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Original article

ISOLATION AND MOLECULAR CONFIRMATION OF *MYCOBACTERIUM AVIUM* SUBSPECIES *PARATUBERCULOSIS* IN CATTLE AND BUFFALOES FROM THREE STATES OF INDIA

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Summary

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In a random survey for identification of *Mycobacterium avium* subsp *paratuberculosis* (MAP) in cattle and buffaloes from states of Telangana, Maharashtra and Gujarat in India, a single time point sampling of 1,008 animals was undertaken. The samples: milk (n=605), faeces (n=381) and necropsy/post-mortem tissues (n=22) were initially processed for isolation of MAP in the MGIT BACTEC 960 system, which detected 22 out of 1,008 as MAP. All 22 samples were sub-cultured in Middlebrook 7H10 agar, Herrold's Egg Yolk medium (HYEM) and 7H9 broth supplemented with Mycobactin J. The three media showed different sensitivities in supporting the growth of field MAP strains. Only 9 out of 22 BACTEC 960 positive cultures grew on Middlebrook 7H10 agar, and 7H9 broth supplemented with Mycobactin J, while 8 out of 22 cultures grew on HYEM. Seven out of 9 positive cultures originated from milk samples from cattle, while the remaining 2 positive cultures were from necropsy and post-mortem tissues of cattle and buffalo respectively. MAP could not be isolated from faecal samples. All cultures grown on 7H10 agar, HYEM, 7H9 broth supplemented with Mycobactin J revealed acid fast bacilli on staining. In a recently developed in-house Real Time PCR targeting the MAP-specific insertion element *ISMav2*, only 8 out of the 9 field isolates were identified as MAP.

Key words: culture, Johne's disease, *Mycobacterium avium* subsp. *paratuberculosis,* Real Time PCR

INTRODUCTION

Mycobacterium avium subsp. *paratuberculosis* (MAP) is responsible for causing paratuberculosis or Johne's disease in domestic and wild ruminants (Douarre *et al.,* 2010). MAP causes significant loss to the dairy industry in terms of decreased milk production and premature culling, increased mortality and decreased reproductive efficiency (Tiwari *et al.*, 2005; 2008). It is an acid-fast bacillus 0.5–1.5 µm in size and an intracellular fastidious pathogen which takes 12–16 weeks to grow (Singh *et al*., 2010; 2014). Animals are infected with MAP early in life by ingestion of bacterium from infected milk or colostrum, or via farm environment, but clinical symptoms appear after 2–5 years (Eltholth *et al*., 2009; Anzabi & Hanifian, 2012). MAP can remain in the field for a long time without multiplication, and such types of fields remain infectious for one year (Eslami, 2011). MAP is chiefly characterised by chronic inflammation of the intestine (Wuhib *et al*., 2005). It causes Crohn's disease in humans (Singh *et al*., 2010). Although the clear association is still not proven, it has been suggested that milk and dairy products could be possible vehicles of transmission of MAP from cattle to humans (Naser *et al.,* 2014).

Efforts have been made in last few decades to develop protocols for the detection of MAP in clinical samples using various methods. Serology and faecal culture are commonly used for confirming paratuberculosis in the field (Collins *et al.,* 2005; OIE, 2014), whereas molecular methods are ideal for rapid identification and confirmation of the pathogen (Lazaro *et al.,* 2005).

The existence of Johne's disease and its serological evidence in different states in India has been documented earlier (Gupta *et al.,* 2012; Trangadia *et al.,* 2012; Narnaware & Tripathi, 2014). Despite such evidence, very few reports are available describing the isolation of MAP from cattle and buffaloes in India. A report has recently described the isolation of MAP from faecal samples (9/184; 4.8%) from cattle and buffaloes in northern India (Chauhan *et al*., 2014). The present study was aimed towards a random survey to detect the direct presence of MAP in bovine and bubaline herds in some of the states in India using various cultural isolation methods, and confirmation of the isolates as MAP by a newly developed and optimised in-house real time PCR.

MATERIALS AND METHODS

Samples

A total of 1,008 samples consisting of milk (n=605), faeces (n=381) and post-mortem tissues (n=22) were included for the random survey in the present study. The samples were from different farms, fields and abattoirs from Gujarat, Maharashtra, and Telangana collected during the period from 2012 to 2014. The tissue samples (n=22) were from young male calves less than one year of age. Out of 22 samples, two were necropsy samples (mesenteric lymph node and tissue from ileo-cecal junction from calves) from a farm in Hyderabad (Telangana). The remaining 20 tissues (mesenteric lymph node) were post-mortem samples from the abattoirs located in Hyderabad (Telangana) and Mumbai (Maharashtra).The remaining 986 samples (milk and faeces) were from adult females. The species-wise and region-wide history/details of milk, faeces and tissue samples are furnished in Table 1.

