Molecular, Serological and Pathological Detection of *Mycobacterium avium* subsp. *paratuberculosis* Infection in Small Ruminants in Peshawar, Pakistan

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ABSTRACT

Paratuberculosis (pTB) also known as Johne's disease (JD), is a chronic infectious disease of animals caused by *Mycobacterium avium* subsp. *paratuberculosis* (MAP). It is also a serious public health concern as MAP is responsible for Crohn's disease in human beings. This infectious disease is so far unexplored in animals in Khyber Pakhtunkhwa (KP) province of Pakistan. Therefore, this study was proposed to investigate the presence of MAP by indirect ELISA (iELISA), associated histopathological lesions and PCR in sheep (*Ovis aries*) and goats (*Capra aegagru hircus)* in district Peshawar. Serum and fecal samples were collected at random both from commercial farms and abattoirs. Additionally, tissue samples (intestine and mesenteric lymph node (MLN)) were collected at random from sheep and goats at abattoirs of district Peshawar. Analyses of serum samples by iELISA revealed the presence of antibodies against MAP in 9% sheep and 5% goats. Ziehl Nielsen (ZN) staining exhibited acid-fast bacilli (AFB) in 31% sheep and 23% goat's fecal impression smears. Gross pathology in intestinal samples (thickness, corrugation) was observed in 30% sheep and 22% goats, while MLN exhibited gross lesions (hemorrhages, edematous swelling) in 35% sheep and 27% goats. Histopathological lesions were observed in intestine and MLN in 26% and 19% sheep, and 17% and 14% goats, respectively. Additionally, PCR revealed the presence of MAP in tissue samples of 5% sheep and 3% goat. This study concluded that MAP is present in the small ruminants of district Peshawar. The infection was confirmed by PCR and iELISA. The presence of MAP could be a serious threat to livestock and public health in the region.

INTRODUCTION

Paratuberculosis (pTB), also known as Johne's disease, is specific infectious granulomatous enteritis of cattle, sheep, goats, deer, camelids and wild ruminants (Thorel *et al.*, 1990). *Mycobacterium avium* subsp. *paratuberculosis* (MAP), slow growing acid fast bacillus, is a causative agent of pTB or JD (Ayele *et al.*, 2001). It is a contagious disease and can affect both domestic and wild ruminants (Khan *et al.*, 2010). Animals become infected early in life but do not develop clinical sign and symptoms, at the age

of 5 months the calves shed MAP in feces. During this subclinical phase of infection, the pathogens can shed in feces and can spread throughout the herd (Hasonova *et al.*, 2009).

Mycobacterium avium subsp. *paratuberculosis* was first reported in Australia in 1895 and isolated in 1910 (Twort, 1910). Generally, the MAP has three strains i.e. Strain I (sheep strain), Strain II (cattle strain) and Strain III (intermittent strain) (Gwozdz, 2010). The disease is wide spread, become more common and has been reported worldwide (Vansnick, 2004). Anyhow the disease is still not endemic in different parts of the world (Okuni, 2013). Some states of Australia and Sweden have been proved to be free of the disease. Among the small ruminants, prevalence of Johne's disease in goats has been reported

from all over the world; in France 62.9% (Mercier *et al*., 2010), in Cyrus 7.9% (Liapi *et al*., 2011), in Argentina 44.1% (Fiorentio *et al*., 2012), in Chile 74.3% (Salgado *et al*., 2007), in USA 76.9% (Manning *et al*., 2002) and in India 79.4% (Singh *et al*., 2013). There are many reports of Johne's disease in sheep and goats from Pakistan. In Punjab 11.19% of ruminants have been previously reported by Sikandar *et al*. (2011).

During clinical phase of infection from one herd to other the pathogen can be transmitted through contaminated equipment, water, feces and contaminated food. Semen and accessory reproductive organs are the common route through which the male animals may carry MAP (Hines *et al*., 2007; Seyyedin *et al*., 2010). Debility and diarrhea are the main symptoms of pTB in ruminants. The initial symptoms can be subtle and may be limited to roughening of the hair coat, decreased milk production and weight loss. Thick diarrhea mucus and epithelial debris are the symptoms in infected animals. During terminal stage of infection, mild and progressive signs are seen in animals (Beard *et al*., 2001; Khan *et al*., 2010). The transmission of MAP to humans may occur through animal contact, meat and raw milk consumption (Eltholth *et al*., 2009). Therefore, MAP could be cause serious inflammatory bowel disease in humans known as Crohn's disease. Milk and its products are a potential source of infection to humans (Greig *et al*., 1999; Khan *et al*., 2010).

