

Molecular detection of *Brucella melitensis* in sheep from district Budgam, Jammu and Kashmir

KHALID BASHIR¹, TUFAIL HUSSAIN¹, NOUSHEEN QADRI², JAVAID AHMAD BABA²,
 IMRAN HAMID SHAH² and MUBASHIR ALI RATHER^{2✉}

Disease Investigation Laboratory, Nowshera, Srinagar, Jammu and Kashmir 190 011 India

Received: 7 May 2024; Accepted: 10 July 2024

Keywords: Abortion, *Brucella melitensis*, PCR, Sheep, Zoonotic

The favourable agro-climatic and geo-physical conditions of Jammu and Kashmir (J&K), India provide excellent opportunity for efficient income generation from sheep husbandry (Baba *et al.* 2020, Rather *et al.* 2020a). This efficient income is determined by average daily gain and ewe reproduction performance, viz. conception rate and litter size at birth and other factors. The litter size at birth is considerably affected by abortions/ stillbirths (Bashir *et al.* 2020) caused by many infectious and non-infectious factors. In Kashmir, the major cause of abortions/stillbirths in ovines is Brucellosis (Ahanger *et al.* 2020). Laboratory diagnosis remains main tool for identification, detection and confirmation of *Brucella* species (Ahanger *et al.* 2020). Various laboratory tests, viz. RBPT (Rose Bengal Plate Test), STAT (Standard Tube Agglutination Test), ELISA (Enzyme Linked Immuno Sorbent Assay), etc. are in vogue for detection of brucellosis in sheep (Rather *et al.* 2020b). Till date, only serological diagnosis of Brucellosis has been carried out in Kashmir. The molecular detection of *Brucella melitensis* by Polymerase Chain Reaction (PCR) offers high sensitivity, specificity, promptness and safety for confirmation of organism.

Farmers from Kralapathri village of Kashmir valley (temperate region) reported abortions and stillbirths in their flock comprising of around 600 sheep including 471 pregnant ewes from January to April 2024. The physical examination of ewes revealed normal physiological parameters. Blood samples were collected from all the pregnant ewes for serological examination. Rose Bengal Plate Agglutination Test was carried out on the serum samples collected from the ewes. Briefly, to 75 µl of serum, 25 µl of Rose Bengal plate coloured antigen solution was added and changes were noted after proper mixing. Post mortem examination of 16 stillborn lambs was conducted at Disease Investigation Laboratory, Nowshera, Srinagar to

ascertain the cause of mortality. On spot heat fixed tissue smears were prepared for Gram's staining. Extraction of bacterial genomic DNA from the abomasal contents, peritoneal fluid, liver and kidney tissue samples collected from stillborn lambs was performed using the Qiagen DNeasy Blood and Tissue DNA extraction kit according to the manufacturer instructions. The obtained DNA extracts were checked by agarose gel electrophoresis and stored at -20°C till further use.

Three primers (GCC Biotech) were specifically used for DNA amplification (Table 1) (Bricker and Halling 1994). The PCR reaction mixture comprised of 12.5 µl GoTaq® Green Master Mix 2×, 1 µl each of the two forward and one reverse primers, 5 µl of the template DNA and 5.5 µl nuclease-free de-ionized water. The amplification was carried out in a thermal cycler with an initial denaturation at 95°C for 3 min, 35 cycles of denaturation at 95°C for 1 min, annealing at 60°C for 2 min, and extension at 72°C for 2 min; and a final extension at 72°C for 5 min (Ntivuguruzwa *et al.* 2022). Following this, the PCR products were processed for electrophoresis in 1.5% agarose gel stained with Ethidium bromide dye and visualized by a Gel Doc (Biorad).

