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Comparative distribution of *Mycobacterium avium* subspecies *paratuberculosis* in the target and non-target tissues of goats and sheep population in India

J VOHRA[1], S V SINGH[2], A V SINGH[3], P K SINGH[4] and J S SOHAL[5]

Central Institute for Research on Goats, Makhdoom, Uttar Pradesh 281 122 India

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ABSTRACT

Target and non-target tissues of 41 goats and 20 sheep (farm and farmer's stocks) were screened by culture and PCR, to know the distribution of MAP in different tissues and organs. Species-wise prevalence of MAP was 42.5 and 40.0% in goats and sheep, respectively, and was 48.1 and 39.3% in animals (goats and sheep) from farm and farmer's stocks, respectively. Of the 215 tissues screened by culture, 47.9% were positive for MAP. Prevalence was 50.8 and 40.0% in tissues of goats and sheep from farm stock, respectively and 60.0 and 44.2% in tissues of young and adult animals, respectively. Tissues-wise, 42.5 and 53.8% farm animals were positive in culture of mesenteric lymph nodes (MLN) and intestine tissues, respectively, whereas, 37.5 and 41.1%, respectively in farmer's animals. Screening of supra-mammary lymph nodes (SMLN) revealed that, 52.5 and 43.1% were positive from farm and farmer's animals, respectively. Inflamed SMLN from farm stocks showed higher infectivity. MAP was recovered from 73.6, 64.2, and 54.5% tissues of uterus, udder and testes, respectively, in farm stocks. Of the MAP cultured from goats, 82.0% were pauci and 17.9% multi-bacillary and from sheep, all the cultures were pauci bacillary. Majority of colonies appeared between 45 and 120 days post inoculation. Decontaminated pellets from tissues and MAP cultures were processed for DNA isolation and screened by IS900 PCR. Positive DNA samples on amplification yielded specific 229bp band from pellets of intestine, MLN, SMLN, udder, uterus and testes. From MAP cultures, 4 of 13 DNA were amplified. Protoplasmic (PPA) antigen from MAP 'Bison type' strain cultured from a terminal case of JD in goat was used in ELISA. Study revealed that MAP was distributed widely in target and non-target tissues of goats and sheep. Of the 3 test used, culture of tissues was most sensitive. This is the first report of recovery of MAP from non-target tissues of goats and sheep in India.

Key words: Culture, ELISA, Goat, Johne's disease, IS 900 PCR, *Mycobacterium avium* subspecies *paratuberculosis*, Sheep, Tissues

Johne's disease (JD) caused by *Mycobacterium avium* subspecies *paratuberculosis* (MAP), is most serious infection of animals and human beings worldwide (Bull *et al.* 2003). Disease has significant impact on livestock economy and losses exceed $1.5 billion to US cattle industry every year. Despite low production per animal in India these losses have not been estimated in the 120 and 62.5 millions goat and sheep, respectively. Johne's disease is quite common and high prevalence has been reported both from farm and farmer's stocks in Mathura region of North India (Singh *et al.* 1996, Singh *et al.* 2006 and Singh *et al.* 2007). Traditionally Johne's is considered mainly a disease of adults and farm animals confined to small intestine and draining lymph nodes (target tissues). Usually intestine near ileo-caecal junction (ICJ) and associated mesenteric lymph nodes (MLN) are examined for the presence of MAP (Tripathi and Parihar 1999, Hajra 2003). Information on distribution of MAP to other tissues and organs (non-target) with respect to any livestock species is not available in India. Presence and distribution of MAP to different tissues was crucial to endemicity of infection within a population. The study is maiden attempt to estimate comparative presence of MAP in target (intestine and mesenteric lymph nodes) and non-target tissues {Supra mammary lymph nodes (SMLN), udder, uterus and testes} of young and adult goats and sheep belonging to farm and farmer's stocks using culture and IS900 PCR.

Present address: [1] M.Sc, Student, [2] Senior Scientist, [3,4] Research Associate, [5] Ph. D. Scholar, Animal Health Division.

