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METHODS AND PROTOCOLS

Multiplex qPCR for differentiation of *Mycobacterium avium* subspecies *paratuberculosis* in active and passive infection of goats

Anjali Pachoori[1] · K. Gururaj[1] · Supriya Sachan[1] · Deepansh Sharma[2]

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Abstract

Mycobacterium avium paratuberculosis (MAP) is causative agent of Johne's disease (JD) in domestic animals and has broad host range. JD infected animals shed viable MAP in their milk, feces, blood, and tissues which get transmitted to human beings directly or indirectly by consumption of animal products, through contact, animal handling and through contaminated environment, aerosols. In this current study, we developed hydrolysis probe based TaqMan® real-time PCR assay where samples were investigated by targeting *IS900* mRNA and *ModD* gene to differentiate live MAP shedders from inactive/dead MAP bacilli shedding animals. The *IS900* mRNA and *ModD* gene primers were designed using discontiguous unique conserved sequences of *IS900* more towards the 3′ end and fibronectin attachment protein (FAP) genes, respectively. Two different reporter dyes Cy5 and TexasRed, with compatible quenchers BHQ-1 and BHQ-2, respectively, were used for probe designing of *IS900* and *ModD* genes. Triplex PCR assay was developed by using serially diluted positive MAP culture in $\log_{10}$ dilution and probe and template titration. TaqMan® probe real-time PCR targeting *IS900* mRNA and *ModD* gene detects the MAP infection at early stage with high sensitivity and specificity. The specificity of developed TaqMan probe real-time PCR was found to be high while validated by using *Escherichia coli* and *Staphylococcus aureus* in addition to the MAP culture as there is no non-specific signal from other microbes. The sensitivity of developed TaqMan® probe real-time PCR was computed based on copy numbers ranged from 4.14×10^{11} to 4.14×10^{4} for *IS900* (FAM), 1.27×10^{11} to 1.27×10^{4} for *IS900* mRNA (Cy5), and 3.68×10^{10} to 3.68×10^{4} for *ModD* (TexasRed), and lowest limit to detect MAP was 4.14×10^{4}, 1.27×10^{4}, and 3.68×10^{4} copies for respective genes. This assay would be of great aid to contain the MAP infection in the large herd, where silent shedders spread active infection can be differentiated from passive shedding by non-infected animals. This test would also be equivalent to culture test in terms of specificity and hence can be able to be undertaken in molecular epidemiological studies to represent the actual disease prevalence in the future.

Key points

- *Multiplex mRNA-based qPCR was developed to identify the actively infective MAP bacilli from passive ones.*
- *ModD and IS900 used as targets to assess active MAP bacilli in fecal samples of suspected animals.*
- *The LOD was computed using copy numbers with 4.14×10^{4} and 3.68×10^{4} copies for IS900 and ModD, respectively.*

Keywords *Mycobacterium avium paratuberculosis* (MAP) · Hydrolysis probe-based TaqMan® real-time PCR · *IS900* mRNA · *ModD* · Fibronectin attachment protein (FAP)

Introduction

Mycobacterium avium paratuberculosis (MAP), the cause of incurable granulomatous enteritis Johne's disease (JD), is endemic in domestic livestock population of the country. This leads to low energy balance consequently making the infected animals unthrift due to poor weight gain and low milk yield. Since infected lactating cows, buffaloes, and goats excrete MAP in their milk it poses greater risk to other healthy animals and contaminates the environment. Since

✉ K. Gururaj
guruvet@gmail.com

[1] Division of Animal Health, ICAR-Central Institute for Research On Goats, Farah (P.O.), Mathura-, 281122 Makhdoom, Uttar Pradesh, India

[2] Amity Institute of Microbial Technology, Amity University Rajasthan, Jaipur, Rajasthan, India

MAP in milk and milk products escapes pasteurization, it is transmitted to human beings and therefore has been associated with number of human autoimmune disorders (Chaubey and Singh 2018). Epidemiological evidence has linked MAP exposure to the occurrence of a variety of human autoimmune disorders such as Crohn's disease, rheumatoid arthritis, type 1 diabetes mellitus (T1DM), Hashimoto's thyroiditis (hypothyroidism), autoimmune arthritis, multiple sclerosis, and others (Naser et al. 2014; Banche et al. 2015). In terms of clinical signs, JD can be similar to many other livestock diseases, and it is necessary to diagnose MAP infection as JD is a chronic disease. In view of the lack of attention and priority, efforts to control Johne's disease in domestic livestock are non-existent in India despite high bio-load in domestic livestock. MAP besides gaining in pathogenicity due to repeated passage from infected parents to new generation, it continues to spread. There are four stages of MAP infection silent, subclinical, clinical, and last advance stage (Karuppusamy et al. 2021). There are several diagnostic tests to detect depending upon the stages of disease along with their limitations which makes the development of a good diagnostic test more challenging. There is no specific test to differentiate live or dead bacilli infection at early stage of disease. As per currently available diagnostic tests, only culture can detect live bacilli, but it takes long time (6–8 weeks) to grow MAP (Karuppusamy et al. 2021). This prompted scientists to look for alternate methods to detect active pathogens in clinical samples. Few authors (Singh et al. 2018) tried to address this issue by the use of silver nanoparticle based nano-immuno rapid assays. In order to achieve this, the present study was conducted to develop multiplexed qPCR assay, while the applicability was evaluated by targeting three genes *IS900 (FAM)*, *IS900 (Cy5)*, and *ModD* in culture and fecal samples of microscopy positive animals.

Material and methods

Collection of biological samples During the period from 2019 to 2020, 30 fecal samples were collected from different breed of goats exhibiting chronic or chronic intermittent diarrhea from herds of different villages of Mathura district of Uttar Pradesh. Fecal samples were collected per-rectally by inserting index finger without any contamination using disposable hand gloves in zip-locks by labeling well. The fecal samples were immediately brought to the laboratory for further processing. About 5 g of fecal sample is homogenized in sterile pestle mortar with 4–5 ml of sterile PBS and transferred to 15-ml falcon tube and top-up with 7–8 ml of PBS. The homogenate was centrifuged at 4500 rpm for 45 min at RT. Semi-solid middle layer is used to prepare smear for ZN staining and DNA isolation.

Cultural and molecular identification of *Mycobacterium avium* subspecies paratuberculosis Herrold's egg yolk medium (HEYM) supplemented with Mycobactin J for isolation of *M. paratuberculosis* was prepared for culture and isolation of *Mycobacterium avium paratuberculosis* as described below:

1. Mineral salt solution (MSS) was dissolved in 870 ml of triple distilled water (TDW) and adjusted pH to 7.6–7.8 with NaOH and added agar powder to it.
2. 2.0 gm malachite green dye was dissolved in 100 ml TDW to make 2% W/V.
3. Both solutions were sterilized in an autoclave for 15 min at 15 lbs (121 °C).
4. MSS was placed on a hot magnetic stirrer at 45–50 °C after autoclaving to prevent from solidifying before media-slant tube preparation.
5. The egg yolk was collected from fresh eggs that had been pre-cleaned with soap and then immersed for 45 min in isopropyl alcohol before being air dried.
6. Egg yolk was gently homogenized at RT under aseptic conditions.
7. By heating and stirring constantly for 2 h, homogenized egg yolk and 5.0 mL malachite green solution were added to MSS at 45–50 °C.
8. Homogenized egg yolk and 5.0 ml of malachite green solution were then added to MSS maintained at 45–50 °C, by heating and stirring continuously for 2 h.
9. Mycobactin J was dissolved in 4.0 ml of absolute ethanol and then added drop by drop to stirring HEYM.
10. The prepared HEYM was then dispensed into sterilized McCartney tubes and allowed to solidify in a slanting position.
11. Slants were incubated at 37 °C overnight after solidification to ensure sterility.
12. For further use, the media tubes were stored at 4 °C.