Diagnosis of MAP by culture is a gold standard but is very laborious because of very slow growth of MAP taking 8-16 weeks *in vitro*. Other test like Johnin could be used in live animals for screening of herd or flock but evidences have accumulated about its lower accuracy (Nielsen, 2008). Beside, diagnosis of pTB can also be achieved through direct detection of the pathogen by conventional ZN staining, and polymerase chain reaction (PCR). Diagnosis of MAP infection could be achieved indirectly using gamma interferon assay, enzyme linked immuno–sorbent assay (ELISA), complement fixation test (CFT) and delayed–type hypersensitivity (DTH). Among all these tests PCR is more sensitive and specific method for the detection of MAP. However in antibiotic treated animals sometime detection of MAP via PCR is rather difficult. Therefore, serological method like ELISA is more reliable method, as it could detect MAP antibodies even in treated animals. Histopathology, postmortem lesions and clinical signs are considered as complementary sources of pTB diagnosis.

We have a dense population of sheep and goats in Pakistan. They are posing high risks for numerous infectious diseases. As pTB is one of the most neglected and unexplored infectious diseases studied so far in KPK Province either in large or small ruminants. This study was undertaken in KPK, Pakistan. We have detected the presence of MAP in small ruminants using molecular and conventional diagnostic tools. The presence of MAP could be a serious threat to livestock and public health in the region.

MATERIALS AND METHODS

Collection of samples

Samples from the small ruminants were randomly collected at the farms and abattoirs of district Peshawar during December 2017 - January 2018. A total of 150 (Sheep n=75, Goats n=75) serum samples from farms and 200 (Sheep n=100, Goats n=100) from abattoirs were collected. A total of 150 (Sheep n=75, Goats n=75) fecal samples from farms and 200 (Sheep n=100, Goats n=100) from abattoirs were collected. Additionally, 100 intestine and 100 MLN tissues from sheep and 100 each from goats were collected at random from abattoirs. Samples for ZN staining, ELISA and PCR were transported at 4°C, while tissue samples for histopathology were transported in 10 % buffered formalin to Histopathology laboratory, Department of Animal Health, The University of Agriculture, Peshawar. Samples were processed directly or stored at -20°C for further analyses.

Serological study

Blood samples (3-4 ml) were collected aseptically from the jugular vein of each animal. Samples were centrifuged at 4000 rpm for 5 min for the separation of sera. The serum samples were subjected to commercially available ID screen paratuberculosis Indirect ELISA Kit (Innovative Diagnostic Lab lillidale, Uk). Samples were analyzed according to manufacturer instructions.

Gross and histopathology

Tissue samples from the animals showing AFB in their impression smears were subjected to histopathology as reported elsewhere (Bancroft and Gamble, 2007). Intestinal samples (ileocecal junction) and mesenteric lymph nodes (MLN) were examined for gross pathology. Gross lesions were recorded in the tissue samples, i.e. hemorrhage, mucosal thickening or corrugations, congestion, and edematous swelling, and scored semi quantitatively (Buergelt *et al*., 2000; Khan *et al*., 2010) by severity using a mild (1), moderate (2), and severe (3) scale of assessment. After which suitable segments were incised from these tissue samples for histopathology. Later on these samples were washed overnight in order to remove formalin. Then dehydration, clearing, infiltration, embedding, sectioning and routine H and E staining were carried out on the sections (Bancroft and Gamble, 2007).

In the mucosa and upper portions of the submucosa of the intestinal sections following histopathological changes were observed i.e. infiltration of mononuclear cells along with mild infiltration of neutrophils and eosinophils, atrophy of mucosal crypts, presence of epithelioid cells, mucosal exfoliation, fatty change in the sub-mucosa and muscularis layer. In MLN, degeneration of lymphocytes showing pyknosis and karyolysis, necrotic foci, and lymphoid cell depletion, vacuoles and less lymphocytes in the lymphoid follicles were examined.