Table 1. Region-wise and species-wise distribution of field samples processed for detection of MAP

Geographical region/ Species	Type of sample		
	Faeces	Milk	Tissue
Telangana			
Cattle	67	148	2
Buffalo	0	0	15
Gujarat			
Cattle	194	423	0
Buffalo	120	34	0
Maharashtra			
Cattle	0	0	0
Buffalo	0	0	5
Total	381	605	22

Maintenance of Mycobacterium avium subsp paratuberculosis reference strain

The *Mycobacterium avium* subsp *paratuberculosis* K-10 (ATCC® BAA-968™) strain obtained from the American Type Culture Collection (ATCC, USA) was propagated and maintained on three different media: 7H10 agar supplemented with 10% albumin dextrose catalase (ADC) supplement, 0.05% Tween 80 (BD, USA) and 2 mg/L Mycobactin J (Synbiotics Europe, France); Herrold's egg yolk medium (HYEM) (Hi-media, India) slopes containing Mycobactin J (2 mg/L); or 7H9 broth containing 10% oleic albumin dextrose catalase (OADC) supplement, 0.05% Tween 80, and 2 mg/L Mycobactin J, and incubated at 37 °C for 10–12 weeks. Maintenance of the strain was performed by continuous sub culturing in the above mentioned media at a minimum interval of 2–3 months.

Processing of clinical samples prior to culture

The milk samples were processed as described earlier by Gao *et al*. (2005). Briefly, 50 mL of milk sample was taken in a Falcon tube, centrifuged at 3100×g for 30 min, then the supernatant was discarded and the pellet was decontaminated in 20 mL of 0.75% hexadecylpyridinum chloride (HPC; Sigma) for 4–5 h. After decontamination the samples were centrifuged at 1000×g for 15 min, and the pellet was dissolved in 1 mL 7H9 broth.

The tissue samples were processed as described by Kent & Kubica (1985). Briefly, 4% NaOH (Merck) was added to an equal amount of homogenised tissue sample, vortexed for 20 min and centrifuged at 2500×g for 20 min. The supernant was disarded and the pellet was neutralised with equal amount of phosphate buffered saline (pH 7.4) and centrifuged at 2500×g for 15 min. The resulting supernantant was discarded and the final pellet was resuspended in 1 mL of 7H9 broth.

Faecal samples were decontaminated as recommended by the manufacturer (Para TB MIGT™ BACTEC960™, Becton Dickinson, USA). Briefly, on day one, 2 g faeces were taken in 50 mL Falcon tube, to this 17.5 mL of sterile distlled

water was added, vortexed and the tube was allowed to stand at room temperature for 30 min. Then 2.5 mL of the supernatant was collected from the above tube and to this 2.5 mL of 15% bacto yeast extract (Sigma) and 0.2 mL of 10% sodium pyruvate (Qualigens) was added, vortexed and incubated at 35–37 ^{o}C for 90 min. For each sample, 0.3 mL of 5% malachite green solution and 25 mL of sterile ½× Brain Heart Infusion (BHI) (BD, USA) – 0.9% HPC solution was added and incubated overnight at 35–37 ^{o}C. On day 2, the supernatant was discarded and 1 mL of antibiotic brew (10 mL of sterile ½×BHI + 40 µL of 2.5% vancomycin + 40 µL of 2.5% nalidixic acid + 25 µL of 1% amphotericin B) was added. This suspension was incubated overnight at 35–37°C. After incubation 0.1 mL of the suspension was inoculated into Para TB medium tubes (Cat. No. 245154, BD, USA).

Isolation by culture

The decontaminated sediments as described above, inoculated into Para TB medium tubes (Cat. No. 245154, BD, USA) were processed in BACTEC MGIT 960 instrument for 49 days at 37 ^{o}C. Prior to inoculation the BACTEC™ MGIT™ Para TB medium tubes (MGIT tubes) were supplemented with OADC enrichment medium (Cat. No. 245156, BD, USA), egg yolk, amphotericin B, nalidixic acid, and vancomycin (Sigma, USA) as indicated in the BD protocol. MGIT tubes prepared as described above were left at room temperature for 18–24 h to allow the egg yolk to equilibrate with culture medium.