Colonies were picked for smear preparation for morphological identification by microscopy, and molecular characterization and confirmation of strain was done by *IS900* PCR (Singh et al. 2010; Millar et al. 1996; Gupta et al. 2017), IS*1311* PCR (Sevilla et al. 2005), and IS*1311* PCR-REA (Whittington and Sergeant 2001). The smears of colonies were examined microscopically under an oil immersion objective lens (× 100) (Singh et al. 2013). *Mycobacterium avium paratuberculosis* (MAP) reference strain "S 5" "Indian Bison Type" was sub-cultured as per (Gupta et al. 2017) MAP colonies were morphologically characterized by Ziehl–Neelsen staining (microscopy) (Singh et al. 2013).

Extraction of RNA MAP RNA was isolated with Tri-reagent protocol. For RNA extraction, 3–4 loop full bacterial growth

from culture/100 µl semisolid fecal slurry was reconstituted to 200 µl of PBS. The reconstituted culture was washed twice with 500 µl of PBS by centrifugation at 10,000 × g for 10 min and then resuspended in 100 µl of NFW. The RNAiso Plus (TaKaRa, Japan Cat. #9108) was added in micro-centrifuge tubes containing MAP culture in NFW to make final volume of 1000 µl. The tubes were briefly vortexed and incubated for 10 min at room temperature and then centrifuged at 12,000 × g for 10 min. at 4 °C. The supernatant was collected in a new micro-centrifuge tube, and 200 µl of chilled chloroform was added followed by vortex for 30 s, and the tubes were incubated at room temperature for 5 min, and then centrifuged again at 12,000 × g for 15 min at 4 °C. Subsequently, the supernatant (without touching interface) was collected into fresh micro-centrifuge tube, and then equal volume of chilled isopropanol was added and allowed to precipitate for 30 min at room temperature. Then, the tubes were gently flicked to mix the content and incubated at room temperature for 30 min and centrifuged at 12,000 × g for 15 min to sediment the pellet. Following this, the supernatant was discarded without disturbing the pellet, and 1 ml of 70% chilled ethanol was added to wash the pellet by centrifugation at 10,000 × g for 5 min. This step repeated with another wash of 70% ethanol. Thereafter, the alcohol was decanted carefully without disturbing pellet and dried for 5–10 min. Finally, the pellet was reconstituted with 30 µl of diethyl pyrocarbonate (DEPC)-treated water. Extracted RNA was tested for its concentration by using low and high standard of QuantiFluor RNA dye (Promega, Cat# E3310) of Quantus Fluorometer® (Promega Technologies, Madison, USA, Cat# E6150) as per the manufacturer's protocol.

cDNA synthesis Total RNA was converted to copy DNA (cDNA) using Primescript™ Ist strand cDNA synthesis kit (TaKaRa, Japan Cat# 6110A) protocol. First reaction mixture was prepared by adding 8.0 µl of RNA (total 1.0 µg/reaction with concentration adjusted to 125.0 ng/µl), 1.0 µl of Random Hexamer Primer, and 1.0 µl of 10 mM dNTP Mix and incubated at 65 °C for 5 min and chilled in ice. In the meantime, 2nd reaction mixture was prepared by adding 4.0 µl 5 × PrimeScript Buffer, 4.5 µl RNase free water, 0.5 µl RNase inhibitor, and 1.0 µl PrimeScript RTase in another set of tube. After incubation, 1st reaction mix was added into 2nd reaction mix followed by thermal incubation for reverse transcription to cDNA in SureCycler 8800 (Agilent Technologies) as given in Figure S2. The synthesized cDNA product was preserved at − 20 °C till further use for real-time PCR.

Probe-based real-time PCR (qPCR)

Probe and primer designing A TaqMan® probe-based real-time PCR method for the specific detection of *Mycobacterium avium paratuberculosis* based on the amplification and detection of sequence in the open reading frame of the *ModD* and *IS900* gene. Primers and probe were designed following alignment of *ModD* gene sequences of MAP organism strain and biovars according to general guidelines and recommendations. The reporter dyes Cy5 (Cyanine-5) and TexasRed were conjugated at the 5′ ends of the probes and quencher dyes, BHQ-1 (Black Hole Quencher) and BHQ-2 at the 3′ ends of *IS900* and *ModD* genes, respectively. All primers and probes were designed using extraction of contiguous/discontiguous unique conservative sequences aligned by using BioEdit software version 7.2.5. Discontiguous conserved regions in different strains of *Mycobacterium avium paratuberculosis* aligned using clustalW multiple alignment. All used primers and probes are listed in Table 1 and primer, and probe binding regions in the Gene sequences of *ModD* and *IS900 mRNA* are shown in Figure S1.

Conventional PCR To assess the activity of designed *IS900* mRNA and *ModD* primers, a conventional PCR was performed by following standard reaction conditions. The components were mixed gently, and amplification was carried out in thermal cycler (Techne, TC-4000) as per the thermal cyclic conditions given in Figure S3. About 5 µl of the PCR amplicons were loaded into each well in 1.8% agarose gel containing ethidium bromide UltraPure™ Ethidium Bromide (0.625 mg/mL; Invitrogen™ USA, Cat# A25645) oriented towards the cathode along with GeneRuler 100 bp ladder (Thermo Scientific, USA, Cat# SM0241). Electrophoresis was carried out at a constant voltage of 120 V for

Table 1 List of primers and probe used in TaqMan.®-based real-time PCR

S. No	Gene	Primer		Probe	Remarks
		Forward	Reverse		
1	*IS900* mRNA	5′GTGGTAGACAGCGTGGTCG3′	5′CTGACCAAGGCCGACAGGT3′	5′-Cy5-TTTCGGTCGTAGTAGGTGC3′-BHQ1	In-house designed
2	*ModD*	5′TCTCACCTCGACTACGGCTC3′	5’TGCGGAAGCCAACAACGCCA3’	5′-TEXASRED-CGCTGCTGAGCAAGGTCACCG3′-BHQ2	In-house designed
3	*IS900*	5′ATCTGGACAATGACGGTTACGGAG3′	5′ATCGCTGCGCGTCGTCGTT3′	5′-6FAM-AAGGCCGACCATTACTGCATGG3′-BHQ1	Gupta et al. (2017)

30–45 min. The DNA products were visualized using a UV trans-illuminator and photographed using gel documentation system.

Simplex probe-based real-time PCR

Standardization of simplex probe-based real-time PCR Standardization of real-time PCR (qPCR) protocol was done by using *IS900* and *ModD* primers and probes, with the positive cDNA. Premix Ex Taq™ (Probe qPCR) master mix (TaKaRa, Japan, Cat# RR390A) was used for quantitative analysis of *IS900* and *ModD* genes. The expression of these genes was assessed using TaqMan probe chemistry (CFX96 real-time PCR system®, Bio-Rad) with the respective primers used at 10.0 pmol final concentration, 1.0 µl of cDNA template equivalent to 50 ng of total RNA, and 2 × master mix in a final reaction volume of 20 µl in duplicates per sample per gene. The reaction condition has been followed as described by the manufacturer with the annealing temperature at 60 °C. Reaction controls like no template control (NTC) and positive controls (PC) have been kept along with unknown samples for interpretation of results. Dynamic threshold had been set by the software according to formula [–d (RFU)/dT] in the Y axis versus the temperature (°C) on the x-axis.

-d (RFU) / dT means [-differential calculus (relative fluorescence units) / differential calculus of temp.]