Ziehl-Neelsen staining

Ziehl-Neelsen staining was performed for recording smear positivity for AFB using fecal impression smears as described previously (Cappuccino and Sherman, 2008). In brief, fecal samples were smeared on glass slide. After air drying, fecal impression smears were heat fixed and subjected to ZN staining. After staining, AFB were examined under microscope (100X).

DNA extraction of MAP from tissue samples

Tissues samples (intestinal, MLN) showing gross and histopathology were processed for DNA extraction of MAP. Commercially available DNA extraction kit from tissue samples (NucleoSpin®, Macherey, Nagel, 2012, Germany) was used for the extraction of DNA.

Table I. Set of primers used for PCR analysis of intestinal tissue infected with *Mycobacterium avium* subsp. *paratuberculosis*.

Target region	Sequence (5′–3′)	Product size
IS900 of MAP		626 bp
IS900-F	GTTATTAACGACGCCCAGC	
IS900-R	ACGATGCTGTGTTGGGCGTTA	
IS1311 of MAP		550 bp
IS1311-F	TGAACGGAGCGCATCACGAA	
IS1311-R	TGCAGCTGGTGATCTCTGAT	

PCR analyses

For the detection of MAP in tissue samples by PCR, two sets of specific primers presented in Table I targeting IS900 and IS1311 of MAP were used as described previously (Khan *et al*., 2010). In brief, PCR was performed using thermal cycler (Bio-Rad, USA). The PCR was conducted in 25 µl reaction mixture containing 1X polymerase buffer 2.5 µl, 1 µl $MgCl_2$, 0.5 µl dNTPs, 0.5 µl of each primer, 0.4 µl Taq polymerase (Thermo scientific, USA) and 0.5 µl of genomic DNA, and remaining volume was of nuclease free H_2O. The amplification was done for

35 cycles at 95ºC for 3 min, 55ºC for 1 min and 72ºC for 5 min. A final extension was done at 72ºC for 10 min. PCR product was run on 1.5% agarose gel in 1X TAE buffer. Product gel was visualized by gel doc system (Fasgene, Germany).

Table II. Serological analyses of serum samples collected from small ruminants at Farm/Abattoir by iELISA.

Species	Serum samples collected at farm and abattoir			
	Total samples	Positive samples	Negative sample	% Prevalence
Sheep	175	16	159	9.0
Goat	175	9	166	5.0
Total	350	25	325	7.0

Statistical analysis

The data collected in the present study were analyzed through descriptive statistics by using SPSS version 20.0 and prevalence was expressed in percentage along with chi square test between the species.

RESULTS

Pathological lesions in tissue samples

Tissue samples including intestinal (ileo-caecal junction) and MLN from both sheep and goats were examined for gross lesions and histopathological lesions. Out of 100 sheep, 40% animals were emaciated, scored on the basis of atrophy of body fat, and 60% were in normal body condition. Diarrhea was noticed in 17.5% emaciated animals. After slaughtering, intestines from animals (30%) and MLN from 35% animals exhibited various different gross pathology, i.e., hemorrhages, mucosal thickening or corrugation, congestion, and edematous swelling (Table III). In a total of 100 goats, emaciation was observed in 30% of animals and 70% goats were in good body condition. Only six emaciated goats were suffering from diarrhea. Intestinal samples from twenty two animals and MLN from 27 animals were showing various gross lesions (Table III).

Histopathological lesions were observed in intestinal and MLN samples from 26 % and 19 % sheep, respectively, whereas 17 % and 14 % goats were showing lesions, respectively, in intestine and MLN.

Presence of acid fast bacilli (AFB) in fecal impression smears

Fecal impression smears were analyzed by ZN staining for the presence of AFB. The percent prevalence of AFB in impression smear from small ruminants

Table III. Apparent body condition and gross lesion of various degrees in tissue samples of examined animals.

