separation steps and thereby reducing procedural variability (Lightbody *et al.*, 1998). Moreover, the availability of portable fluorescence polarization analyzers enables on-site testing and provides results within minutes, making it highly suitable for field applications (Surujballi *et al.*, 2002). The use of receiver operating characteristic (ROC) curve analysis further strengthens assay evaluation by providing an objective measure of diagnostic performance across a range of cutoff values (Zweig & Campbell, 1993). For example, in bovine brucellosis, FPA demonstrated excellent diagnostic performance, with sensitivity and specificity values of 99.0% and 95.5%, respectively (Marcelo *et al.*, 2017), highlighting its broader applicability in infectious disease diagnostics.

In the context of mycobacterial infections, FPA has shown promising results. Detection of antibodies against *Mycobacterium bovis* using fluorescein-labeled MPB70 protein successfully identified all skin test-positive animals and the majority of skin test-negative animals, with no cross-reactivity observed in MAP-infected sera (Surujballi *et al.*, 2002). Similarly, Torres-Velez *et al.* (2019) reported that FPA outperformed ELISA in the diagnosis of paratuberculosis in bovines when using a 35 kDa MAP-derived protein, demonstrating improved sensitivity and specificity. Furthermore, Lin *et al.* (1996) observed a substantial increase in fluorescence polarization in sera from *M. bovis*-infected animals across multiple species, confirming the reliability of FPA in detecting specific antibody responses.

The findings of the present study further support the utility of FPA for detecting MAP-specific antibodies in cattle and buffaloes. In this study, FPA identified 35 positive samples compared to 20 detected by ELISA, with an optimal cutoff value of optimal cutoff of 39.15 mP (operational threshold >39 mP) yielding a relative sensitivity of 85% and specificity of 71.1%. The higher number of positives detected by FPA suggests that it may identify additional seropositive animals that are not detected by ELISA, potentially due to its ability to detect early or low-level antibody responses. This is particularly important in the context of paratuberculosis, a chronic and often subclinical disease, where early detection plays a critical role in controlling transmission within herds.

However, the relatively lower specificity observed in the present study indicates a higher rate of false-positive results, which may limit the use of the assay as a standalone confirmatory test. This limitation could be attributed to the use of a whole-protein antigen, which contains multiple conserved epitopes that may cross-react with antibodies generated against other mycobacterial species. Ruminants are frequently exposed to environmental mycobacteria, particularly members of the *Mycobacterium avium* complex (MAC), which share antigenic similarities with MAP and may induce cross-reactive antibody responses. In addition, antigenic similarities with *Mycobacterium bovis* may also contribute to cross-reactivity, particularly in regions where bovine tuberculosis is prevalent (Chiu *et al.*, 2026). Such cross-reactive immune responses may lead to false-positive reactions, thereby reducing the specificity of the assay. Therefore, further optimization of antigen composition, including the use of purified or recombinant MAP-specific proteins, may improve assay specificity and minimize cross-reactivity.

Overall, FPA represents a promising complementary diagnostic tool for paratuberculosis and other mycobacterial infections. Its advantages, including rapid turnaround time, operational simplicity, and suitability for field deployment, make it particularly valuable for herd-level screening and epidemiological surveillance. Future studies should focus on validating the assay across diverse geographical regions and host species, improving antigen specificity, and integrating FPA into routine herd health management programs to enhance early detection and control of mycobacterial diseases.

CONCLUSION

The fluorescence polarization assay (FPA) demonstrated a sensitivity of 85% and a specificity of 71.1% for the detection of antibodies against *Mycobacterium avium* subsp. *paratuberculosis* (MAP). Notably, FPA identified a greater number of seropositive samples than the ELISA comparator assay, which may reflect differences in antigen composition or analytical performance between the two serological tests. However, in the absence of bacteriological culture or molecular confirmation, the additional FPA-positive samples cannot be classified as true positives or false positives. Cross-reactivity with antibodies against environmental mycobacteria or *Mycobacterium bovis* may also have contributed to the observed discordance. Nevertheless, FPA offers practical advantages, including rapidity and simplicity, and may serve as a useful complementary screening assay alongside established diagnostic methods. Further validation using culture and/or molecular methods is warranted to establish its diagnostic utility.

ACKNOWLEDGEMENT AND FUNDING

The authors express their gratitude to the Director of Research, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana, for providing the necessary research facilities. The authors also acknowledge the Department of Biotechnology (DBT), Government of India, for financial support under the “Mycobacterial Diseases in Animals Network (MyDAN) Programme” (BT/AVD/Bovine Tuberculosis/2018).

Conflict of Interests

The authors declare that they have no conflict of interests.

REFERENCES

Aitken, J.M., Aitken, J.E. & Agrawal, G. (2024). *Mycobacterium avium* ssp. *paratuberculosis* and Crohn's Disease – Diagnostic microbiological investigations can inform new therapeutic approaches. *Antibiotics* **13**: 158. <https://doi.org/10.3390/antibiotics13020158>

Barkema, H.W., Orsel, K., Nielsen, S.S. & Koets, A.P. (2023). Paratuberculosis in ruminants: Progress in diagnostics, epidemiology and control. *Frontiers in Veterinary Science* **10**: 1215489. <https://doi.org/10.3389/fvets.2023.1215489>

Chaubey, K.K., Gupta, R.D., Gupta, S., Singh, S.V., Bhatia, A.K., Jayaraman, S., Kumar, N., Goel, A., Rathore, A.S., Sahzad, et al. (2016). Trends and advances in the diagnosis and control of paratuberculosis in domestic livestock. *Veterinary Quarterly* **36**: 203-227. <https://doi.org/10.1080/01652176.2016.1196508>