Optimization of probe and primer concentration for simplex real-time PCR Probe was titrated with different concentrations as 2 pmol/rxn, 4 pmol/rxn, 6 pmol/rxn, 8 pmol/rxn, 10 pmol/rxn, where primer concentrations were constant (10 pmol/rxn) as given in Table S1. Likewise, primers (*IS900* and *ModD*) were titrated at the same concentrations mentioned for probes, where probe concentration was constant (10 pmol/ rxn) as tabulated in Table S1. All the reactions were set in 20 µl of reaction volume in 8 strip tubes in duplicates. Premix Ex Taq™ (Probe qPCR) master mix (TaKaRa, Japan, Cat# RR390A) was used in the real-time PCR. Based on the amplification efficiency of the titrations, the final reaction mix contained primers and probes at a concentration of 10 pmol/µl for each target. The amplification was performed on CFX96™ real-time PCR system®, Bio-Rad, with final reaction mix given below in Table 2. Real-time PCR was performed by using the following thermal cycles which is given in Figure S4. The assay was repeated at least 2 times with each template and the negative control.

Table 2 Quantity and concentration of reagents used in probe-based real-time PCR

S. No	Reagent		Volume	Conc. used
1	Premix Ex Taq Probe qPCR Master Mix	:	10.0 µl	2x
2	Forward primer	:	1.0 µl	(10 pmol/µl)
3	Reverse primer	:	1.0 µl	(10 pmol/µl)
4	Probe	:	1.0 µl	(10 pmol/µl)
5	Template	:	1.0 µl	(~5 ng)
6	Nucleus free water	:	6.0 µl	-

Multiplex probe real-time PCR

Standardization and optimization of probe, primer, and template for multiplex probe real-time PCR Standardization of real-time multiplex probe PCR (qPCR) was done by using *IS900* and *ModD* primers and probes, with the positive cDNA. Probes were titrated with constant primer and template concentration. Template were titrated where both probe and primer concentrations were kept as a constant, and the other parameter is used as a variable to check the best optimized concentrations as given in Table S2.

Standardization of multiplex probe real-time PCR All the reactions were set in 8 strip tubes in duplicates in 25 µl of total reaction volume. Premix Ex Taq™ (Probe qPCR) master mix (TaKaRa, Japan, Cat# RR390A) was used in the real-time PCR (CFX96 real-time PCR system®, Bio-Rad). The assay was performed by using the same thermal cyclic conditions given in Figure S4, and the final reaction mix for all three genes *IS900 (FAM)*, *IS900 (cy5)*, and *ModD (TexasRed)* is given in Table 3.

Limit of detection assays using copy number for the multiplex target genes To assess the range of detection (ROD) and limit of detection (LOD) for the current mRNA-based TaqMan® real-time assay targeting multiple genes, we used the purified amplicon concentrations of each of the target genes (*IS900* (FAM), *IS900* (CY5), and *ModD* (Texas Red)). The amplicons generated for the genes, viz., *IS900* (FAM), *IS900* mRNA (Cy5), *ModD* (TexasRed) were gel purified, and their concentrations were calculated using Quantus Fluorometer (Promega) ONE dsDNA dye (Promega, Cat# E4871) following manufacturer's protocol. Based on concentrations, length of amplicon (bp), they were converted into copy number using the online tool, viz., http://cels.uri.edu/gsc/cndna.html. The reactions and thermal conditions were performed as per the previous protocols for the respective genes described in Figure S4.

Specificity assays The current mRNA-based TaqMan® probe real-time PCR assay for *IS900* (cy5) and *ModD* (TexasRed) was verified for its specificity by incorporating non-related microbes by following the thermal conditions as described in

Table 3 Optimized concentrations of primer, probe, and templates in the final multiplex TaqMan probe real-time PCR reaction mix

S. No	Reagent			Volume	Conc. used
1	Premix Ex Taq™ (Probe qPCR)Master Mix		:	10.0 µl	2x
2	*IS900 (FAM)*	Forward primer	:	0.20 µl	(2 pmol/µl)
		Reverse primer	:	0.20 µl	(2 pmol/µl)
		Probe	:	0.20 µl	(2 pmol/µl)
3	*IS900 (cy5)*	Forward primer	:	1.0 µl	(10 pmol/µl)
		Reverse primer	:	1.0 µl	(10 pmol/µl)
		Probe	:	1.0 µl	(10 pmol/µl)
4	*ModD (TexasRed)*	Forward primer	:	1.0 µl	(10 pmol/µl)
		Reverse primer	:	1.0 µl	(10 pmol/µl)
		Probe	:	1.0 µl	(10 pmol/µl)
5	Template		:	3.0 µl	(~5 ng)
6	Nucleus free water		:	5.4 µl	-
Total			:	25 µl	

Figure S4 and reagents as described in Table 2. Separate reactions in duplicates were used in a total reaction volume of 25 µl for *E. coli* (ATCC-25922), *S. aureus* (ATCC-29213), and MAP S5 strain, respectively, along with no template controls (NTC). This was done to identify whether if any unrelated non-specific amplification occurs from these standard microbes which are commonly present in fecal samples and other bio-samples. The machine used in the current study is CFX96™ real-time thermal cycler (Bio-Rad), and the software used for machine call of results is CFX96 manager. The positive call is based on a combination of RFU cutoff limit and Cq, where the RFU cutoff for positive should be above the prescribed limit, and the Cq should be below the prescribed limit for the genes in question. The "RFU" denotes the fluorescence intensity of the reporter dye as measured by the real-time thermo cycler after each cycle, and "Cq" denotes the cycle quantification in the form of a sigmoidal curve with its log phase crossing the basal threshold line.

Results

Diagnostic techniques including ZN smear fecal microscopy, conventional PCR, IS1311 REA, and culture techniques were done to identify the MAP in animals suspected of Johne's disease. The results are presented for these established diagnostic techniques. However, there is a need for development of a robust diagnostic assay that could sensibly detect the MAP infection.

Microscopy and culture identification Out of the 30 J.D. suspected animals sampled, their fecal samples were subjected to ZN smear microscopy, IS900 cPCR, IS1311PCR-REA, and culture tests (Table 4). In ZN staining, 30 fecal samples were assessed out of which 5 samples were found positive where *Mycobacterium avium paratuberculosis* stained pinkish of carbol fuchsin (primary stain) and appeared as "comma"-shaped short bacilli on bright pale blue background of methylene blue (counter stain) in the smear by light microscopy (Fig. 1). The fecal samples which were positive by microscopy were decontaminated and processed for culture in HEYM media along with Mycobactin J using the protocol as described in the "Methods" section. Out of the samples cultured, only one sample could grow into colonies after 5 months which was confirmed by ZN microscopy, IS900 cPCR, and IS1311 REA using restriction enzymes, viz., HinfI (Takara Bio Cat#1238A) and MseI (Takara Bio Cat#1247A) as "Bison" type, whereas other samples could not be recovered by culture method.