MATERIALS AND METHODS

Animals, tissues, vaginal secretions and serum samples

Sixty-one goats and sheep from farm stocks (44 necropsied at the Central Institute for Research on Goats CIRG), Mathura and farmer's stocks (17 sacrificed at slaughterhouse Mathura) between 29.01.05 to 01.04.05 were sampled. The 215 tissues (intestine near ileo-caecal junction, MLN, SMLN, udder, uterus and testes) were collected from 41 goats and 20 sheep. Of 61 animals, 16 goats, 17 kids, 10 sheep and 1 lamb were from farm stock and 8 goats and 9 sheep were from farmer's

stocks. Animals sacrificed at Mathura were driven from farmer's stocks in villages. Of the 44 animals necropsied at CIRG (farm stock), 40, 39, 22, 19, 19, 14, and 11 tissues were collected from intestine, mesenteric lymph node (MLN), left supra mammary lymph node (L-SMLN) right SMLN (R-SMLN), uterus, udder and testes, respectively. The 16, 17, 9, and 9 tissues from MLN, intestine, L-SMLN and R-SMLN, respectively were from 17 sacrificed animals (Mathura).

Culture of tissues

Fecal culture being 'Gold standard' (Sweeney *et al.* 1992) standardized and followed at Microbiology laboratory (Singh *et al.* 1996), was adopted for screening of tissues (Singh *et al.* 1996) and used in the present study. Briefly, modified Herrold's egg yolk medium (HEYM) was used for cultivation and hexa-decyl pyrindinium chloride (HPC) as decontaminant. About 2 cubic cm of freshly collected tissue was finely ground with sterile sand and normal saline and allowed to stand for 5–6 h at room temperature. Supernatant (5–6 ml) was layered onto decontaminant (0.9% HPC) and kept undisturbed for 12–24 h at room temperature. About 0.2 ml of sediment was inoculated on slants of HEYM (with mycobactin J and without antibiotics/fungicide). Cultures were examined at weekly intervals. MAP colonies were primarily identified on the basis of mycobactin J dependency, acid fastness and slow growth.

DNA isolation and IS900 PCR (decontaminated pellets/ cultures)

A modified method of isolation of DNA was used; wherein the decontaminated sediment (<1.0 ml) left after inoculation of HEYM medium was pelleted after centrifugation at 10000 rpm for 10 min. The pellet was re-suspended in phosphate buffered saline, vortexed to mix it well and centrifuged. Washed pellet was stored at–20°C, till further processing. This pellet was the starting material for the isolation of DNA. Pellets and cultures were processed for DNA isolation as per van Soolingen *et al.* (1993). DNA was amplified by PCR using specific IS900 primers (Vary *et al.* 1990). Briefly, in a volume of 60 µl of reaction mixture, 1 µM of each primers (forward primer: 150 C 24 mer, 1 µl; reverse primer: 921, 25 mer, 1 µl, Taq PCR master mix: Qiagen, 30 µl and deionised water, 26 µl) and 2 µl of template DNA was added. Total of 35 cycles were performed in a thermocycler (M J research) for complete amplification reaction. Reaction conditions were: initial denaturation at 94°C for 3 min followed by 35 cycles of de-naturation at 94°C for 10 sec, annealing at 61°C for 10 sec, extension at 72°C for 10 sec and final extension at 72°C for 3 min. Presence and yield of specific PCR product (229 bp) was analyzed by 1.8% agarose ethidium bromide gel electrophoresis.

An animal was considered positive for JD if any of the tissues (intestine, MLN, SMLN, udder, uterus and testes and vaginal swab) was positive in culture or PCR.

RESULTS AND DISCUSSION

Presence of MAP in tissues by culture

Of the 215 target and non-target tissues processed from farm and farmer's stocks, live cultivable MAP was recovered from 47.9% tissues (45.5% target and 50.4% non-target tissues). Prevalence of MAP was 51.0, and 41.8% in tissues of goats and sheep, respectively. MAP was isolated from 49.3 and 43.1% tissues from farm and farmer's animals, respectively. Screening of tissues of young and adult animals, 60.0 and 44.2% yielded MAP, respectively. Species-wise prevalence of MAP was 54.9 and 41.8% in goats and sheep population (farm and farmer's stocks), respectively. Prevalence of MAP was 52.5 and 43.1% in animals from farm and farmer's stocks, respectively (Tables 1–5).

Target tissues

MAP was isolated from 45.5% target tissues (48.1% farm and 39.3% farmer's stocks).