The identification of MGIT tubes positive for MAP was according to the algorithm prescribed for BACTEC 960 (Becton Dickinson, USA).The MAP positive MGIT were sub-cultured. According to the diagnostic protocol, readouts of the results returned by the machine from the MGIT tubes signalling/flagging positive (audio-signal) due to the algorithm of BACTEC 960 expressed in terms of 'Growth Units' and 'Time to detection' were noted. The MGIT tubes that were detected positive were re-entered into the BACTEC 960 for confirmation of initial (first) positive signal. The results obtained after second and final audio-signals were considered as confirmatory for detection of MAP as per the BACTEC 960 protocol. The +T in readout indicated that the machine had detected a signal above the positive threshold value as determined by the algorithm of the instrument, while the readout T indicated a positive value lying on the threshold.

One tube of each of the following media: 7H10 agar supplemented with 10% albumin dextrose catalase (ADC) supplement, 0.05% Tween 80 and 2 mg/L Mycobactin J (Synbiotics Europe, France); Herrold's egg yolk medium (HYEM) slopes containing Mycobactin J (2 mg/L) or 7H9 broth containing 10% oleic albumin dextrose catalase (OADC) supplement, 0.05% Tween 80, and 2 mg/L of Mycobactin J, were inoculated with 0.1 mL of culture from MGIT culture positive tubes and incubated at 37 ^{o}C for 12–16 weeks.

Identification of MAP from cultures by staining for acid-fast bacilli (AFB)

Heat fixed smears prepared from MGIT cultures declared positive by the BACTEC 960 system, and typical growths on 7H10 agar and HEYM slopes or 7H9 broth cultures showing turbidity were screened for presence of AFB organisms. The smears were examined for AFB organisms by using modified Ziehl-Neelsen and AFB fluorescent staining as per the vendor's protocol (Becton Dickinson, USA).

Identification of contaminating bacteria and fungi in MGIT cultures

The Soybean Casein Digest Medium (SCDM) (Hi-media, India) was prepared as prescribed by the manufacturer. After the end of 49-day incubation of cultures in the MGIT BACTEC 960, 0.1 mL of sample from each MGIT tube was inoculated in SCDM.

Molecular confirmation of MAP field isolates

The positive MGIT cultures were subcultured into 7H9 broth. Ten to fifteen days cultures in 7H9 broth were used for DNA extraction. DNA extraction was performed according to the protocol of a commercial MAP Extraction kit (Tetracore®, Rockville, U.S.A).

An optimised in-house real time TaqMan PCR targeting a MAP specific *ISMav2* gene recently developed in our laboratory (unpublished data) was used for confirmation of isolates as MAP. The *ISMav2* was selected because of the specific diagnostic potential of the region (Strommenger *et al.*, 2001; Startmann *et al.,* 2002). The threshold Cq of <40 was determined as the cut-off value in the real time PCR for classifying samples as positive.

Statistical analysis

The data for 'days to detection' and 'growth units' obtained from MGIT BACTEC 960 machine readouts were subjected to statistical analysis employing Microsoft Excel software version 2010. The 'T' in the 'Growth Unit' readout value was considered as 1 in this study for statistical considerations, since this number was the least readout value returned by the BACTEC 960.

RESULTS

Twenty-two out of 1,008 samples were detected as MAP by the MGIT BACTEC 960. The results of the confirmatory final readouts indicated that the mean±SD time to detection was 23.11±10.97 days and the mean±SD growth units: 2096±5416.46 (Table 2). Only 9 out of 22 BACTEC 960 positive cultures grew on Middlebrook 7H10 agar, and 7H9 broth supplemented with Mycobactin J, while 8 out of 22 cultures grew on HYEM. Seven out of 9 positive cultures originated from milk samples from cattle, while the remaining 2 positive cultures were from necropsy and post-mortem tissues of cattle and buffalo, respectively (Table 2). MAP could not be isolated from faecal samples. All cultures grown on 7H10 agar, HYEM, 7H9 broth supplemented with Mycobactin J revealed acid fast bacilli on staining, however only 9 out of 22 samples from MGIT were positive by AFB staining (Table 2).

It was also observed that an animal may not shed MAP simultaneously in milk and faeces (Table 3). However, when a recently developed in-house real time PCR targeting the MAP specific insertion element *ISMav2* was used for confirmation, only 8 out of the 9 field isolates were identified as MAP (Table 2).

Results of sterility testing in SCDM medium indicated that 13 samples positive by MGIT BACTEC 960, but negative by AFB staining were contaminated with Gram negative rods, Gram negative cocci and sometimes Gram positive cocci.