Molecular detection Fecal samples found positive in microscopy were tested for conventional PCR targeting IS900 and IS1311 gene. In gel electrophoresis, 5 samples showed specific

Table 4 Results of all tests performed for collected samples

S. No	Type of diagnostic tests used for screening	Positive	Positivity percent	Remarks
1	ZN acid fast Microscopy ($n=30$)	5	16.66%	-
2	IS900 cPCR ($n=30$)	5	16.66%	-
3	IS1311 PCR-REA ($n=5$)	5	100	Sheep type(1) Bison type (4) (Whittington et al. 2001)
4	Culture ($n=5$)	1	20%	-

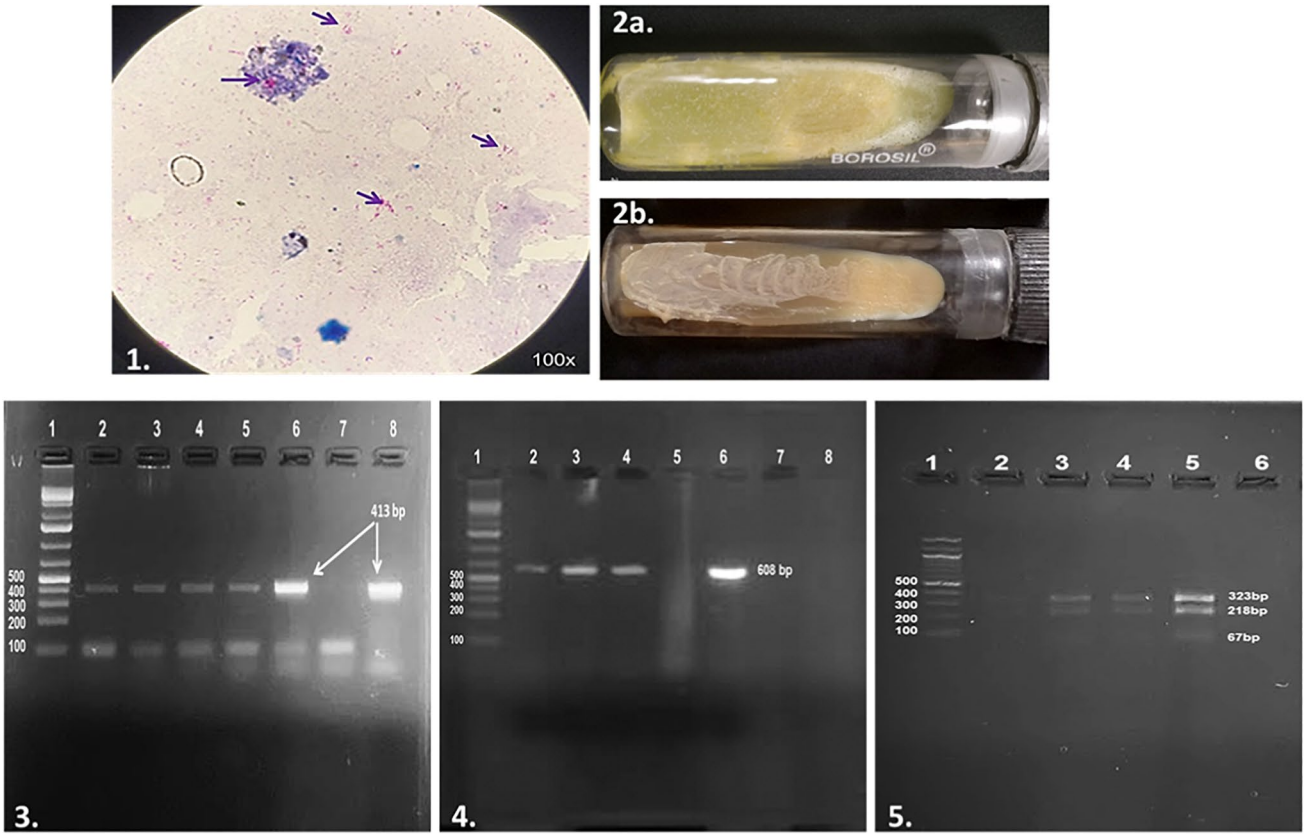


Fig. 1 **1** Fecal smear stained from clinically affected Johne's disease animal using Ziehl Neelsons' technique showing acid fast bacilli (indicating arrows). **2** *Mycobacterium avium paratuberculosis* isolation in Herrold's egg yolk medium (HEYM) slant supplemented with Mycobactin J. **2a** Unknown fecal sample from goat showing colonies on HEYM slant; **2b** MAP positive isolate (S5 native strain) maintained in the laboratory. **3** Gel-electrophoresis of conventional PCR targeting gene *IS900* (413 bp). Lane 1: 100 bp ladder, Lane 2, 3, 4, 5, 6 positive unknown sample, Lane 7: NTC (no template control) Lane 8: PC (positive MAP DNA control). **4** Gel-electrophoresis of conventional PCR targeting gene *IS1311* PCR (608 bp). Lane 1: 100 bp ladder, Lane 2, 3, 4 positive sample, Lane 5, 7 negative samples, Lane 8: NTC (no template control), Lane 6: PC (positive MAP DNA control). **5** *IS1311* PCR-REA: Lane 1: 100 bp ladder, Lane 2: sheep type, Lane 3–5 Bison type

amplification of 413 bp and 608 bp for IS900 and IS1311 gene, respectively (Fig. 1). To confirm the MAP types, we subjected the IS1311 PCR amplicon for restriction endonuclease digestion analysis (REA) using restriction enzymes, viz., HinfI (Takara Bio Cat#1238A) and MseI (Takara Bio Cat#1247A). After double digestion, restriction fragments were then visualized through gel-electrophoresis, and genotypes were interpreted as described by Whittington. The current set of bio-samples when analyzed by IS1311PCR-REA, restriction fragments corresponding to 'Bison' type (67 bp, 218 bp, and 323 bp) was found in 4 goat samples and the fragments corresponding to 'Sheep' type (285 bp and 323 bp) was found in one goat sample.

These positive samples screened by the above tests were later used as positive controls for RNA extraction to assess the presence of viable MAP organism using the mRNA-based TaqMan® probe real-time PCR targeting various genes of MAP. Before the actual screening of viable MAP in these clinical samples, we developed and standardized the aforementioned diagnostic assay, and the results are given in Fig. 1.

Development of mRNA *TaqMan.*® probe-based real-time PCR (qPCR) assay for detection of MAP

In present study, we designed primers for genes expressed more frequently in metabolically active *Mycobacterium avium paratuberculosis*, allowing us to test for live MAP by developing multiplex qRT-PCR technique using the more sensible marker *ModD*. Before this, various standardization techniques were followed including checking the primers by conventional PCR, simplex TaqMan® probe PCR using positive MAP cDNA as discussed in the following "Results" sections.

Conventional PCR for newly designed *ModD* and *IS900* primers for development of multiplex TaqMan probe real-time assay To check the workability of the primers designed for multiplex PCR, conventional PCR was carried to check

the conditions and amplicon size. The gel photographs for newly designed IS900 and ModD primers' amplified products are given in Fig. 2.

Probe and primer standardization Probe was titrated with different concentrations as 2 pmol/rxn, 4 pmol/rxn, 6 pmol/rxn, 8 pmol/rxn, 10 pmol/rxn, while primer concentrations were constant (10 pmol/rxn). Likewise primers (*IS900* and *ModD*) were titrated at the same concentrations mentioned for probes, where probe concentration was constant (10 pmol/rxn) and analyzed to determine the optimal concentration. The early exponential and log linear phases were ideal at all titrations (Fig. 3 (1a and 1b, 2a and 2b)), but the plateau phase was better at primer dilution 10 pmol and probe dilution 10 pmol; thus, these concentrations were used further in the assay.

Simplex PCR First, we checked all three target genes, viz., ModD (TexasRed), IS900 (FAM), and IS900 mRNA (cy5), by performing simplex PCR assay individually with final optimized probe primer concentration using standard thermal conditions given in Fig. S4. All real-time PCR assay reactions were set in duplicates by using final reaction mixture given in Table 2. Positive fecal cDNA showed late amplification for IS900 (FAM), IS900 (Cy5), and ModD (Texas Red) in comparison to positive MAP culture cDNA. On the contrary, positive MAP culture cDNA showed good amplification for IS900 (FAM) target only, but IS900 (Cy5) and ModD (Texas Red) did not produce any positive signal for their respective fluorophores (Fig. 4). This implies that the latter two targets are efficient only in transcriptional form reiterating their role in actively replicating MAP cells.