Farm animals

MAP was present in 48.1% tissues of animals (goats: 50.8%; sheep: 40.0%). In goats, MAP was recovered from 48.3% adults and 53.5% young animals. From MLN and intestine, MAP was isolated from 43.7 and 53.3% goats and 42.8 and 64.2% kids, respectively. In sheep, MAP was present in 33.3 and 100.0%, each of MLN and intestine, in adults and lambs, respectively. Comparative distribution of MAP in MLN and intestine (target tissues) was evaluated using

Table 1. Distribution of MAP in target tissues (MLN and intestine) of goats and sheep from farm stock by culture

	Species	Animals sampled	Per cent positives MLN	Intestine
Farm Stock	Goats	16	43.7	53.3
	Kids	17	42.8	64.2
		33	43.3	58.6
	Sheep	10	33.3	33.3
	Lambs	1	100.0	100
		11	40.0	40.0
Cumulative total		44	42.5	53.8

Table 2. Comparative distribution of MAP in target tissues (MLN and intestine) in farm stock by culture

Tissues	Combinations			
	1	2	3	4
MLN	+	–	+	–
Intestine	+	–	–	+
Total (39)	11 (28.2)	12 (30.7)	06 (15.3)	10 (25.6)

Table 3. Distribution of MAP in non-target tissues of goats and sheep from farm stock by culture

	Species	Animals sampled	Per cent positives				
			L-SMLN	R-SMLN	Uterus	Udder	Testes
Farm stock	Goats	16	36.3	36.3	66.6	55.5	00.0
	Kids	17	50.0	00.0	100	50.0	60.0
		33	**38.4**	**33.3**	**76.9**	**54.5**	**54.4**
	Sheep	10	33.3	44.4	60.0	33.3	—
	Lambs	1	100	—	100	—	—
		11	**40.0**	**44.4**	**66.6**	**33.3**	**54.4**
Cumulative total		44	36.3	38.0	73.6	50.0	54.5

culture. MAP was recovered from 69.2% farm animals of the total 39 farm animals screened. MAP was recovered from both the tissues in 28.2% animals and independently in MLN and intestine of 15.3 and 25.6% animals, respectively (Tables 1 and 2).

Farmer's animals

MAP was recovered from 39.3% tissues of farmer's animals (goats: 34.2% and sheep: 47.0%). From MLN and intestine, MAP was present in 37.5 and 25.0% tissues of goats and 37.5 and 55.5% tissues of sheep, respectively. Comparatively MAP was distributed in the 58.8% target tissues (MLN and intestine) of animals. MAP was recovered from both the tissues in 17.6% animals and separately in MLN and intestine of 17.6 and 23.5% animals, respectively (Tables 4, 5).

Table 4. Distribution of MAP in target and non-target tissues of goats and sheep in farmer's stock by culture

	Species	Animals sampled	Per cent positive		
			MLN	Intestine	SMLN
Farmer's stock	Goats	8	37.5	25.0	75.0
	Sheep	9	37.5	55.5	30.0
Total		**17**	**37.5**	**41.1**	**50.0**

Total tissues 51.

Table 5. Comparative distribution of MAP in target tissues (MLN and intestine) by culture in farmer's stock

Tissues	Combinations			
	1	2	3	4
MLN	+	-	+	-
Intestine	+	-	-	+
Total (17)	3 (17.6)	6 (35.2)	3 (17.6)	4 (23.5)

Non-target tissues

Of the 103 non-target tissues processed, MAP was recovered from 50.4% tissues (50.5% farm and 50.0% farmer's animals-Tables 2 and 4).

Farm animals

Goats: Of the 39 tissues screened from goats, 48.7% were positive for MAP in culture. MAP was recovered from 36.3, 36.3, 66.6, 55.5% and none, tissues of L-SMLN, R-SMLN, uterus, udder and testes, respectively. In kids, of 19 tissues screened, 63.1% were positive for MAP. MAP was recovered from 50.0, none, 100, 50.0 and 60.0% tissues of L-SMLN, R-SMLN, uterus, udder and testes, respectively (Table 3).

Sheep: Of 26 tissues screened from sheep, 42.3% were positive. MAP was recovered from 33.3, 33.3, 33.3, 44.4, 60.0, 33.3% and none tissues of MLN, intestine, L-SMLN, R-SMLN, uterus, udder and testes, respectively. Of the 1 lamb screened, the intestine, MLN and uterus were positive for MAP in culture (Table 3).