Chiu, S.J., Bugde, P., Kinga, A., Bannantine, J.P., Liggett, S., Dukkupati, V.S.R. & Furuya, Y. (2026). Multiplex biomarker-based ELISA enables early detection of *Mycobacterium avium* subspecies *paratuberculosis*-specific antibodies comparable to IFN- γ testing. *Frontiers in Veterinary Science* **12**:1749510. <https://doi.org/10.3389/fvets.2025.1749510>

Collins, M.T. (2011). Diagnosis of paratuberculosis. *Veterinary Clinics of North America: Food Animal Practice* **27**: 581-591. <https://doi.org/10.1016/j.cvfa.2011.07.013>

Garcia, A.B. & Shalloo, L. (2015). The economic impact and control of paratuberculosis in cattle. *Journal of Dairy Science* **98**: 5019-5039. <https://doi.org/10.3168/jds.2014-9241>

Jameson, D.M. & Ross, J.A. (2010). Fluorescence polarization/anisotropy in diagnostics and imaging. *Chemical Reviews* **110**: 2685-2708. <https://doi.org/10.1021/cr900267p>

Jolley, M.E. & Nasir, M.S. (2003). The use of fluorescence polarization assays for the detection of infectious diseases. *Combinatorial Chemistry & High Throughput Screening* **6**: 235-244. <https://doi.org/10.2174/138620703106298419>

Lightbody, K.A., Skuce, R.A., Neill, S.D. & Pollock, J.M. (1998). Mycobacterial antigen-specific antibody responses in bovine tuberculosis: an ELISA with potential to confirm disease status. *Veterinary Record* **142**: 295-300. <https://doi.org/10.1136/vr.142.12.295>

Lin, M., Sugden, E.A., Jolley, M.E. & Stilwell, K. (1996). Modification of the *Mycobacterium bovis* extracellular protein MPB70 with fluorescein for rapid detection of specific serum antibodies by fluorescence polarization. *Clinical Diagnostic Laboratory Immunology* **3**: 438-443. <https://doi.org/10.1128/cdli.3.4.438-443.1996>

Marcelo, I., Hernan, B., Ruth, S., Milena, G., Judith, G., Julio, P., David, H., Jorge, M., Martin, C. & Byron, P. (2017). Determining a diagnostic cut-off on fluorescence polarization assay (FPA) for bovine brucellosis in Carchi, Ecuador. *Open Journal of Animal Science* **7**: 425-432. <https://doi.org/10.4236/ojas.2017.74033>

Martinez, C.A.G., Santillán, F.M.A., Guzmán, R.C.C., Favila, H.L.C., Córdova, L.D., Díaz, A.E., Hernández, A.L. & Blanco, O.M. (2012). Development of an enzyme-linked immunosorbent assay (ELISA) for the diagnosis of bovine paratuberculosis. *Revista Mexicana de Ciencias Geológicas* **3**: 1-18.

Pfiffer, G.E. (2015). *Mycobacterium*: General characteristics, laboratory detection, and staining procedures. In: Manual of Clinical Microbiology, Jorgensen, J.H., Carroll, K.C., Funke, G., Pfaller, M.A., Landry, M.L., Richter, S.S. & Warnock, D.W. (editors) 11th edition. Wiley, pp. 536-569. <https://doi.org/10.1128/9781555817381.ch30>

Roller, M., Hansen, S., Knauf-Witzens, T., Oelemann, W.M.R., Czerny, C.P., El Wahed, A.A. & Goethe R. (2020). *Mycobacterium avium* subspecies *paratuberculosis* infection in zoo animals: a review of susceptibility and disease process. *Frontiers in Veterinary Science* **7**: 572724. <https://doi.org/10.3389/fvets.2020.572724>

Silva, D.A.V., Siconelli, M.J.L., Burger, K.P. & Keid, L.B. (2018). Comparison between tests for tuberculosis diagnosis in slaughtered bovines. *Arquivos do Instituto Biológico* **85**: 1-8. <https://doi.org/10.1590/1808-1657000652016>

Singh, S.V., Gupta, S., Chaubey, K.K., Rawat, K.D., Kumar, N., Sohal, J.S., Singh, S., Tiwari, R., Chakraborty, S. & Dhama, K. (2014). Johne's disease (JD) in a high yielding Holstein Friesian cattle dairy farm in India. *Journal of Biological Sciences* **14**: 195-203. <https://doi.org/10.3923/jbs.2014.195.203>

Surujballi, O.P., Romanowska, A., Edward, A.S., Turcot, C. & Jolley, M.E. (2002). A fluorescence polarization assay for the detection of antibodies to *Mycobacterium bovis* in cattle sera. *Veterinary Microbiology* **87**: 149-157. [https://doi.org/10.1016/s0378-1135\(02\)00044-5](https://doi.org/10.1016/s0378-1135(02)00044-5)

Torres-Velez, R., Santillan-Flores, M.A., Cordova-Lopez, D., Martinez-Martinez, O.L. & Guzmán-Ruiz, C.C. (2019). Comparison of fluorescence polarization assay and enzyme-linked immunosorbent assay for the diagnosis of bovine paratuberculosis. *Journal of Veterinary Medicine and Animal Health* **11**: 94-99. <https://doi.org/10.5897/JVMAH2019.0784>

Whittington, R.J. & Sergeant, E.S.G. (2001). Progress towards understanding the spread, detection and control of *Mycobacterium avium* subsp. *paratuberculosis* in animal populations. *Australian Veterinary Journal* **79**: 267-278. <https://doi.org/10.1111/j.1751-0813.2001.tb11980.x>

Zweig, M.H. & Campbell, G. (1993). Receiver-operating characteristic (ROC) plots: a fundamental evaluation tool in clinical medicine. *Clinical Chemistry* **39**: 561-577. <https://doi.org/10.1093/clinchem/39.4.561>