Multiplex mRNA TaqMan® probe real-time PCR

Application of multiplex PCR assay in the 5 MAP positive fecal RNA Out of the five MAP positive fecal RNAs assayed for mRNA-based TaqMan® probe multiplex real-time PCR assay, most showed good amplification of IS900 (FAM) and few showed IS900 (Cy5), while one showed very mild amplification of ModD (Texas Red) (Fig. 4), which eventually was a "Bison" type that came positive in HEYM media. Hence, it is also evident that culture has highest specificity compared to that of conventional PCR, which can amplify anything of the likes of dead bacilli to naked DNA. Also, it can be interpreted from the above findings that triplex mRNA real-time PCR is equivalent to culture in terms of specificity, but highly advantageous with time, labor, and sensitivity.

Limit of detection (LODs) of *ModD* (TexasRed), *IS900* (FAM), *IS900* mRNA (cy5) genes of MAP multiplex mRNA TaqMan® (three colors) probe PCR assay The LOD assay was conducted using this TaqMan® probe PCR for the multiple gene targets to assess the lowest copy number of MAP mRNA that can be detected in a given sample. The amplicons generated for the genes, viz., *IS900* (FAM), *IS900* mRNA (Cy5), and *ModD* (TexasRed), were gel purified, and their stock concentration were 4.98 ng/µl, 2.0 ng/µl, and 0.6 ng/µl for *IS900* (FAM), *IS900* mRNA (Cy5), and *ModD* (TexasRed), respectively. Further, their copy numbers calculated were 4.14×10^{10}(*IS900*, FAM), 1.27×10^{10} (*IS900*, Cy5), 3.68×10^{9} copies (*ModD*, Texas Red) which were estimated as per the methods described.

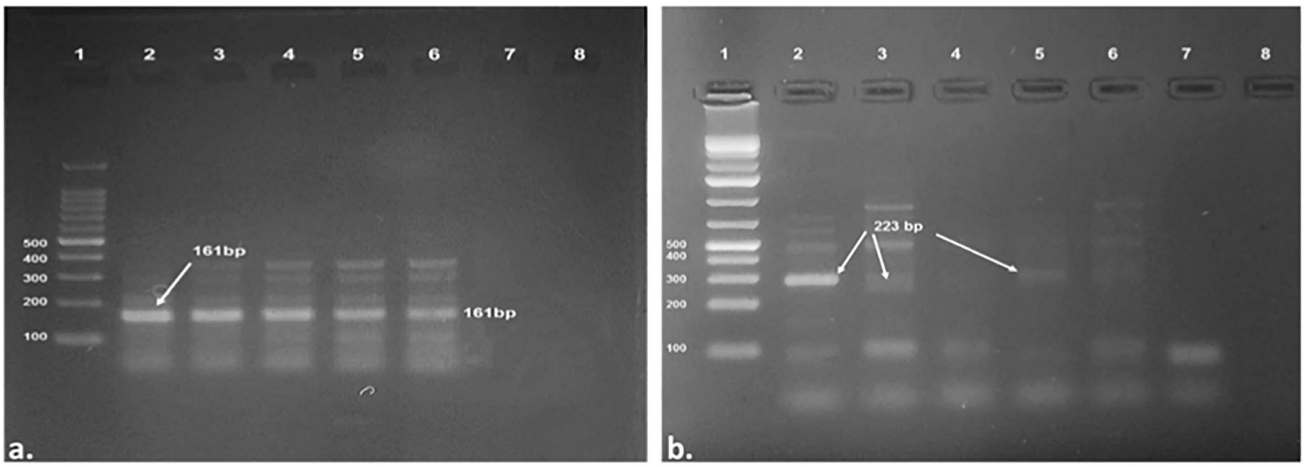


Fig. 2 **a** Gel-electrophoresis of conventional PCR targeting gene IS900 mRNA (161 bp). Lane 1: 100 bp ladder, Lane 2, 3, 4, 5 positive unknown sample, Lane 6: PC (positive control), Lane 7: NTC (no template control). **b** Gel-electrophoresis of conventional PCR targeting gene ModD (223 bp). Lane 1: 100 bp ladder, Lane 3, 4, 5, 6 unknown sample, Lane 2: PC (positive MAP DNA control), Lane 7: NTC (no template control)

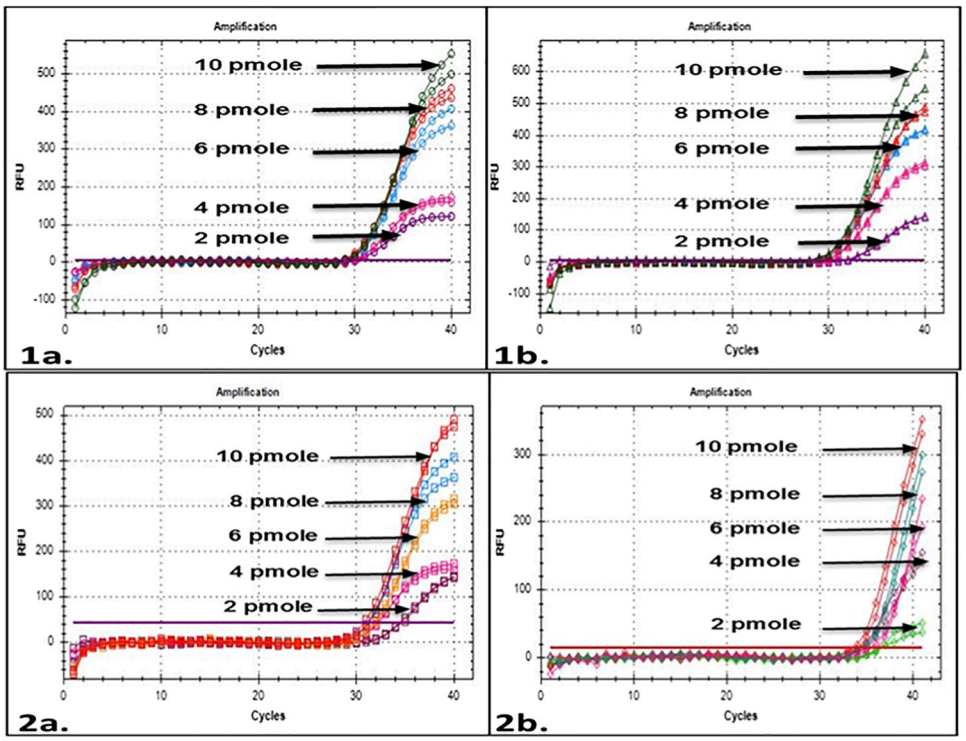


Fig. 3 **1a** and **1b** Probe and primer titration for *IS900* mRNA (CY5) gene of MAP for mRNA probe PCR. **2a** and **2b** Probe and primer titration for *ModD* (TexasRed) gene of MAP for mRNA probe PCR

The sensitivity was computed based on copy numbers ranged from 4.14×10^{11} to 4.14×10^{4} for *IS900* (FAM), 1.27×10^{11} to 1.27×10^{4} for *IS900* mRNA (Cy5), and 3.68×10^{10} to 3.68×10^{4}. The standard curve, on the other hand, was shown to be linear over these dilutions making it their range of detection (ROD) for the respective genes. The LOD for the targets *IS900* (FAM), *IS900* (CY5), and *ModD* (Texas Red) are 4.14×10^{4}, 1.27×10^{4}, and 3.68×10^{4} copies, respectively. LOD is the lowest copy that can be detected using this assay for the above target genes (Fig. 5).

