

Serological and molecular identification of *Mycobacterium avium* subsp. *paratuberculosis* and associated risks in bovine

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Abstract

Discrepancies in sensitivity and specificities of tests (enzyme-linked immunoassay (ELISA) and tuberculin skin test (TST)), prevalence, and potential risk factors associated with *Mycobacterium avium* subsp. *paratuberculosis* (MAP) of bovine were hypothesized to affect its control. Keeping PCR as the gold standard test, 101 cattle and 39 buffaloes maintained at Livestock Experiment Station were tested for MAP with ELISA and TST. The incidence of MAP was 13.57% (19/140), 12.85% (18/140), and 8.57% (12/140), based on PCR, ELISA, and TST. Discrepancies in the identification of MAP in buffalo were 7.62% and 2.79% for cattle, based on ELISA compared with TST. The highest discrepancies in MAP prevalence were noted in brown buffalo (22.22%), whereas the lowest were recorded from crossbred cattle (2.63%). Status of milking was a potential risk factor ($P < 0.05$) if MAP was identified by TST, whereas breed showed significant association ($P < 0.05$) with MAP only on its identification by ELISA. Considerable variations were noted in the sensitivity of ELISA (94.4%) and TST (66.7%). On mutual comparisons, considering TST as a fixed diagnostic tool, the specificity and sensitivity of ELISA were 95.31% and 100%. Similarly, fixing ELISA as diagnostic tool, TST showed 100% specificity and 66.67% sensitivity. The conclusion of the study was that because of rising MAP in bovine, significant discrepancies in routine diagnostic tools, and variations in the declaration of significant risk factors, stern measures must be taken to avoid exaggerated outcomes in testing for this pathogen.

Keywords: bovine, discrepancies, paratuberculosis, risk factors

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Introduction

Paratuberculosis (Johne's disease) is a long lasting and infectious granulomatous disease that disturbs mainly ruminant, non-ruminant (Foxe *et al.*, 2020) and wild animal species across the world (Singh *et al.*, 2014). The disease is produced by *Mycobacterium avium* subsp. *paratuberculosis* (MAP). Currently, Johne's disease is regarded as being among the top serious threats disturbing the world's dairy industry (Chiodini *et al.*, 2012). Exposure to infection seems to be higher in younger animals (less than 6 months old) compared with old ones (Sergeant *et al.*, 2003). A clear clinical picture of the disease appears after 2–4 years of exposure (Harris *et al.*, 2001). The bacteria can survive longer in a harsh environment, but cannot

propagate (Raizman *et al.*, 2007). The intermittent shedding of the organism in the calving areas and the milk might cause Crohn's disease in humans because this bacterium is viable even after pasteurization (Gupta *et al.*, 2012; McNees *et al.*, 2015). The long-term duration of the disease lies in its subclinical nature, resistance to unfavourable environments, slow growth, and intermittent spread (Agrawalet *et al.*, 2021).

Paratuberculosis has significant importance throughout the world because of its economic and health concerns. The production loss from paratuberculosis in the USA dairy sector is approximately \$200 million yearly (Rasmussen *et al.*, 2021). Prevalence of Johne's disease reaches 40% in some countries, for example Venezuela (South America). Prevalence in dairy cows in Australia and the US was reported at 22% and 75.8% (Ruiz *et al.*, 2020). In Belgium and Austria, the prevalence rate was 41% and 7%. In Pakistan, cattle and buffalo were identified as being 6.67% and 12.5% positive for MAP (Sikandar *et al.*, 2012). The ELISA-based study reported an overall 1% prevalence of MAP in cattle and buffalo (Rehman *et al.*, 2017b), whereas at public livestock farms buffaloes showed 1.3% prevalence (Rehman *et al.*, 2018).

This disease is diagnosed through TST, ELISA, ZN staining, culture isolation and PCR (Whittington & Sergeant, 2001). Among these tests, TST and ELISA are easily available and used routinely for preliminary diagnosis of the disease. Purified protein derivative (PPD) involves delayed type hypersensitivity in the form of inflammation and production of specific cytokines (*al.*, 2000). Constant screening through PPD is routine for eradication of disease (Schiller *et al.*, 2010). On the other hand, ELISA is a more sensitive tool for screening animal herds (Sikandar *et al.*, 2012)). But every technique has its own discrepancies in identification. It is necessary to study the effects of these discrepancies on the spread of diseases and their determinants so that precise measures may be adopted. Therefore, the current study was planned to highlight the discrepancy in identification of MAP by ELISA and TST, keeping PCR as gold standard, and to investigate variations in declaration of disease determinants.