Farmer's animals

MAP was isolated from 75.0 and 30.0% tissues of goats and sheep, respectively. In goats, MAP was recovered from 37.5, 25.0 and 75.0% tissues of MLN, intestine and SMLN, respectively. In sheep, MAP was recovered from 35.7, 55.5 and 30.0% tissues of MLN, intestine and SMLN, respectively (Tables 4 and 5).

Gross lesions and culture in target tissues

Farmer's stocks: In young animals prevalence of MAP was 45.4 and 33.3% in normal and inflamed MLN, respectively. The 61.5% tissues were positive from normal and one tissue from inflamed intestine. In adult animals, MAP was recovered from 43.7% normal and 38.4%, inflamed MLN tissues. The 14.2% normal and 60.0% inflamed intestinal tissues were positive (Tables 1 and 2).

Gross lenins and culture in non-target tissues

Supra-mammary lymph nodes: In farm stock, the 37.2% of 43 SMLNs screened were positive for MAP. Recovery of

Table 6. Distribution of MAP in target tissues of goats and sheep (young and adults) in farm stock by PCR

Animals	Percent positive	
	MLN	Intestine
Adult goats	12.5	13.3
Kids	14.2	00.0
Adult sheep	22.2	11.1
Lambs	00.0	00.0
Total	15.0	7.6

MAP was 65.0 and 32.2% from inflamed and normal SMLN, respectively (fig. 1). In Farmer's stock, MAP was isolated from 50.0% of 18 SMLN screened (Normal SMLN–71.4% and inflamed–36.3%) (Table 4).

Uterus, udder and testes: In farm stock, MAP was cultured from 73.6, 50.0, and 54.5% of uterus, udder and testes tissues, respectively. Prevalence of MAP was 76.9, 72.7 and 54.5% in uterus, udder and testes of adult goats, respectively. In kids prevalence was 100.0, 100.0 and 60.0% in uterus, udder and testes, respectively. MAP was recovered from 66.6 and 33.3% of uterus and udder, respectively. Tissue from uterus of a lamb was positive (Table 5).

Colony forming units (cfus): In goats, of the 67 positive cultures, 82.0 and 17.9% were pauci and multi-bacillary (10–100 colonies–17.9%, 5–10 colonies–25.3%, >3 colonies–44.7%, 1 colony–11.9%). Only few goats (12) were super shedders (Fig.2). In sheep, of the 32 positive cultures, all were pauci-bacillary (5–10 colonies–37.5%, >3 colonies-56.8% and single colony–15.6%).

Appearance of colonies: MAP colonies appeared between 45 to 120 days post inoculation. Colonies from sheep grew more slowly than from goats. The 51.9, 28.4, 14.7 and 6.8% colonies appeared between 30–40, 45–60, 60–90 and 90–120 days post inoculation, respectively.

Screening of tissues by IS900 PCR

Target tissues: In intestine, of 50 decontaminated pellets, DNA was recovered from 12 and out of these 3 were amplified by IS 900 PCR. Two MAP cultures from intestines also amplified in IS 900 PCR. In MLN of 56, decontaminated pellets, DNA was isolated from 12 and 8 (66.6%) were amplified in IS900 PCR and of the 4 cultures, 1 was amplified (Tables 6, 7 and 8).

Table 7. Per cent distribution of MAP in non-target tissues of goats and sheep (young and adults) from farm stock by PCR

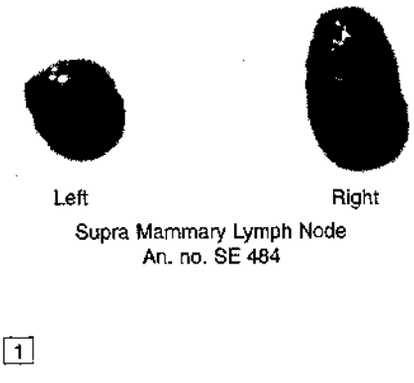
Testes	Percent positive				
	Animal	L-SMLN	R-SMLN	Uterus	Udder
Adult goats	27.2	11.1	11.1	22.2	100
Kids	50.0	00.0	25.0	00.0	00.0
Adult sheep	11.1	22.2	20.0	66.6	—
Lambs	—	—	00.0	—	—
Total	22.7	15.8	15.8	28.5	9.0