Specificity assays Similarly, the newly developed assay was verified for its specificity using a Gram-negative bacteria *Escherichia coli* (ATCC25922) and a Gram-positive bacteria *Staphylococcus aureus* (ATCC259213) along with the positive MAP culture (S5 strain) and was done to ensure there is no non-specific signal generated from other microbes while conducting the real-time PCR assay (Fig. 6). Based on the machine call, there were no signal (Cq or RFU) produced for *E. coli* and *S. aureus* for both the target fluorophores. RFU lower cutoff limit was 150 RFU & 450 RFU and Cq upper cutoff limit was 33 for specific target genes, IS900 (Cy5) and ModD (Texas Red), respectively, based on real-time PCR.

Discussion

Control of Johne's disease in farmed livestock and other animal populations is becoming increasingly difficult due to horizontal spread and lack of effective control measures. To apply the control measures, a robust diagnostic technique which could tick all the key boxes is essential. The lack of sensitive and specific testing capable of detecting early MAP infection makes control operations difficult. Although PCR-based screening approaches based on (pooled) fecal samples are now available in some countries, veterinary diagnostic laboratories still mostly use a combination of direct examination of ZN-stained smears, serological tests, and culture to identify infected farms (Harris and Barletta 2001, L.M. Irenge et al. 2009). To make it clear, the major issue is in identifying active and silent spreaders, which do not show any clinical sign, but act as a major hub of infection, and on the converse passive spreaders that is not a part of the infectious cycle needs to be identified. Passive shedders are animals that are actually not infected with MAP, but MAP ingested through feed material or water and other sources gets excreted through the fecal route eventually identified by ZN stain acid fast smear. This confuses the diagnostic part, as microscopy cannot differentiate between actively replicating infective/live MAP from inactive MAP. Hence, a proper diagnostic tool is required to address the aforesaid issue. In this context, the current study evaluated the use of multiplex mRNA-based real-time PCR, which can specifically be employed to address this concern. While most authors still consider culture to be the gold standard for MAP identification, it is still a time-consuming method that takes up to 16 weeks to incubate and has a low sensitivity. Furthermore, MAP dependence on Mycobactin J should be interpreted with caution, as some MAP strains can grow

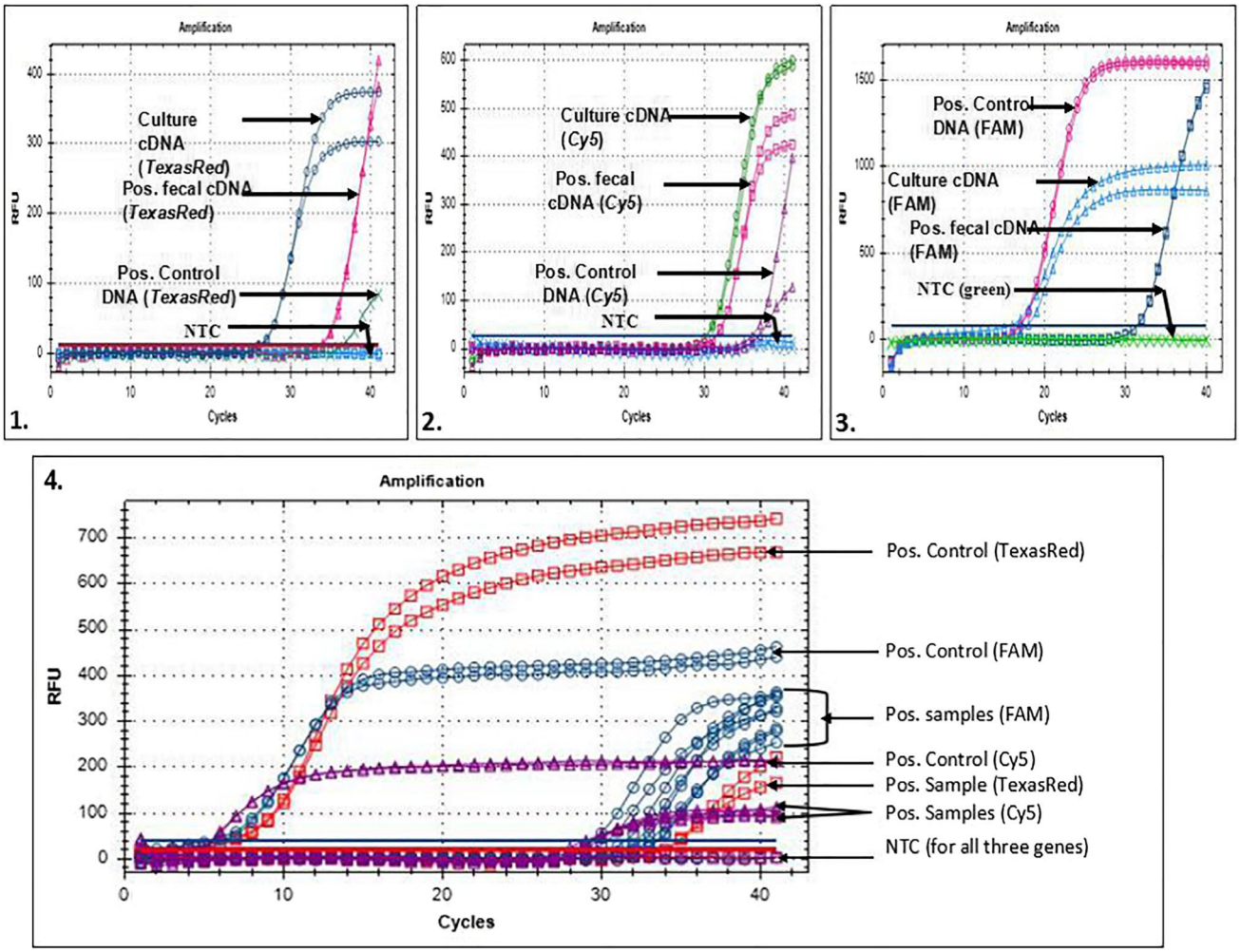


Fig. 4 **1** mRNA probe PCR for ModD TexasRed gene of MAP for culture cDNA and fecal cDNA, positive DNA control, and NTC. **2** mRNA probe PCR for IS900 Cy5 gene of MAP for culture cDNA and fecal cDNA, positive DNA control, and NTC. **3** mRNA probe PCR for IS900 FAM gene of MAP for culture and fecal cDNA, positive DNA control and NTC. **4** Multiplex mRNA TaqMan® (three colors) probe PCR assay for three genes, *ModD* (TexasRed), *IS900* (FAM), *IS900* mRNA (cy5), for MAP positive fecal RNA samples, positive control (MAP culture spiked fecal RNA), and NTC (no template control)

without it. As a result, culture restrictions are of particular importance when it comes to the specificity and sensitivity of MAP detection in animals. Earlier investigations revealed increased sensitivity of microscopy compared to ELISA, fecal and blood PCR; higher identification of MAP by fecal smear microscopy may be attributable to screening of clinical JD animals. However, the specificity of microscopy is heavily dependent on the user's expertise. By comparing IS900 blood PCR with fecal microscopy for the diagnosis and estimation of prevalence of *Mycobacterium avium paratuberculosis* (MAP), SV Singh and coworkers in 2013 discovered that fecal microscopy was cost effective, easy to adopt, and repeatable for screening the domestic ruminant population against MAP infection. Direct PCR, rather than culture, is used to treat pooled environmental samples, which decreases processing time and costs (Bölske and Herthnek 2010, Barkema et al. 2018). Another advantage of employing PCR over culture is that it has increased sensitivity and does not rely on live MAP bacteria in the sample. The quality of results however for PCR depends on many factors which includes DNA extraction process and optimization of primers and other reagents (Mita et al. 2016). On the other hand, quantification of MAP bacilli is not possible by conventional PCR, but off late semi quantification PCR (densitometry) or qPCR (real-time PCR) aids in the quantification of starting template or quantification by copy numbers or by interpolation using standard curve generated by assaying MAP spiked fecal samples (Laurin et al. 2015, Barkema et al. 2018). Hence, in the current study, we report an assay that can detect transcript copies of live MAP ranging from 4.14×10^{11} to 4.14×10^{4} for *IS900* (FAM), 1.27×10^{11} to 1.27×10^{4} for *IS900* mRNA

