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Clinicopathological studies of *Pasteurella multocida* B:2 experimental infection in rabbits

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ABSTRACT

The present study was carried out to study pathology and virulence of *Pasteurella multocida* B:2 isolated from natural outbreak of *Pasteurella* infection in rabbits. Healthy *Pasteurella multocida* B:2 free rabbits (12) were divided in control and challenged group. The challenged group rabbits were inoculated intra-nasally by spraying of 0.5 ml of inoculum in each nares, containing 1×10^5 CFU of *Pasteurella multocida* B:2 in BHI. The control group of rabbits was inoculated with 0.5 ml uncultured BHI broth in each nares in the same manner. Live animals were observed for atleast 14 days. The main clinical signs observed in infected rabbits comprised of conjunctivitis, nasal discharge, pyrexia, sneezing, dyspnoea and abdominal breathing. The pathomorphological changes in lungs comprised of acute fibrinous and/or fibrino suppurative bronchopneumonia/pleuropneumonia. Trachea showed acute inflammatory changes with multifocal erosion/ulcer and necrosis. Nasal cavity showed acute inflammation with accumulation of cellular debris in nasal meatus. Three infected rabbits showed meningitis characterized by congestion, infiltration of heterophils and minimal oedema. *Pasteurella multocida* B:2 was successfully detected in blood and tissues of infected rabbits by PCR. The organisms were also demonstrated in blood/impression smear from different organs.

Key words: Hemorrhagic septicemia, *Pasteurella multocida* B:2, Pneumonia, Rabbit

Hemorrhagic septicemia (HS) is a very important killer disease of young adult buffalo and cattle (OIE 2012), caused by *Pasteurella multocida* (*Pm*), with economic importance as it causes heavy morbidity and mortality leading to loss of milk production as well as milk producing animals. The serotypes of *Pm* prevalent in India are B:2, A:1, A:1,3, A:3, A:4, A:3,4,12, F:3, D:1, D:3, F:1, F:4 and F:4,12 (Hemandri and Hiremath 2011). Clinically, HS caused by *Pm* B:2 strain is characterized by fever, respiratory distress with nasal discharge, and frothing from the mouth, eventually leading to recumbency and death, while serotype A:1 or A:3 has been implicated in fatal pneumonia of cattle (OIE 2012). In recent outbreaks of HS caused by *Pm* B:2 strains in Gujarat, typical symptoms and signs like edema of neck or labored breathing were not seen, instead animal died after a short clinical illness and showed only fever and marked pneumonia at necropsy. Case fatality approaches 100% if treatment was not followed at the initial stage of infection.

There are several published reports available on HS in domestic animals, however, there seems to be very few reports available with regards to experimental pathology in rabbits by *Pm* B:2 strain isolated from HS outbreak in buffalo. Looking to the different clinical signs and paucity of information, especially on experimental pathology of *Pm* B:2 in rabbits, the present research work was carried out.

MATERIALS AND METHODS

IAEC approval: Protocol used in this study was approved by the Institutional Animal Ethics Committee, College of Veterinary Science and Animal Husbandry, SDAU, Sardarkrushinagar

Procurement of culture: Pasteurella multocida B:2 live culture was procured on 5% blood agar slant in duplicate from the Department of Microbiology of the university. The bacteria were isolated from the tissues collected from two buffaloes that died of HS at a village. The isolates thus received were streaked on 2 different blood agar plates to get isolated colony for further use in experiment.

Procurement of animals: The study was conducted on 12 healthy HS free, 11 to 12 weeks age (weighing 1 to 1.5 kg) adult New Zealand White (NZW) rabbits of either sex. Rabbits and two mice were procured and were maintained under standard management conditions. For feeding,

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conventional standard laboratory diet was used with an unlimited supply of drinking water via automatic watering bottle. Animal care, housing, and environmental conditions (temperature, humidity and light dark cycle) were according to recommendation stated in the Guide for Care and Use of Laboratory Animals before and during the study period. Identification of animals was done by ear marking with permanent marker.

Pre inoculation testing: Before inoculation, the rabbits were checked for *Pm* free status. Sterile swab was deeply inserted in either nares of rabbits and cultured on 5% blood agar media. The grown culture on blood agar was subjected to Gram staining. Gram negative samples were confirmed by PCR not to be *Pm*. To rule out the presence of antibody against *Pm* B:2, ELISA was applied to test the serum samples. The antigen of *Pm* B:2 was prepared by extraction with potassium thiocyanate as per the protocol described by McKinney *et al.* (1982).