Table 1. List of primer pairs used in the PCR assay

Primers	Sequence
Bm-F	AAATCGCGTCCTTGCTGGTCTG
Ba-F	GACGAACGGAATTTTCCAATCCC
IS711-R	TGCCGATCACTTAAGGGCCTTCAT

The stillborn lambs were apparently normal with normal growth *in utero* as revealed by PM examination. The cornea had developed opacity in two lambs. The subcutaneous tissues were edematous and congested. The peritoneal cavity was filled with sero-sanguinous fluid / blood (Fig 1A). All the visceral organs were severely congested (Fig 1B). The lambs had hepatomegaly with rupture of liver and loss of normal architecture in two lambs (Fig 1C). The kidneys were severely haemorrhagic (Fig 1D, E). The pleural cavity was also filled with sero-sanguinous fluid

Present address: ¹Department of Sheep Husbandry, Srinagar, Jammu and Kashmir. ²Disease Investigation Laboratory, Nowshera, Srinagar, Jammu and Kashmir. ✉Corresponding author email: mubashir.70011@gmail.com

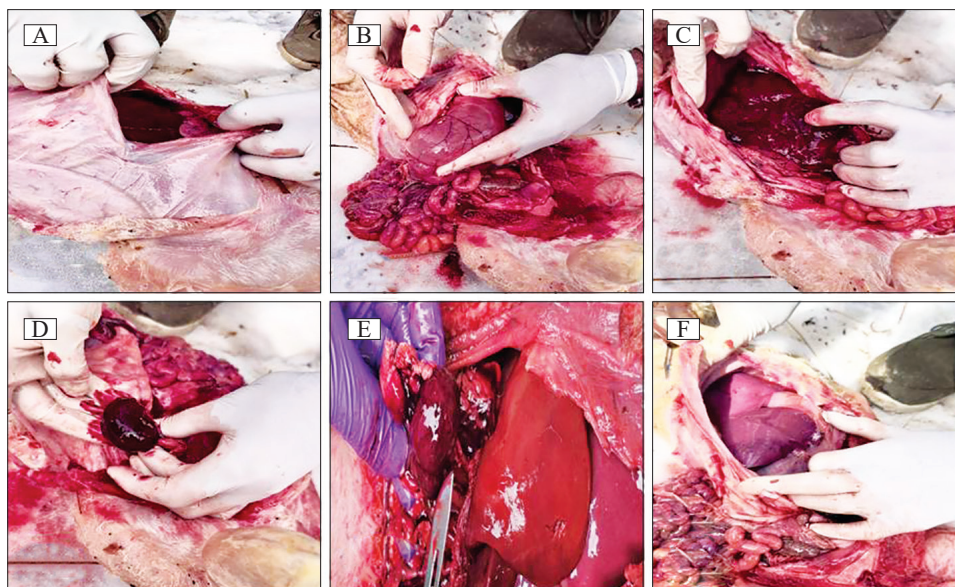


Fig. 1. Gross postmortem findings of still-born lamb: (A) Peritoneal cavity filled with blood-tinged fluid; (B) Severely congested visceral organs; (C) Hepatomegaly with rupture of liver, with loss of architecture of liver parenchyma; (D) & (E) Hemorrhagic kidney; (F) Cobble stone pattern of lungs.

and lungs were atelectic and displayed typical cobblestone appearance (Fig 1F). Rose Bengal Plate Antigen test (RBPT) of the dams showed that the dams were positive for Brucellosis. The lambs thus were infected *in utero* as the organism is well-known for its placental transmission (Keogh *et al.* 1958).

Brucellosis in sheep is highly prevalent disease with high clinical correlation in Kashmir (Ahanger *et al.* 2020). Seroprevalence of 3.2%, 14.14% (unorganized sector), 3.3% (organized sector), 35.87%, 17.38% and 20.17% were reported by Bandey *et al.* (1989), Lone *et al.* (2013), Hussain *et al.* (2017), Ahanger *et al.* (2020) and Rather *et al.* (2020b), respectively in sheep in Kashmir. The difference in reported seroprevalences may be due to the sampling methods and study design employed by each researcher. On Gram staining of abomasal fluid, peritoneal fluid, impression smears from liver and kidney, Gram positive cocci and bacilli and Gram negative bacilli were observed. Although *Brucella* is Gram-negative coccobacillus (Anonymous 2017), variability in Gram's staining has been reported (Chow 2022). Since both *Brucella abortus* and *Brucella melitensis* are responsible for abortion/stillbirths in sheep, PCR for detection of both the agents was carried out as per the AMOS PCR (Bricker and Halling 1994, Ntivuguruzwa *et al.* 2022). The PCR conducted on the abomasal, peritoneal contents and liver and kidney samples revealed 731 bp band after the gel electrophoresis confirming *Brucella melitensis* as cause of stillbirths in the flock (Fig. 2). No band specific for *Brucella abortus* was detected. *B. melitensis* has been reported in sheep previously in other parts of India (Sonekar *et al.* 2018, Shome *et al.* 2018) but this is the first report of molecular confirmation of *B. melitensis* in sheep from Jammu and Kashmir. The postmortem signs like