DISCUSSION

The MGIT BACTEC 960 system has been extensively used for the isolation of mycobacteria in recent years (Hillemann *et al.,* 2006). The present study confirms the

Table 2. Results of identication of MAP field samples by BACTEC MGIT 960 system, Acid Fast Bacilli (AFB) staining, Traditional culture media (Middlebrook 7H9 broth, Middlebrook 7H10 agar, HYEM) and *ISMav2* Real-time PCR

Lab ID	Geographical origin/State	Species	Type of sample	Culturing by BACTEC MGIT 960		AFB staining	Sub-culture in traditional medium			AFB staining^A	RT-PCR^B
				Days to detection	Growth Units		Middlebrook 7H9 broth	Middlebrook 7H10 agar	HYEM		
1	Telangana	Cattle	Milk	19.3	310 +T*	+	+	+	+	+	+
2	Telangana	Cattle	Milk	21.2	21 +T*	+	+	+	+	+	+
3	Telangana	Buffalo	Tissue	10.21	4167	+	+	+	+	+	+
4	Telangana	Cattle	Tissue	17.23	11 +T*	+	+	+	+	+	+
5	Telangana	Cattle	Milk	12.16	14322	+	+	+	+	+	+
6	Gujarat	Cattle	Milk	25.8	85 +T*	+	+	+	+	+	+
7	Gujarat	Cattle	Milk	10.21	1 +T*	+	+	+	+	+	+
8	Gujarat	Cattle	Milk	16.9	T*	+	+	+	+	+	+
9	Gujarat	Cattle	Milk	4.7	T*	+	+	+	–	+	+
10	Telangana	Buffalo	Tissue	18.21	22665	–	–	–	–	–	–
11	Telangana	Buffalo	Tissue	38.17	552	–	–	–	–	–	–
12	Maharashtra	Buffalo	Tissue	20.23	1409 +T*	–	–	–	–	–	–
13	Telangana	Cattle	Milk	20.7	1 +T*	–	–	–	–	–	–
14	Telangana	Cattle	Milk	37.21	T*	–	–	–	–	–	–
15	Telangana	Cattle	Milk	25.8	85 +T*	–	–	–	–	–	–
16	Telangana	Cattle	Milk	42.15	1849 +T*	–	–	–	–	–	–
17	Gujarat	Cattle	Milk	16.14	117	–	–	–	–	–	–
18	Gujarat	Cattle	Milk	38.5	165	–	–	–	–	–	–
19	Gujarat	Cattle	Milk	41.15	80	–	–	–	–	–	–
20	Gujarat	Cattle	Milk	38.6	7 +T*	–	–	–	–	–	–
21	Gujarat	Cattle	Milk	17.15	270 +T*	–	–	–	–	–	–
22	Gujarat	Cattle	Milk	16.9	T*	–	–	–	–	–	–
			Mean ± SD	23.11 ±10.97	2096.49 ±5416.46						

+T* = above positive threshold fluorescence value, T* = on positive threshold fluorescence value, T* value returned by MGIT BACTEC 960 was considered as 1, whereas for +T* results the prefixed numerical values in the column was considered for statistical consideration; ^A = Result of acid fast bacilli staining of culture subcultured in 7H9 broth, 7H10 agar, HYEM; ^B = Result of *ISMav2* Real-Time PCR using DNA extracted from growth of sub-cultures in Middlebrook 7H9 broth

Table 3. Comparison of distribution of MAP isolated from various field samples

Laboratory ID of samples	Faecal samples	Milk samples	Tissue samples
1	NA	Positive	NA
2	Negative	Positive	NA
3	NA	NA	Positive
4	Negative	NA	Positive
5	Negative	Positive	NA
6	NA	Positive	NA
7	NA	Positive	NA
8	NA	Positive	NA
9	NA	Positive	NA

NA – not available

utility of the MGIT BACTEC 960 culture system for quicker diagnosis of MAP by culture as compared to the traditional media. In this report, for the first time, we confirm the presence of MAP by culture in milk samples of cattle from Gujarat and Telangana employing the BACTEC MGIT 960. Out of 9 MAP field isolates recorded in this study 7 (77.77%) were from unpasteurised milk samples of cattle in Gujarat and Telangana. In an earlier report MAP was isolated from 44% (7/16) of unpasteurised and 67% (18/27) pasteurised milk samples of cattle from states of northern India (Shankar *et al.,* 2009). Since MAP has been associated with Crohn's disease in humans, the zoonotic implications of consumption of cattle milk infected with MAP in the regions studied in India could be of serious concern for public health.