Materials and Methods

This research was reviewed for ethical consideration the by Research Scrutiny Committee and Director Graduate Studies of the University of Agriculture, Faisalabad, and was carried out at Livestock Experiment Station where animals were reared with optimum feeding and living requirements. The health and management of animals was monitored by professionals employed at the farm. The farm is located 500 meters from the veterinary teaching hospital of the university. Hence health services included routine monitoring. This study included 140 adult animals (101 cattle and 39 buffalo) more than two years old.

Blood samples (5 mL) were drawn aseptically directly from the jugular vein of the cattle and buffaloes in clean gel-coated vacutainers and were processed for serum collection to run indirect ELISA (Salgado *et al.*, 2007) for MAP. LSIVet ruminant serum paratuberculosis advanced antibody kit (Lot No. 2-VETPTRS-007) was used according to the instructions of the manufacturer.

The TST was performed by injecting 0.1 mL PPD antigen (Veterinary Research Institute, Lahore, Pakistan) at the left side of the neck area (cervical) adopting the intradermal route. The site was washed, cleaned, and dried before the injection of PPD. Skin thickness was measured by Vernier callipers at this site before performing the test. Similarly, skin thickness was measured 72 hours post PPD administration. The cattle and buffaloes were considered positive for the intradermal tuberculin test if the net thickness of skinfold was greater than 4 mm at the injection site. A negative control was run alongside to rule out prick response in the animal.

Faecal samples were collected and processed for DNA extraction through the boiling method (Alexopoulou *et al.*, 2006). PCR was performed for rapid diagnosis of MAP following Stanley *et al.*, 2007. A set of primers (P90 GTTCGGGGCCGTCGCTTAGG, P91 GAGGTCGATCGCCACGTGA, IS 1245 F GTGGCAATCTGCCCTGCACTTCGG, IS 1245 R GCCCGCACGCTCACAGTTAAGCCGT) reported by Bartos *et al.* (2006) was used with the recipe as DNA (3 µL) master mix (Enzynomics) 20 µL, primer (F+R) 1 µL each. Thermocycler (Qantarus) was set at preliminary denaturation (94 °C for 2 minutes), 35 cycles of denaturation for 30 seconds, annealing (65 °C for 2 minutes), and three elongations (3 minutes at 72 °C). At the end of the last cycle, 30 seconds of denaturation, two minutes of annealing at 65 °C and a final extension at 72 °C for 10 minutes were carried out (Stanley *et al.*, 2007).

Information about the species, breed, age of animal (years), weight (Kg), milk yield, lactation number, and milking status was documented. A chi-square test was applied to calculate the epidemiological association. SAS statistical software (SAS Institute Inc., Cary, North Carolina, USA) was used for all analyses. Differences were declared significant at the 5% level of probability and remarked upon at the 10% level.

The data were evaluated using frequency analysis, stratified analysis at 95% confidence interval. A chi-square test was applied to calculate the epidemiological association. SAS statistical software (SAS Institute Inc., Cary, North Carolina, USA) was used for all analyses and differences were declared significant at the 5% level of probability.

Results and Discussion

Of the animals tested for MAP, 12.85% tested positive based on ELISA, 8.57% were positive with TST, and the PCR technique (Figure 1) identified 13.57% positive animals. Buffaloes were twice as likely to be positive for MAP compared with cattle ($P=0.093$) (Table 1). Brown buffaloes were five times more likely to be positive for MAP than black buffaloes, although the numbers of observations were small.