Passage in mice for virulence: Loopful of culture was dissolved in 0.5 ml sterile PBS and injected intra-peritoneally with insulin syringe in mice. After 32 h, mice died, postmortem was carried out following all aseptic precautions. Blood collected from heart was streaked on 5% blood agar. The culture was inoculated in other mice second time as described earlier. After 18 hours, mice died and from left femur, bone marrow was collected and streaked on 5% blood agar. After incubation of 18 h, separate colonies were observed on blood agar medium. The virulent culture obtained was used for final inoculation in healthy *Pm* B:2 rabbits. The uncultured broth was used as sham for control group. The obtained culture was confirmed with *Pm* B:2- PCR.

Preparation of inoculum: Three separate uniform colonies were picked and transferred to BHI broth and incubated in Shaker Incubator, 60 RPM for overnight. From the overnight grown culture, approximately 0.5 ml broth was transferred in new BHI broth and incubated for 5 h in same incubator with same RPM. OD of broth was measured at 600 nm with spectrophotometer three times against zeroing with uncultured broth. The average OD of 0.744 was taken as measurement of CFU in BHI. The cultured broth was serially diluted 10 fold from 10^2 to 10^7. Each dilution was transferred in 100 mL pre warmed BHI, mixed well and poured in three different plates and incubated at 37º C for 24 h. Numbers of CFU were counted as per the standard formula (Miles *et al.* 1938). At measured OD, one mL broth contained 1.053×10^8 CFU. The number of bacteria in inoculum (1×10^5) was adjusted by addition of uncultured BHI in cultured broth.

Experimental design: Healthy *Pm* B:2 free rabbits (12) were divided in two different groups, each having six animals. 0.5 mL of prepared inoculum was sprayed in each nares by inserting the insulin syringe without needle, keeping the head of rabbit in upward position for 2 min so that inoculum reach deep in nasal cavity. The control group of rabbits was inoculated with 0.5 ml uncultured BHI broth in each nares in the same manner. Live animals were observed for atleast 14 days.

Parameters evaluated

Clinical observations: All the experimental rabbits were closely observed for any behavioral changes, clinical signs or symptoms and mortality during the entire 14 days of study period. The rectal temperature was recorded daily with digital thermometer.

Sample collection, gross necropsy and histopathology: Before necropsy, blood was collected from ear vein with insulin syringe in sterile anticoagulant vials for hematology, cultural isolation and molecular detection. Two mL of blood was collected directly from heart of moribund rabbits. Animals were necropsied as soon as possible after the time of death. A systemic and detailed necropsy was conducted on the rabbits which died during experiment. The remaining animals including control group were euthanized with high dose of sodium thiopentone at the end of experiment on day 15. During the necropsy, lung, liver, spleen, heart, kidney and brain were collected in sterile PBS for molecular detection and in 10% neutral buffered formalin (NBF) for histopathology. Other organs like stomach, intestine, lymph node, nasal cavity, trachea, esophagus, thymus and salivary glands were also collected in 10% NBF. Impression smears were prepared before fixation from lungs, liver, spleen, heart, and kidneys and fixed in methanol and stained with Eosine Methylene Blue (EMB) stain for examination of bipolar organisms. Bone marrow impression smears were collected from long bones and fixed by heating. Fixed tissues were processed routinely, histological sections of the tissues were stained with Hematoxylin and Eosine (H & E) (Suvarna *et al.* 2013).

Molecular detection: Blood samples from live rabbits and tissue samples during necropsy were cultured on blood agar and incubated at 37 ºC for 24 h. The grown colonies were subjected to PCR. DNA from blood and tissues was extracted using blood and tissue kit and heat method was used for the extraction of DNA from culture. For genomic DNA extraction from colony of *Pasteurella multocida* by heat method, isolated colony was mixed with 200 µl of nuclease free water in centrifuge tube and dissolved with vortex. The tube was kept in float rack in boiling water bath at 100°C for 20 min. Tubes were centrifuged at 12,000 rpm for 10 min and supernatant was collected in a separate collection tube and stored in deep freeze (–20°C) for further use as template. As described by Townsend *et al.* (1998, 2001) primers were procured from GeNei^{TM} Mumbai, and Eurofins genomic, Bengaluru. Specifications of primers are as follow. Species specific (460 bp):-1) KMT1SP65'-GCTGTAAACGAACTC GCCAC-3'; 2) KMT1T7 5'-ATCCGCTATTTACCCAG TGG-3'; and Serotype B:2 specific (760 bp):- 1) CAP B-F 5'- CATTTATCCAAG-CTCCACC-3'; and 2) CAP B-R 5'-GCCCGAGAGTTT-CAATCC-3'. The PCR was performed on cycler, with an initial denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 95°C for 1 min, annealing at 55°C for 1 min, extension at 72°C for 2 min, and a final extension at