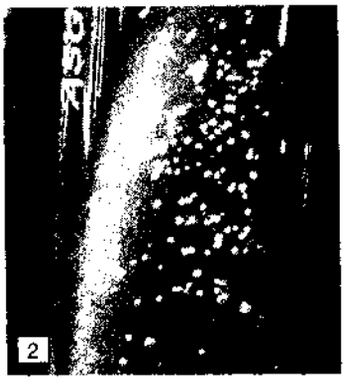
Table 8. Distribution of MAP in target and non-target tissues of goats and sheep from farmer's stock by PCR

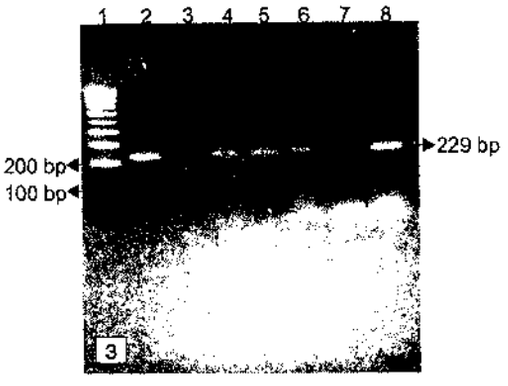
Animal	MLN	Intestine	L-SMLN
Adult goats	25.0	00.0	12.5
Adult sheep	12.5	00.0	30.0
Total	18.7	0.0	22.2

Non-target tissues: In SMLN of 61 decontaminated pellets; DNA was isolated from 16 and 12 (75.0%) were amplified (Fig. 3). Of the 5 MAP cultures, 1 (pauci-bacillary), was amplified. In udder of 14 decontaminated pellets, DNA was isolated from 5 and 3 (60.0%) were amplified. From cultures none of the DNA was amplified. In uterus of 19 decontaminated pellets, DNA was isolated from 8 and 3 (37.5%) were amplified. In testes of 11 decontaminated pellets, DNA were recovered from 6 and 1 were amplified. From culture none was amplified (Tables 6, 7 and 8).

Sampling of sacrificed animals in the slaughterhouses is difficult in India. Most of the slaughterhouses are in poor condition and practically any efforts to collect samples are met with strong resistance by butchers. Screening of target and non-target tissues by culture revealed high prevalence of MAP in goats and sheep population in Agra region including Mathura (North India). Species-wise, prevalence was higher in goat tissues and were probably more susceptible to JD as compared to sheep. Though prevalence was high in goats and sheep but there were few super shedders. Goats and sheep belonging to farm stocks had higher susceptibility to MAP infection as compared to farmer's stocks sacrificed in slaughterhouses. Higher prevalence of MAP in tissues showed the higher infectivity, pathogenicity and endemicity in animals. Screening for MAP using fecal examination, fecal culture. ELISA (serum and whey) and PCR, farm stocks of CIRG, Mathura were found to be endemic for MAP infection (Kumar *et al.* 2007). Using direct microscopy, Tripathi and Parihar (1999) reported low (3.5%) prevalence of MAP in goats. Prevalence of MAP was high in kids and lambs as compared to adult animals (Kumar *et al.* 2007). They also reported high prevalence in kids from Agra region (farm and farmer's). Screening of both MLN and intestine tissues provided better estimates of MAP prevalence. In goats (both adults and young) recovery of MAP was higher in intestinal tissues as compared to MLN (Tables 3–5). Per cent distribution of MAP in non-target tissues of farm and farmer's stocks were either comparable or were higher than in target tissues. Prevalence of MAP was higher in uterus and udder than in target and supra mammary lymph nodes (SMLN) tissues. Sweeney *et al.* (1992) detected 27.0 and 11.6% positives in culture of SMLN and milk samples from cows, respectively. Disseminated infections have been documented







Figs 1–3. **1.** Swollen right supra mammary lymph node and positive for MAP in culture and PCR; **2.** Typical multibacillary colonies (super shedder) of MAP on HEY medium **3.** Screening of supra mammary lymph nodes for MAP by IS 900 PCR; Lane 1 Marker (100 bp ladder); Lane 2 Positive Control (Map S 5 DNA); Lane 3 Negative Control; Lane 4 R 159 (Positive); Lane 5 R 81 (Positive); Lane 6 R 171 (Positive); Lane 7 R 8 (Negative); Lane 8 R 18 (Positive).

in sheep (Carrigan and Seaman 1990). Study showed wide distribution of MAP in various tissues of animals which was correlated with high prevalence and endemicity of MAP in these herds (Singh *et al.* 1996).