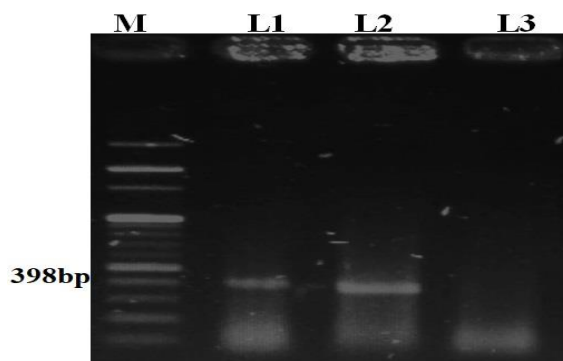


Figure 1 Molecular identification of *Mycobacterium avium subsp. paratuberculosis* at 398 bp product size M shows the molecular ladder, L1 and L2 indicate positive samples, and L3 indicates a negative sample

The findings of the current study contradict those of studies in which Rehman *et al.* (2017b) found prevalence of MAP was 5.66%, and Haji Hajikolaie *et al.* (2006) observed a 2% incidence. The results of an abattoir-based study conducted at Faisalabad showed the prevalence rate to be higher in cattle compared with buffaloes (Rehman *et al.*, 2017a). Recently an ELISA-based study revealed 14.2% positive animals for MAP antibodies in Tai'an city of China (Cheng *et al.*, 2020).

The prevalence rate was comparatively higher in young animals, those with low bodyweight and high lactating animals (Table 2). It seems the chances of finding the infection in cattle and buffalo with low bodyweight might be attributable to the severe diarrhoea that debilitates the body condition of affected animals (Kudahl *et al.*, 2009). The results are in line with those of Borujeni *et al.* (2021), who reported high disease prevalence in low bodyweight animals. The prevalence of MAP in animals that weighed less than 350 kg was numerically greater based on the ELISA test than on the TST. The salient assumed risk factors had P -values indicating significant variability (Table 2). Milking status (dry vs lactating animals) had a significant effect ($P=0.03$) with MAP prevalence based on the TST, whereas its effect only approached significance ($P=0.059$) when MAP was diagnosed with the ELISA test. The difference in P -values for milk yield potential was more significant ($P=0.07$) when ELISA was used whereas it was non-significant ($P=0.72$) for TST (Table 2). A higher percentage of milking animals testing positive for MAP was noted with the ELISA test, and TST showed higher prevalence of MAP in animals that were not lactating. In addition, cows with fewer than three lactations were more prone to MAP being identified by ELISA than TST (Table 2).

Table 1 Comparisons of species and breed for the prevalence of paratuberculosis based on enzyme-linked immunoassay and tuberculin skin diagnostic tests

| | | Diagnostic test | | | | | | | | <i>P</i> -value ¹ |
|---------|------------------|---------------------------|--------------------|--------------------|-----------------|------------------|--------------------|--------------------|-----------------|------------------------------|
| | | Enzyme-linked immunoassay | | | | Tuberculin based | | | | |
| | | Number negative | Number positive, % | 95% CI, % positive | <i>P</i> -value | Number negative | Number positive, % | 95% CI, % positive | <i>P</i> -value | |
| Species | Buffalo | 31 | 8 (20.51) | 10.01–35.26 | 0.093 | 34 | 5 (12.8) | 4.85–26.15 | 0.264 | 0.362 |
| | Cattle | 91 | 10 (9.90) | 5.14–16.94 | | 94 | 7 (6.93) | 3.08–13.23 | | 0.477 |
| | Sahiwal cattle | 57 | 6(9.52) | 3.95–18.76 | | 59 | 4 (6.30) | 2.05–14.61 | | 0.510 |
| Breeds | Crossbred cattle | 34 | 4(10.53) | 3.44–23.47 | 0.016 | 35 | 3 (7.80) | 2.05–20.00 | 0.055 | 0.692 |
| | Brown buffalo | 4 | 5(55.56) | 24.04–83.95 | | 6 | 3 (33.30) | 9.27–66.76 | | 0.343 |
| | Black buffalo | 27 | 3(10.00) | 2.61–24.85 | | 28 | 2 (6.66) | 1.13–20.32 | | 0.640 |
| | Total | 122 | 18 (12.85) | 8.05–19.19 | | 128 | 12 (8.57) | 4.73–14.12 | | 0.246 |

¹Comparison of the ELISA test with the tuberculin based test; CI: confidence interval