72°C for 6 min. Amplification products were separated by agarose gel electrophoresis (2% agarose in 1× TAE) at 4 V/cm for 1 h and stained with ethidium bromide (0.5 µg/ml). DNA fragments were viewed by UV illumination and photographed.

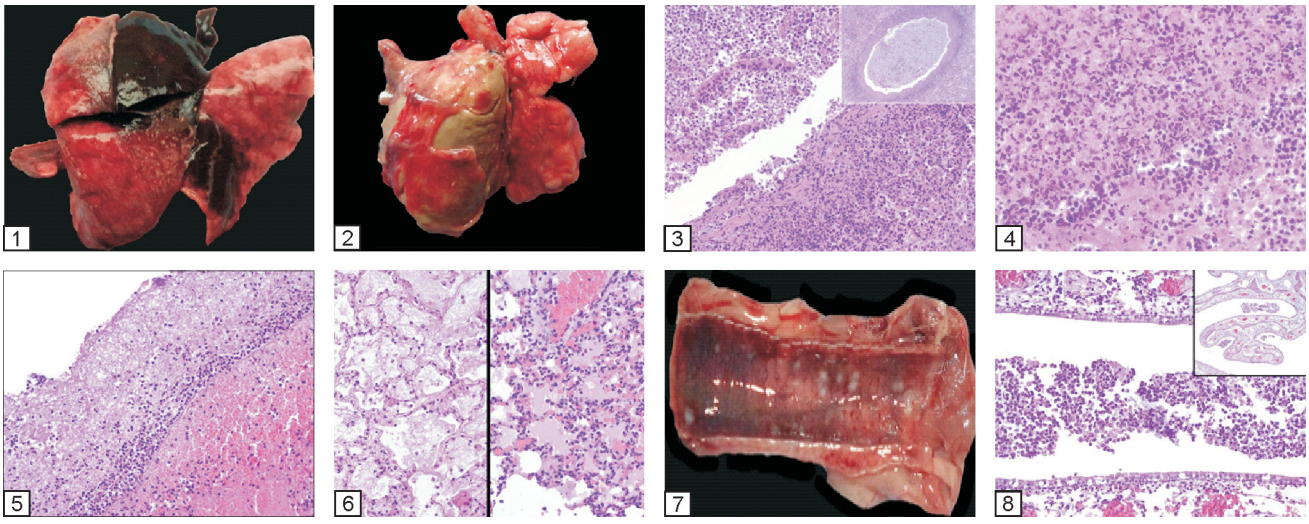
RESULTS AND DISCUSSION

Before the use of the rabbits in the experiment, all the rabbits were confirmed as *P. multocida* free and were negative for IgG antibodies against *Pm* by ELISA. During the experiment, all the rabbits belonging to the negative control group 1 remained *Pasteurella* free and serologically negative, and no clinical signs, mortality and post mortem lesions were observed among them.

After 24 h post inoculation (PI), all six infected rabbits of group 2 showed nasal rubbing with fore legs. There was minimal mucus discharge from nasal cavity in two animals with soiling of the hair of nose and forepaw, severe hyperemia of nasal mucosa and occasional sneezing. All infected rabbits showed pyrexia with rectal temperature ranging from 103.5 to 106.4 ºF with erected hair. Three animals showed variable degree of mucus to mucopurulent discharge from eyes and conjunctivitis. After 36 h of intranasal inoculation of *Pm* B:2, 1 rabbit was found dead. The signs of dyspnoea and abdominal breathing were observed in terminal stage. Four animals from experimentally challenged group died at interval of 48, 72, 96 and 108 h PI respectively. One rabbit survived till the study period of 14 days which was euthanized on 15th day with control group animals by over dose of sodium thiopentone I/V.