Animal Species	Body condition/no. of animals(%)	Types of Tissue sample	No. of samples having gross lesion, n (%)	Observed gross lesion in tissue samples, n (%)	Tissue samples having gross lesion of various degrees (%)		
					Mild	Moderate	Severe
Sheep (100)	Emaciated/40 (40)	Intestine MLN	30 (30) 35 (35)	Hg and Cr ES and Cg	6(20) 9(25.7)	9(30) 12(34.2)	15(50) 14(40)
	Normal/60 (60)	Intestine MLN	0(0) 0(0)	Hg and Cr ES and Cg	0(0) 0(0)	0(0) 0(0)	0(0) 0(0)
Goat (100)	Emaciated/30 (30)	Intestine MLN	22 (22) 27 (27)	Hg and Cr ES and Cg	4(18.1) 6(22.2)	6(27.2) 10(37.03)	12(54.5) 11(40.7)
	Normal/70 (70)	Intestine MLN	0(0) 0(0)	Hg and Cr ES and Cg	0(0) 0(0)	0(0) 0(0)	0(0) 0(0)

Hg and Cr hemorrhage and corrugation observed in the intestinal tissue (terminal ileum) of emaciated and normal sheep and goats, ES and Cg edematous swelling and congestion noticed in the MLN of emaciated and normal sheep and goats.

Table IV. Detection of acid fast bacilli (AFB) through ZN-staining in fecal impression smear.

Species	Fecal samples collected at farm and abattoir			
	Total samples	Positive samples	Negative samples	% Prevalence
Sheep	175	54	121	31
Goat	175	40	135	23
Total	350	94	256	27

collected both at farm and abattoirs is presented in Table IV. The total number of fecal impression smears examined was 350 (Sheep n=175, Goats n=175). Among the total 175 fecal samples from sheep 54 were found positive for AFP, while 40 out 175 samples from goats were positive for AFB. The total percent prevalence was about 31% for sheep and 23% for goats respectively. There was no statistical difference in the presence of AFB in impression smears between sheep and goats (P>0.05).

Detection of MAP antibodies in serum samples by iELISA

Serological analysis was carried on serum samples from either sheep or goats for the presence of anti-MAP antibodies collected both at farms and abattoirs of district Peshawar. Overall, the percent seroprevalence of MAP in small ruminants using commercial iELISA was recorded and presented in Table II. Out of total 175 serum samples from sheep 16 samples were found positive for anti-MAP antibodies, whereas only 9 out 175 serum samples from goats were positive for antibodies against MAP. The total percent seroprevalence of MAP was recorded 9% in sheep and 5% in goats. There was no significant statistical difference in seroprevalence between sheep and goats (P>0.05).

Table V. PCR analyses revealed the presence of MAP in tissue samples of sheep and goats.

Species	Tissue samples collected at abattoir				P value
	Total samples	Positive samples	Negative samples	% Prevalence	
Sheep	100	5	95	5.0	
Goat	100	3	97	3.0	
Total	200	08	192	4.0	0.268

PCR analyses revealed the presence of MAP in tissue samples from small ruminants

Molecular diagnosis of MAP in tissue samples (intestinal) was carried out by PCR targeting IS900 and IS1311 specific regions of MAP using 2 sets of primers presented in Table I. Only five samples from sheep and three samples from goats were found positive by PCR (Table V) using primer set 1 and primer set 2 and generated an amplicon of 626 bp (Supplementary Fig. 1) and 550 bp (Supplementary Fig. 2), respectively.

DISCUSSION

Paratuberculosis is a chronic granulomatous, debilitating bowl disease responsible for drastic decrease in production of dairy and meat animals. However, in the province of Khyber Pakhtunkhwa it has never been reported till date. Therefore, we aimed in this study to detect seroprevalence, identify MAP and its associated lesions in small ruminants of district Peshawar and produce baseline data for the future intervention regarding the control and mitigation of this disease. Paratuberculosis is not only dangerous for animals but it can pose serious threats to public health as well. As pTB is very chronic

disease, therefore its diagnosis is rather cumbersome. The organism shed in the milk during clinical and sub clinical infection and have the potential to survive at pasteurization temperature (Grant *et al*., 2001). Debate on the role of the organism in the etiopathology of human Crohn's disease is gaining importance as bacterium has been isolated from the breast milk of a patient with Crohn's disease (Naser *et al*., 2000).

In the present study serum samples from small ruminants were analyzed by indirect ELISA. Antibodies against MAP were found in 9 % sheep and 5% goats in samples collected both at commercial farms and abattoir. The low prevalence percentage at farm level may be due to long survival rate of the disease and insensitive diagnostic tests. Moreover, because the MAP may go in latent stage (12 to 16 weeks) of infection and the levels of detectable antibodies through ELISA are significantly reduced, the ability to identify a truly positive paratuberculosis remains limited (Abbas *et al*., 2011). The current study shows similarity to the findings of (Hussain *et al*., 2018). He reported that the prevalence at an abattoir is higher than at the farm and the reason is quite understandable that mostly the low producer or untreatable animals are sold out by the farmers and those come to slaughter at the abattoirs. Sikandar *et al*. (2013) conducted a study to evaluate the effectiveness of conventional diagnostic tools including histopathological examination, ELISA and ZN staining for the prompt diagnosis of ovine paratuberculosis, the animals were randomly selected, from abattoirs of Jhang and 10.63% animals were declared as positive for MAP through ELISA.