Table 2 Association between levels of factors characterizing the animals and incidence of paratuberculosis based on enzyme-linked immunoassay and tuberculin skin diagnostic tests

| Factor | Level | Diagnostic test | | | | | | | |
|------------------|------------|---------------------------|------------------------------|-------------|----------------|------------------|------------------------------|-------------|----------------|
| | | Enzyme-linked immunoassay | | | | Tuberculin based | | | |
| | | Number negative | Number infected (Positive %) | 95% CI | <i>P-value</i> | Number negative | Number infected (Positive %) | 95% CI | <i>P-value</i> |
| Age | < 5 years | 8 | 7 (46.67) | 23.20–71.32 | 0.001 | 10 | 5 (33.33) | 13.38–59.21 | 0.004 |
| | 5-10 years | 95 | 6 (5.94) | 2.44–11.94 | | 97 | 4 (3.96) | 1.27–9.28 | |
| | > 10 years | 19 | 5 (20.83) | 8.06–40.30 | | 21 | 3 (12.50) | 3.28–30.36 | |
| Weight | < 350 kg | 83 | 13 (12.26) | 6.95–20.28 | 0.720 | 88 | 8 (8.33) | 3.95–15.21 | 0.880 |
| | > 350 kg | 39 | 5 (11.36) | 4.28–23.40 | | 40 | 4 (9.09) | 2.96–20.49 | |
| | Dry | 14 | 5 (26.32) | 10.34–49.06 | | 16 | 3 (15.7) | 4.18–37.21 | |
| Milk yield | 1–5 L | 37 | 3 (7.50) | 1.94–19.07 | 0.070 | 37 | 3 (7.50) | 1.94–19.07 | 0.720 |
| | 5.1–8 L | 65 | 7 (9.72) | 4.35–18.28 | | 67 | 5 (6.94) | 2.59–14.72 | |
| | > 8 L | 6 | 3 (33.33) | 9.27–66.76 | | 8 | 1 (11.1) | 0.56–43.86 | |
| Lactation number | < 3 | 54 | 6 (10.00) | 4.15–19.64 | 0.380 | 57 | 13 (5.26) | 1.84–13.21 | 0.208 |
| | > 3 | 68 | 12 (15.00) | 8.39–24.11 | | 71 | 9 (11.25) | 5.63–19.64 | |
| Status | Dry | 14 | 5 (26.32) | 10.34–49.06 | 0.059 | 15 | 4 (21.00) | 7.07–43.32 | 0.030 |
| | Milking | 108 | 13 (10.74) | 6.11–17.24 | | 113 | 8 (6.61) | 3.12–12.17 | |

CI: Confidence interval

The technique-based variation with PCR throughout the trial was most sensitive followed by ELISA and TST (Table 3). Association of ELISA and TST by replacing each other as a fixed diagnostic tool showed shifting sensitivity and specificity (Table 3). Considering TST as a standard tool, the ELISA specificity and sensitivity were 95.31% and 100% in the current study. On the other hands keeping ELISA as fixed diagnostic tool, the specificity and sensitivity of TST were observed 100% and 66.67% (Table 3). Higher sensitivity results of ELISA for MAP identification were also noted by Cihan *et al.* (2012), who did studied the efficiency of diverse diagnostic approaches paratuberculosis. The TST-based studies showed this test is a routine diagnostic tool but cross-reactivity with other mycobacteria hampered the accuracy of results (Marassi *et al.*, 2005). Vargas *et al.* (2009) also reported tuberculin skin testing affecting immune system mechanism of animal's body resulting in false positive outcomes. A serological survey on 19627 cattle from Italy reported MAP identification through ELISA an easy, reliable, economical, and useful in dairy herds (Lillini *et al.*, 2005). On the other hand, as the disease progresses, the humoral response of animals can vary resulting in discrepancies in the outcomes of serological diagnostic tests (Collins *et al.*, 2005).

Table 3 Association of the tuberculin skin test with the enzyme-linked immunoassay test for detection of paratuberculosis

| Tuberculin test | Enzyme-linked immunoassay test | | |
|-----------------|--------------------------------|-----------------------|------------|
| | Negative | Infected (positive %) | 95% CI |
| Negative | 122 | 6(4.29) | 1.75-8.71 |
| Positive | 0 | 12(8.5) | 7.73-14.12 |

CI: confidence interval

The apparent discrepancies between the ELISA test and TST showed the percentage changes in the identification of paratuberculosis for various species and breeds. In comparing ELISA to TST for buffaloes 7.62% of the results were disparate, whereas for cattle the numbers of cases in which the tests produced conflicting results were only 2.97% of the total number of animals evaluated (Figure 1a). The highest rate of discrepancies between the tests was noted for brown buffalo (Figure 1b). However, the total number of brown buffalo that were sampled was small, so even a discrepancy of results for a single animal produced a very large percentage change.