In the present study, virulence of the organism was judged by high morality in challenged rabbits even at low dose. Some other investigators had used quite higher challenge doses (10^6–10^{10} CFU) in intra-nasal experimental infections of rabbits with *Pm* serogroup A strains (DiGiacomo *et al.* 1987, Glávits and Magyar 1990, Lu *et al.* 1991). Clinical signs, viz. conjunctivitis, nasal discharge, pyrexia, sneezing, dyspnoea and abdominal breathing as observed in the present study were also reported by some earlier workers in rabbits (Al -Haddawi *et al.* 2001, Jaglic *et al.* 2006, Rameshkumar *et al.* 2006).

At necropsy, variable lesions of pneumonia ranging from haemorrhagic to fibrinous/purulent/fibrinopurulent, were seen in rabbits who died in the early stage up to 108 h PI. One rabbit that died after 36 h PI showed hemorrhagic pneumonia characterized by marked consolidation with red hepatisation of cranial lobes lung (Fig. 1). Trachea showed hyperaemic mucosa with petechial to ecchymotic haemorrhages. Four rabbits that died between 48 to 108 h PI showed the most severe and prominent lesions in lungs and trachea. The lesions were characterized by diffused necrosuppurative fibrinous pneumonia involving different lobes of lung with highest severity in cranial lobes. There was deposition of thick fibropurulent exudate involving the entire lobes of lung (Fig. 2). Three rabbits showed fibrinous pneumonia characterized by moderate to severe pleural thickening, multifocal to diffuse haemorrhage, fibrin capsule deposition on lungs and adhesions with chest wall. Thoracic cavity contained serosanguinous exudate. Pericardium and other thoracic organs of all affected rabbits exhibited variable degree of fibrin deposition admixed with pus. Mediastinal lymph nodes were moderately enlarged and dark in color in all affected rabbits.



Figs. 1–8. **1.** Lung from infected rabbit with severe red hepatization and hemorrhage. **2.** Lung of infected rabbit showing suppurative pneumonia. **3.** Lung showing marked infiltration of heterophils in the alveolar and bronchiolar lumen with sloughing of bronchiolar mucosa and mild necrosis. (H & E 200×; Inset 50×). **4.** Lung showing oat shaped macrophages with elongated streaming nuclei in alveoli. (H & E 400×). **5.** Lung showing thick fibrin capsule infiltrated with minimal inflammatory cells over the lung parenchyma with marked haemorrhages in subcapsular area (H & E 400×). **6.** Left Panel: Lung showing fibrinous exudate in the alveoli; Right Panel: Lung showing marked accumulation of proteinaceous fluid in alveoli. (H & E 200×). **7.** Trachea: mucosa showing hyperemia, petechial to ecchymotic hemorrhages and multifocal white patches of necrosis. **8.** Nasal cavity showing accumulation of cellular debris in nasal meatus with submucosal congestion, minimal edema and heterophilic infiltration. (H & E 200×; Inset H & E 50×).

The microscopic changes in rabbit intranasally inoculated with *Pm* B:2 comprised of acute fibrino-necro hemorragic bronchopneumonia to fibrinosuppurative bronchopneumonia. The fibrino-necrotic bronchopneumonia was characterized by multifocal to diffuse necrosis, severe infiltration of heterophils with few lymphocytes and necrotic debris mixed with fibrin in alveoli and bronchioles (Fig. 3). There was desquamation of bronchiolar epithelium with presence of necrotic debris mixed with heterophils and other inflammatory cells in the bronchiolar lumen. At places, there was "Oat" shaped elongated and streaming nuclei evident in and around necrotic area (Fig. 4). Besides these changes, alveoli contained proteinaceous eosinophilic oedematous fluid with congestion of alveolar capillaries. Lungs of rabbits with fibrino-hemorrhagic pneumonia showed thick fibrin capsule infiltrated with minimal inflammatory cells over the lung parenchyma with marked haemorrhages in subcapsular area (Fig. 5). Deeper parenchyma showed extensive accumulation of fibrinous exudate (Fig. 6, left panel), protein rich oedema fluid (Fig. 6, right panel) and abundant erythrocytes in alveoli with mild to moderate infiltration of heterophils, lymphocytes and few alveolar histiocytes. Rabbits that showed fibrinous pleuritis showed marked congestion, oedema, fibrin deposition and minimal infiltration of inflammatory cells predominantly heterophils. The pathological changes in suppurative pneumonia were characterised by severe infiltration of heterophils in alveoli as well as in wall of artery, arterioles and capillaries. Tunica media showed heterophilic infiltration and necrosis. At places, lung tissue showed regeneration, characterized by basophilic cuboidal cells lining the alveoli and bronchioles with minimal congestion and infiltration of inflammatory cells.