Ziehl Nielsen (ZN) staining of fecal impression smears samples collected at commercial farms and abattoir exhibited AFB in 31% sheep and 23% goats. This study shows similarity with the findings reported elsewhere (Rehman *et al*., 2017). They reported that poor hygienic conditions at local farms increase the chances of occurrence of the disease. Along with this higher number of animals in a limited area, constant shedding of *Mycobacterium* by sick animals and ability of the bacteria to remain for longer time in the environment are the major predisposing elements of pTB. Emaciation and diarrhea were also mentioned in JD (Delgado *et al*., 2009). The shedding of MAP bacilli in fecal samples frequently precedes the onset of serum antibody responses (Stewart *et al*., 2006; Whittington *et al*., 2001). Hence, the current study revealed the very early onset of the antibodies response in the infected lambs, it would have been interesting to examine fecal shedding during the early post infection period, but restrictions on the availability of culturing facilities precluded these samples from being test. The preeminence of intestinal samples over mesenteric lymph nodes for the detection of MAP using ZN staining was indicated previously (Sikandar *et al*., 2013).

Postmortem examination revealed pathological lesions including thickness and corrugation in 30% and 22% of intestinal samples, while hemorrhages, edematous swelling was observed in 35% and 27% in MLN samples in sheep and goats respectively. Our results are in accordance with the finding reported elsewhere (Alharbi *et al*., 2012; Maxie *et al*., 2007). They observed that advanced cases of JD were typified by diffused intestinal thickening coupled with longitudinal and transverse corrugations that gave rise to asymmetrical folds having red surfaces but no ulcerations. A histopathological study in India revealed inflammation of intestine, thickening and corrugation of small intestine and granulomatous inflammation (Sivakumar *et al*., 2006). In the present study the mucosa and upper portions of the submucosa of the intestinal section, there was an increase infiltration of mononuclear cells along with mild infiltration of neutrophils and eosinophils. There was sloughing of the mucosal lining of the intestine. The mucosal gland of the intestine shows atrophy because of heavy infiltration of inflammatory cells. The epithelioid macrophage cytoplasm was darker and foamy in appearance. Another study also revealed that in cattle main lesions of JD usually confined to the intestine and associated lymph nodes (Tiwari *et al*., 2006). The thickening of the intestinal wall up to three or four times than normal with corrugation of the mucosa was characteristic for pTB. There was a thick capsule of fibrous connective tissue (FCT) in the mesenteric lymph nodes. In the cortical and parcortical regions variable sized calcified and variable sized necrotic areas by thin layer of FCT. It has also been revealed that thickening and corrugation of intestine along with enlargement of MLN were prominent in diseased animals (Koets *et al*., 2015).

The percent prevalence of MAP confirmed by PCR on abattoir level both for sheep and goats was 5% and 3% respectively. Although PCR is more sensitive technique than acid-fast staining, however the result obtained in this study showed a higher number of positive samples with acid-fast staining compared to PCR (Khan *et al*., 2010). This significant difference between PCR and ZN staining might be due to low specificity of ZN staining. Paratuberculosis might be hindering factors present in the fecal samples or their might be a chance of missing of MAP DNA in 4-6 µl of the sample collected from tissue samples for PCR, while ZN staining revealed the presence of AFB in larger sample spread over the slide or there could be some more unknown factors involved (Hussain *et al*., 2018).

Various conventional and molecular techniques were used in this study for the detection of *Mycobacterium avium* subsp. *paratuberculosis* (MAP) in samples taken

from sheep and goats. Sensitivity and specificity of conventional methods (ZN staining and Postmortem) were found lower compared to PCR and iELISA. Since ZN staining could detect acid fast bacilli (AFB) and postmortem only examined gross and histopathological lesions could be possible. It is well known that all AFB are not MAP, therefore most of the samples found positive by ZN staining were negative by PCR and iELISA. In this study, less number of samples were found positive by iELISA and PCR. No doubt these techniques are more sensitive and specific for the detection of pathogens, however the reason of lower sensitivity of these tools might be less number of samples analyzed in this study at limited geographical locations. Therefore, we recommend extensive epidemiological investigation throughout the province to devise a comprehensive therapeutics and control strategies for MAP infection in small ruminants.