Although this laboratory had earlier used fluorescence based commercial liquid culture system to identify an MAP isolate from faecal samples (n=20) from chronically infected crossbred (as determined by Johnin test and milk and serum ELISA) dairy cows in a farm located in Hyderabad, it was unable to identify MAP from any of the 20 milk samples (unpublished results). In the study cited above the animals were over 4 years of age. We report in the current study, isolation of MAP from necropsy as well as post-mortem tissues of young male beef and buffalo calves less than one year of age, thus confirming an ealier observation that animals are infected very early in life but exhibit clinical symptoms after 2–5 years (Eltholth *et al.*, 2009; Anzabi & Hanifian, 2012).

The detection rate varied from 2.18% to 0.79% in the different media used in this study (MGIT BACTEC 960, 7H10 agar, HYEM and 7H9 broth – all media supplemented with Mycobactin J). By employing the MGIT BACTEC 960 for identification of MAP by culture, we were able to advance the mean TTD in this study to 23.11 days post inoculation; and in one occasion the confirmation was recorded as early as 4.7 days. These results indicated the obvious diagnostic advantage of the BACTEC 960 fluorescence based liquid culture over tradition culture media such as HYEM, 7H10 agar or 7H9 broth with respect to turn around time. From unpublished data, the faecal isolate was detected 19 days post inoculation in the MGIT 960 system. The TTD is crucial for diagnosis. In an earlier study, positive detection of MAP on HYEM was ob-

served between 45 to 600 days (Shankar *et al*., 2009). The liquid culture media have been favoured for the cultivation of MAP from clinical samples because of their greater analytical sensitivities than solid media (Whittington, 2009). In the current study MAP could not be detected in any of the faecal samples. This may have been due to the low sensitivity of faecal culture or a result of intermittent shedding of MAP in faecal samples (Laurin *et al.*, 2014; Kauffman *et al.*, 2014). The success of culture from faeces is clearly related to the sufficient number of MAP being shed from the intestinal lesions (Vansnick *et al*., 2002). Linked to these observations, the single point sampling could have contributed to the non-recovery of MAP from faecal samples in this study. Although the number of observations was small, at least in three cases, MAP could be isolated from milk but not from the faeces of the same animal, indicating that at one point in time, MAP may not be shed simultaneously in the faeces and milk.

In this study we found that 13 out of 22 samples identified as MAP positive cultures by MGIT BACTEC 960 were contaminated by other bacteria, even when recommended decontamination protocols were performed. This observation emphasises that meticulous care is essential during collection of field samples while ensuring the adherence to far more stringent aseptic conditions. It also appears that decontamination protocols practiced in this study need further modifications to suit the local conditions for recovery of pure MAP cultures from field samples. The degree of contamination in the 13 MGIT BACTEC 960 positive cultures may have affected the AFB staining results.

Molecular confirmation of MAP isolates employing the most widely used IS900 gene target has been recommended (Donaghy *et al.*, 2010; Selim *et al.*, 2013). However, there are evidences of false positive results using IS900 gene target by PCR (Cousins *et al.,* 2000; Harris & Barletta, 2001; Bolske *et al*., 2002; Englund *et al*., 2002). Alternatively, the MAP specific genetic element *ISMav2* found in at least three copies have been evaluated to improve the specificity and reliability of detection of MAP by PCR (Strommenger *et al*., 2001; Stratmann *et al*., 2002; Schonebrucher *et al*., 2008; Selim *et al*., 2013). We also experienced the non-specificity of IS900 PCR while optimising an in-house Real Time TaqMan PCR and found that the *ISMav2* based TaqMan Real Time PCR was specific for the identification of MAP (unpublished data). We therefore used the *ISMav2* in-house Real Time TaqMan PCR for confirmation of the 9 MAP field isolates using DNA extracts from sub-cultures in Middlebrook 7H9 broth and found that it had a sensitivity of 88.88%.

CONCLUSION

The sensitivity of detection of MAP from field samples by the fluorescent based liquid culture system (BACTEC MGIT 960) (2.18%) was just slightly better than that of traditional media (0.789 to 0.89%). The most obvious advantage of employing the BACTEC MGIT 960 is the quicker identification of MAP from field samples (Mean TTD = 23.11 days post inoculation) compared to the traditional media. Together, the strategy of adopting BACTEC MGIT 960 for intial cultures followed by sub-culturing of positive isolates in Middlebrook 7H9 broth, and molecular confirmation of sub-cultures using the Real time *ISMav2* PCR can be suggested as a robust and specific MAP iden-

tification system. The AFB staining of cultures grown in MGIT 960 or traditional media seems to have a very significant role in this strategy as it provides specificity to morphological identification. Multiple time point sampling rather than the single time point sampling that was employed in this study may further aid in improving the efficacy of the adopted strategy.

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