MAP was isolated both from normal and inflamed tissues. Buergelt *et al.* (2004) reported that unlike intestinal tissues, other organs did not elicit typical inflammatory response to the presence of MAP. Inflammatory lesions in this study were observed in MLN, SMLN (left or right or both and intestinal tissues (Fig. 1). SMLN was comparable tissue to estimate JD in female animals. Presence of MAP in testes of young kids showed the need for examination of semen of these animals in breeding age. Buergelt *et al.* (2004) reported MAP in semen of cattle. MAP in udder tissues correlated with the excretion of MAP in milk. Presence of MAP in the tissues of young kids (<6 months), confirmed the infection of kids in fetal life and distribution of MAP in different tissues. Majority of colonies were pauci-bacillary (82.0–100.0%). Only 17.9% goats were multi-bacillary (super-shedder). Shedding of MAP in this study was lower than reported for symptomatic cows by Sweeney *et al.* (1992). Hines *et al.* (1987) reported dissemination of MAP from enteric sites to other organs. Despite long growth period and higher requirement of MAP shed into feaces (10^6 cfu/gram) culture from tissues has been largely recommended. Detection of large number of paucibacillary cases also showed sensitivity of culture. The 42.5 and 12.7% colonies appeared around 45 and 60 days post inoculation, respectively and continued to appear up to 120 days. Comparatively, colonies from sheep grew more slowly than from goats. MAP has been recovered from *in-utero* infection of pregnant cow and amniotic fluid of cattle (Buergelt and Williams 2003, Buergelt *et al.* 2006). Similar findings were reported in cattle (Ayele *et al.* 2001 and Seitz *et al.* 1989).

This study showed that decontaminated material of tissues left after inoculation of HEY medium (0.5–1.0 ml) could be good starting material for isolation of DNA (A modified method was used). IS900 PCR was standardized using Vary *et al.* (1990) primers, IS 900/150C and IS900/921. Buergelt *et al.* (2004), standardized nested PCR for detection of MAP in blood and semen using two sets of primers, P90/P91 and J1/J2). Positive reaction in IS900 PCR from all type of tissues indicated wide distribution of MAP in tissues of animals, as was also seen in culture. Though in this study sensitivity of PCR was lower than that of culture but it identified few new animals separately. PCR performed better in MLN and SMLN tissues than in other tissues. This may be due to less contamination of DNA with inhibitory ions from these tissues as compared to intestine, uterus and udder. Poor sensitivity might have been due to presence of fewer bacilli in tissues or insufficient samples (pellet) volume. Contamination of DNA by PCR inhibitors interferes with amplification processes (Gowzdz *et al.* 1997). Sensitivity of PCR could be improved by amplifying all the DNA samples, irrespective of quality of DNA and also by diluting template DNA. PCR screening was important since it detected many of tissues missed by culture. In this study DNA isolation was standardized for very small quantities ($<10^{1-3}$ colonies/g of tissue). Specific amplification of DNA from MAP cultures using IS 900 PCR confirmed the identity of these cultures as MAP. Detection of MAP DNA by PCR in target and non-target tissues and vaginal secretions showed transmission by routes other than traditional faecal–oral. Extra-intestinal pathway such as milk (udder and SMLN), in-uterus (semen and vaginal secretions) should be addressed when considering the epidemiology and control of the disease through test and cull method.

Besides intestine and MLN (target tissues) MAP was distributed to non-target tissues (udder, SMLN, uterus and testes). MAP was equally prevalent in farm and farmer's stocks and young and adult animals. Tissues culture was highly sensitive method as compared to IS900 PCR. Goat and sheep stocks located in Agra region had high prevalence of MAP and MAP was endemic in the small ruminant

population. Presence of MAP in uterus tissues of females and testes of males can be potential source of infection to the fetus and animal handlers. Study confirmed that MAP could spread both vertically and horizontally in animals.

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