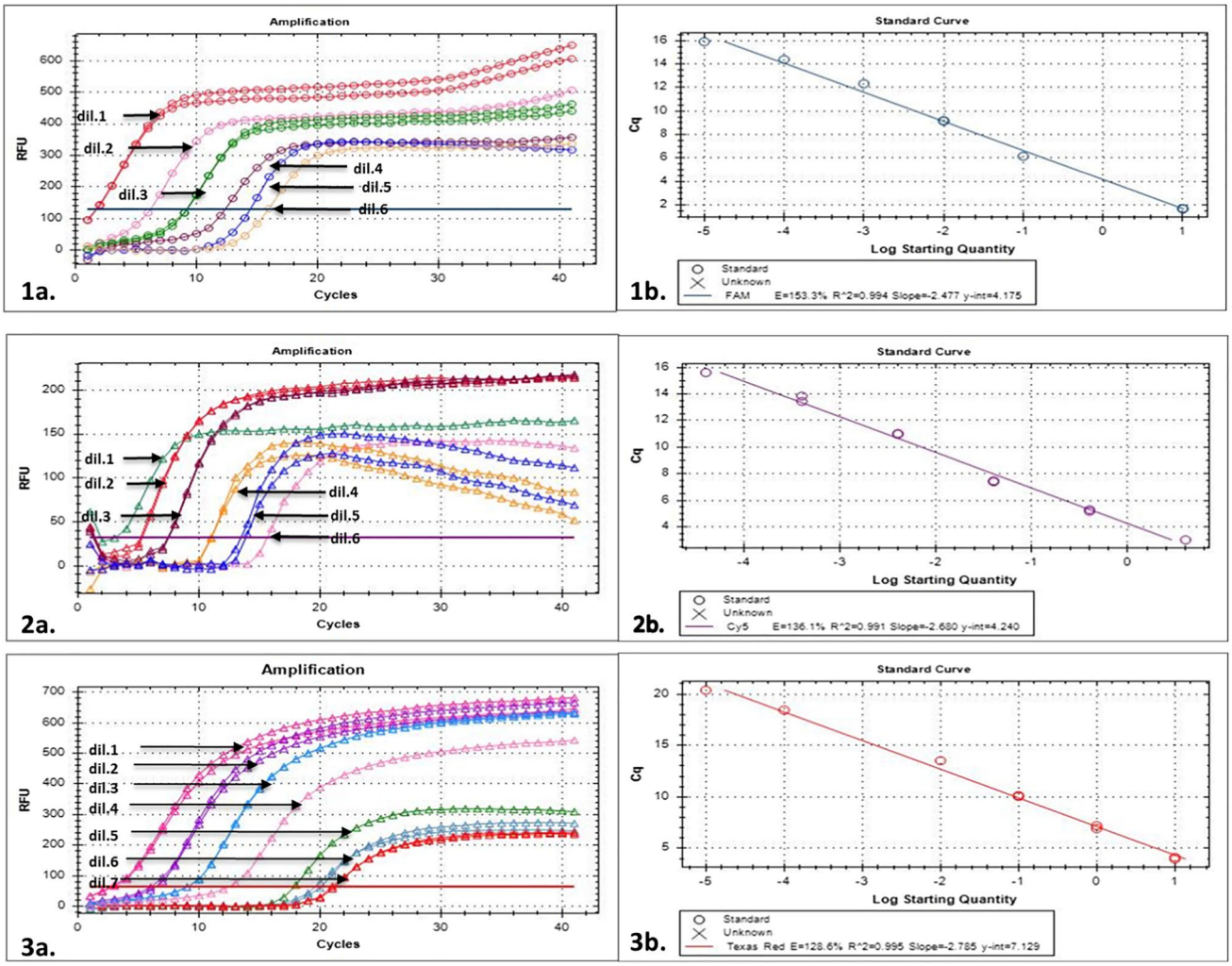
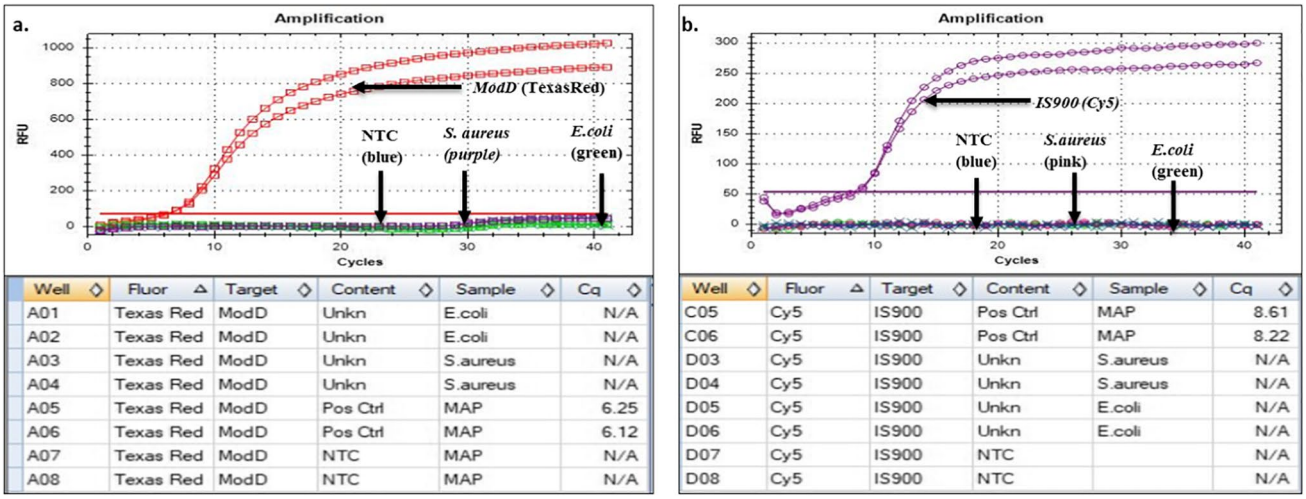


Fig. 5 **1a** Amplification plot of serially diluted MAP amplicon of IS900 FAM gene, descending concentrations (by a magnitude of Log10) ranging from 4.14×1011 (dil.1) to 4.14×104 (dil.6) for determination of LOD by TaqMan® probe-based real-time PCR assay. **1b** The standard curve for a tenfold serial dilution series plotted as the threshold cycle on the Y-axis, against the target concentration of cDNA per assay (X-axis) with E value = 153.3%, correlation coefficient (R^2) = 0.994, slope = −2.477, and Y intercept = 4.175. **2a** Amplification plot of serially diluted MAP amplicon of IS900 Cy5 gene, descending concentrations (by a magnitude of Log10) ranging from 1.27×1010 (dil.1) to 1.27×104 (dil.6) for determination of LOD by TaqMan® probe-based real-time PCR assay. **2b** The standard curve for a tenfold serial dilution series plotted as the threshold cycle on the Y-axis, against the target concentration of cDNA per assay (X-axis) with E value = 136.1%, correlation coefficient (R^2) = 0.991, slope = −2.680, and Y intercept = 4.240. **3a** Amplification plot of serially diluted MAP amplicon of ModD TexasRed, descending concentrations (by a magnitude of Log10) ranging from 3.68×109 (dil.1) to 3.68×104 (dil.7) for determination of LOD by TaqMan® probe-based real-time PCR assay. **3b** The standard curve for a tenfold serial dilution series plotted as the threshold cycle on the Y-axis, against the target concentration of cDNA per assay (X-axis) with E value = 128.6%, correlation coefficient (R^2) = 0.995, slope = −2.785, and Y intercept = 7.129