Moreover, the low prevalence of MAP by PCR compared to ELISA might be due long incubation period of MAP and extensive use of antibiotics in small ruminants in the region of study. These results were found in similarity index with the findings reported elsewhere (Hajikolaei *et al*., 2006). Another study reported that primers targeted insertion sequence against IS900 and IS1311 can magnify DNA regions of MAP (Sevilla *et al*., 2005).

In this study analyses of serum samples by iELISA revealed the presence of antibodies against MAP in 9% sheep and 5% goats. Additionally, PCR revealed the presence of MAP in 5% sheep and 3% goat's tissue samples. Compared to studies conducted elsewhere in Pakistan revealed 11% sero-prevalence of MAP in small ruminants by iELISA (Sikandar *et al*., 2013). Moreover, molecular techniques confirmed the presence of MAP in 12.8% and 14.2% samples from ruminants (Khan *et al*., 2010). Sero-molecular prevalence of MAP in small ruminants at Peshawar district during specific time period of the study was found lower compared to other parts of the country (Sikandar *et al*., 2013; Khan *et al*., 2010).

An iELISA is an appropriate method for the detection of chronic granulomatous infections like pTB as these types of diseases are mostly taking long time to exhibit clinical signs due to very slow growth of causative agent. In this investigation, presence of MAP in small ruminants at district Peshawar is indicated by the resultant 9% and 5% seroprevalence both in sheep and goats using a specific commercially available iELISA Kit. The present study concluded that the presence of anti-MAP antibodies in small ruminants, its associated lesions and AFB in tissue samples, and later on confirmation by PCR implicated that MAP could be a serious threat to livestock industry, as well as to public health at district Peshawar.

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Supplementary material
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Statement of conflict of interest
The authors have no conflict of interest to disclose.

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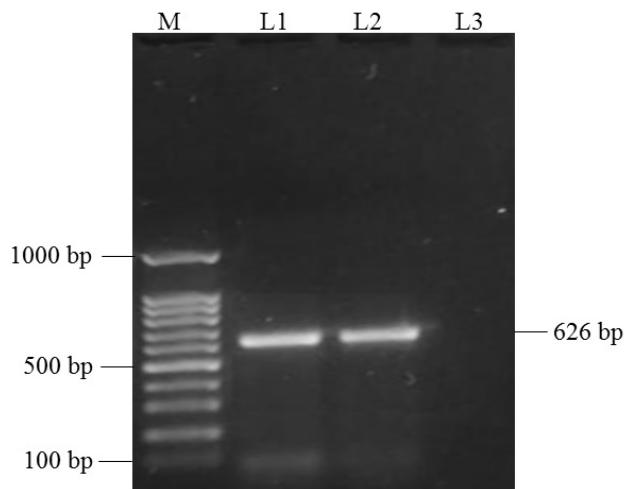
Supplementary Material

Molecular, Serological and Pathological Detection of *Mycobacterium avium* subsp. *paratuberculosis* Infection in Small Ruminants in Peshawar, Pakistan

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Supplementary Fig. 1. Polymerase chain reaction targeted specific IS900 region of MAP.
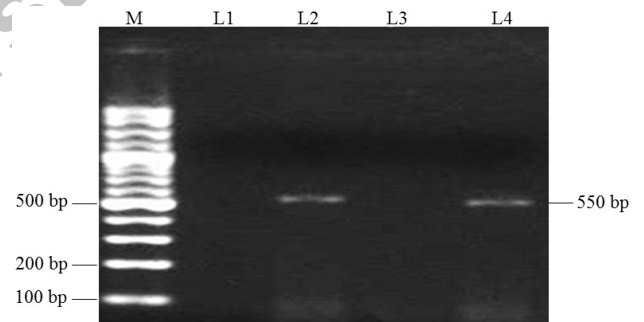
Supplementary Fig. 2. Polymerase chain reaction targeted specific IS1311 region of MAP.

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