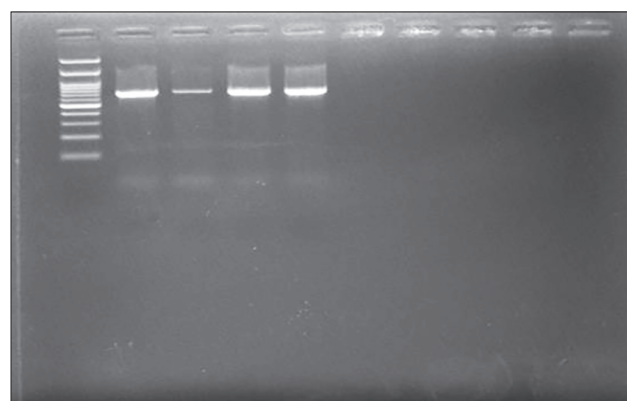


Fig. 2. Gel electrophoresis of AMOS PCR products [Lane 1: 100 bp ladder, Lane 2: 731 bp band (Abomasal fluid), Lane 3: 731 bp band (Peritoneal fluid), Lane 4: 731 bp band (Liver fluid), Lane 5: Kidney fluid)].

subcutaneous oedema, blood-stained fluid in the thorax and abdomen, splenomegaly and hepatomegaly in aborted/stillborn lambs have been reported in animals suffering from Brucellosis infection (Mazlan *et al.* 2021). The lesions are due to colonization of the bacteria in almost all foetal organs (Higgins *et al.* 2017). In addition to this, liver in one of the lambs was ruptured with jelly like consistency of the liver parenchyma. The lesions in kidney were in concurrence with the earlier study by Mazlan *et al.* (2021). The atelectic lungs and blood-stained fluid in the peritoneal and thorax cavities indicated that the lamb might have died *in utero* one to two days before parturition (Menzies 2007). Brucellosis remains major and most important cause of pre and perinatal mortality in ovines in Kashmir (Lone *et al.* 2013, Ahanger *et al.* 2020, Bashir *et al.* 2020). However, till date only seroprevalence has been determined in the Kashmir with no information regarding the species

involved. This study provided first molecular confirmation of *Brucella melitensis* as responsible agent for ovine pre and perinatal mortality in Jammu and Kashmir, India. Saravanan *et al.* (2021) also confirmed *Brucella melitensis* as etiological agent for abortion in Mecheri sheep around fourth month of gestation. Lonkar *et al.* (2023) generated evidence on the distribution of *B. melitensis* in goat milk in the country.

It is concluded that *Brucella melitensis* is prevalent cause of stillbirths and abortions in sheep flocks of Kashmir. Appropriate diagnostic techniques remain pre-requisite for the control and eradication of brucellosis in sheep. Being a zoonotic disease which is risking both economy (through loss of lamb crop and germplasm) and also human health, immediate steps may be taken to check the spread and further control the disease.

SUMMARY

Farmers reported abortions and stillbirths in 600 sheep including 471 pregnant ewes. A complete investigation including physical examination of livestock, postmortem examination of stillbirths, serological examination of ewes and molecular study of abomasal contents, peritoneal fluid, liver and kidney tissue collected from still born lambs was done to ascertain the cause of mortality at Disease Investigation Laboratory, Nowshera, Srinagar. Also, on spot heat fixed tissue smears were prepared for Gram's staining and accordingly observed for presence of organism. Extraction of bacterial genomic DNA from samples from still born lambs was performed and obtained DNA extracts were checked by agarose gel electrophoresis and stored at -20°C till further use. The PCR conducted on the abomasal, peritoneal contents and liver and kidney tissues revealed 731 bp band after the gel electrophoresis, confirming *Brucella melitensis* as cause of stillbirths in the flock. The investigation revealed that *Brucella melitensis* was cause of stillbirths and abortions in sheep flocks.

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