(Cy5), and 3.68×10^{10} to 3.68×10^{4} for *ModD* and lowest limit of detection (LOD) to amplify the targets *IS900*-FAM, *IS900*-CY5, and *ModD* Texas Red are 4.14×10^{4}, 1.27×10^{4}, and 3.68×10^{4} copies, respectively. There is dearth of information in the literature as far as mRNA-based multiplex real-time PCR for MAP is concerned. Few of the multiplex assays are available for MAP and that too are DNA-based. The triplex qRT-PCR assays were developed a decade earlier (Schonenbrucher et al. 2008) using three targets, viz., *IS900*, ISMAP02, and f57, or another robust Triplex TaqMan® real-time PCR amplifying the F57 and the ISMav2 (Léonid M. Irenge et al. 2009) following combined internal amplification control (IAC), as a stand-alone application for the detection of MAP from bovine fecal samples without additional PCR confirmation tests. Few of the works (Foddai and Grant 2020) were based on phage-based RT-PCR assay for detection of MAP using D29 mycobacteriophage-coated tosylactivated paramagnetic beads to capture MAP cells directly from milk samples. Evidence of passive fecal shedding of *Mycobacterium avium* subsp.



Well	Fluor	Target	Content	Sample	Cq
A01	Texas Red	ModD	Unkn	E.coli	N/A
A02	Texas Red	ModD	Unkn	E.coli	N/A
A03	Texas Red	ModD	Unkn	S.aureus	N/A
A04	Texas Red	ModD	Unkn	S.aureus	N/A
A05	Texas Red	ModD	Pos Ctrl	MAP	6.25
A06	Texas Red	ModD	Pos Ctrl	MAP	6.12
A07	Texas Red	ModD	NTC	MAP	N/A
A08	Texas Red	ModD	NTC	MAP	N/A

Well	Fluor	Target	Content	Sample	Cq
C05	Cy5	IS900	Pos Ctrl	MAP	8.61
C06	Cy5	IS900	Pos Ctrl	MAP	8.22
D03	Cy5	IS900	Unkn	S.aureus	N/A
D04	Cy5	IS900	Unkn	S.aureus	N/A
D05	Cy5	IS900	Unkn	E.coli	N/A
D06	Cy5	IS900	Unkn	E.coli	N/A
D07	Cy5	IS900	NTC		N/A
D08	Cy5	IS900	NTC		N/A

Fig. 6 **a** Amplification plot of MAP DNA and other non-related bacteria *Escherichia coli* (ATCC25922) and *Staphylococcus aureus* (ATCC259213) along with NTC to test specificity of *ModD* (Texas Red) TaqMan® probe-based real-time PCR assay assays and table showing Cq values and target sample replicates for Texas Red fluorophores targeted for *ModD* gene. **b** Amplification plot of MAP DNA and other non-related bacteria *Escherichia coli* (ATCC25922) and *Staphylococcus aureus* (ATCC259213) along with NTC to test specificity of *IS900* mRNA (cy5) TaqMan® probe-based real-time PCR assay assays and table showing Cq values and target sample replicates for Cy5 fluorophores targeted for *IS900* mRNA gene

paratuberculosis in a Limousin cattle herd was previously studied (Petr Kralik et al. 2014), which is caused by the ingestion of MAP in contaminated feed and passive passage through GIT without active infection that might produce false-positive results in microscopy or PCR. Another challenge is early diagnosis of viable MAP infection in farmed livestock that can aid to break the cycle of MAP transmission by vaccination or quarantine of active silent shedders. But there are no tools to effectively detect the dose–response to infection in terms of active/dead MAP in animals treated by drug or vaccine prototypes. In the past, many workers tried to address these issues by developing different methodologies for assessing the viability of MAP in milk samples (Grant 2021) using peptide-mediated magnetic separation (PMS) phage assay followed by liquid culture (PMS culture) and direct *IS900* qPCR, which was way complex and involve multiple techniques to assess. Another work to assess the viable and dead MAP in milk samples is a combination of two techniques: peptide magnetic bead separation followed by propidium monoazide qPCR (Ricchi et al. (2014). But through this, we can only estimate probability of viable and nonviable MAP cells in milk samples rather than accurate enumeration of viable MAP bacilli. Later, few more tests were compared with the above test by another set of workers (Hannah B. Pooley, et al. 2016) which includes three commercially available bacterial viability assays, viz., resazurin viability dye assay, Live/Dead BacLight bacterial viability kit, propidium monoazide viability dye–based assay. The researchers compared these tests with a modified liquid culture method coupled with high-throughput quantitative PCR to differentiate live and dead MAP and their accuracy at low bacterial concentrations. It was found that the modified liquid culture-based method was sensitive enough to detect MAP at low concentrations compared to the gold standard culture technique. Botsaris et al. (2013) described the use of phage-PCR to detect live MAP cells in powder milk products which is more sensitive and faster than culture with specificity matching that of PCR with an added advantage of detecting only live cells (Emma C. Stanley, et al. (2007). Singh et al. (2018) developed a nano-immuno test for detection of live MAP bacilli in milk samples through MAP specific antibody-conjugated magnetic nano-particles in presence of resazurin chromogen. Although the test is simple and suitable for wide-scale screening, this test is time-taking, and it takes around 10 h to give results. Many of the authors reported less specificity of IS900 for MAP detection due to non-specific amplification of IS900-like sequences and also highlighted the fact that IS900 can still found in soil after MAP removal from animal farms or field, so the epidemiological pertinence of IS900-only positive shedding shows requirement of further investigations (Cook and Britt 2007, L.M. Irenge et al. 2009). So we designed primers for genes expressed more frequently in metabolically active *Mycobacterium avium paratuberculosis*, allowing us to test for live MAP by developing triplex qRT-PCR technique using the more sensible marker *ModD*. *ModD* gene encodes for the fibronectin attachment protein (FAP) that aids the MAP bacilli to colonize the host cells by binding to soluble cell integrin: fibronectin (FN) by attaching to extracellular matrix. In a

way FN acts as a link between MAP bacilli and cellular cytoskeleton and helps internalization into host epithelial cells. The FAP is antigenic and induces protective immunity because any specific antibody against this protein could effectively block the internalization. It has been observed that *IS900* transcripts are constitutively expressing despite the stage of infection or in inactive/dead MAP cells due to its abundance, while *ModD* are a functional gene whose expression is modulated in live MAP cells and will be absent in dead or inactive/passive organism. Thus, our current triplex real-time PCR test will be aiding in detecting the viable MAP organisms and also can differentiate between active and passive MAP shedders.

The passive ingestion of MAP bacilli which appears in the fecal material often confuses the microscopical findings that cannot differentiate between active and passive MAP organism. To address this issue, the currently developed diagnostic tool will be aiding in the differentiation of actively replicating MAP organism from that of passive one. This study presents a first of its kind multiplex mRNA-based TaqMan® qPCR assay that can differentiate live MAP shedding animals from dead/inactive MAP shedders.

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Author contribution GK conceived and designed the experiments and wrote the manuscript. AP performed the experiments and analyzed the data. DS helped in designing experiments. SS helped in performing experiments. The manuscript was critically examined and contributed to all authors, who also approved the final version.

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Declarations

Ethics approval This article does not contain any studies with human participants or animals performed by any of the authors.

Conflict of interest The authors declare no competing interests.

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