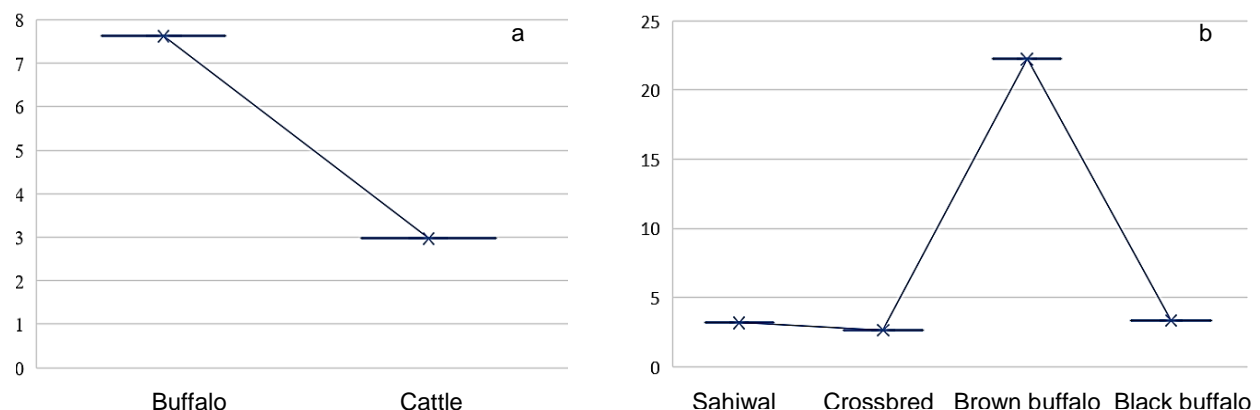


Figure 1 Percentage discrepancies (vertical axis) of enzyme-linked immunoassay in context to tuberculin for paratuberculosis identification in cattle and buffalo

Percentage discrepancies = (Elisa positive-tuberculin positive/total tested) x 100

Considering PCR as fixed diagnostic/gold standard test, the current study found variable sensitivity and specificity of ELISA and TST (Table 4). The specificity of TST was similar to that of ELISA indicating that these tests were similarly and highly precise in screening negative samples. However, the sensitivity of TST was low compared with that of ELISA, indicating that TST was not reliable in identifying true positive MAP in bovine (Table 4).

Table 4 Sensitivity and specificity of enzyme-linked immunoassay and tuberculin skin test for diagnosis of paratuberculosis when evaluated against diagnosis using the polymerase chain reaction

| Methods | Number of tests | Positive (%) | 95% CI | <i>P</i> -value | Sensitivity, % (True positive) | Specificity, % (True negative) |
|------------|-----------------|--------------|------------|-----------------|--------------------------------|--------------------------------|
| ELISA | 140 | 18 (12.8) | 8.05–19.19 | | 94.4 | 99.2 |
| Tuberculin | 140 | 12 (8.5) | 4.73–14.12 | 0.378 | 66.7 | 100.0 |
| PCR | 140 | 19 (13.6) | 8.62–20.01 | | | |

ELISA: enzyme-linked immunoassay, PCR: polymerase chain reaction

Conclusion

A higher than expected prevalence of MAP was found in bovine maintained at an experimental station where reasonable healthcare and management were provided. There was considerable variation in the detected prevalence of MAP based on diagnostic techniques, and a significant association of risk factors with MAP also showed discrepancies between the test results. Significant variation in sensitivity and specificity of TST and that of ELISA were observed. Moreover, a significant association of assumed risk factors varied with diagnostic tests. Thus, immediate measures are needed to identify a suitable diagnostic test to facilitate the prevention of MAP.

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Authors contributions

AR (<https://orcid.org/0000-0003-4665-781Xcc>) and MTJ conceived the idea of research; AR collected the samples and was involved in sample testing and lab activity; MFQ drafted the skeleton; AIA analysed the data; IA, AS (<https://orcid.org/0000-0001-7966-772>) and MR prepared the draft of the manuscript; MK, LA and MN drafted the details of the manuscript and helped in data editing, data analysis and construction of tables; MKR, TH and MFAK contributed to proofreading, added references and checked reference styling, and helped in revising the draft and editing. All authors read and interpreted the data critically and approved the final version.

Declaration of conflicting interests

The authors received no financial support for the research, authorship, and publication of this article; and they declare there are no potential conflicts of interest.

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