Grossly, tracheal mucosa was markedly reddened and showed multifocal white necrotic patches (Fig. 7). Minimal amount of mucus and/or pus admixed with necrotic debris was present overlying mucosa. Pharynx and larynx mucosa was severely reddened due to congestion and haemorrhages. Nasal mucosa was congested with presence of mucus. Microscopically, the lesions in trachea comprised of focal extensive to multifocal mucosal necrosis, erosion/ulcer, submucosal oedema, severe congestion and infiltration or inflammatory cells in mucosa and submucosa. The microscopic changes in nasal cavity were characterized by accumulation of cellular debris in nasal meatus with submucosal congestion and oedema along with mild angiectasis (Fig. 8). At places, there were increased numbers of goblet cells in respiratory epithelium with submucosal congestion. Submucosa of ethno turbinate showed mild congestion and minimal infiltration of inflammatory cells with little degenerative changes in submucosal gland

Grossly, meninges of infected animals were thickened and congested. Microscopically, three rabbits showed meningitis characterized by congestion, diffuse infiltration of heterophils and minimal oedema (Fig. 9 left panel). Occasionally, bacterial emboli were seen in blood vessels (Fig. 9 right panel).

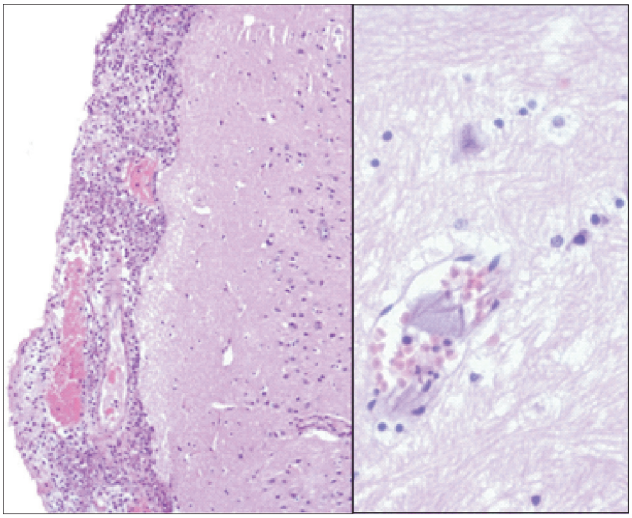


Fig. 9. Left panel: Brain showing diffuse infiltration of inflammatory cells in meninges, congestion and submeningial edema. (H & E 200×); Right panel: Brain showing bacterial emboli in blood vessels. (H & E 400×).

At the time of necropsy, spleen did not show any pathological changes. Microscopically, the lesions in spleen consisted of severe lymphoid necrosis characterized by karyorrhexis and karyolysis of lymphocytes in white pulp with basophilic aggregates of bacteria in the centre of necrotic area. The architecture of thymus was found completely changed with dark densely stained medulla and lightly stained cortex. The lesions comprised of marked necrosis of lymphoid cells of cortical region with mild congestion and haemorrhages.

Grossly, liver of all infected rabbits were diffusely congested. Microscopically, there were no appreciable lesions in liver of experimental rabbits except congestion, however, in one rabbit microgranuloma characterized by necrosis of hepatocytes at the centre with infiltration of mononuclear cells and fibrosis was observed. No significant pathological lesions were seen in other organs except scattered haemorrhages and minimal infiltration of inflammatory cells. Other organs showed minimal serosal congestion and/or petechiae.

In rabbit pathogenicity of serotype *Pm* A has been extensively studied by earlier workers, and they reported fatal fibrinopurulent or fibrinohemorrhagic pneumonia (Dillehay *et al.* 1991, Esquinas *et al.* 2013, Percy *et al.* 1986, Yarim *et al.* 2005). Dagleish *et al.* (2007) in their studies on experimental infection of *Pm* A:3 in calves observed edema and inflammatory cells infiltration within 24 hours and abscess formation in lung after 7 day PI. In other experimental study, infection of *Pm* A:3 in calves resulted in to abscessation after 4 day PI (Dowling *et al.* 2002). An experimental infection of *Pm* A:1 in buffalo calves produced fibrinous and suppurative bronchopneumonia with focal areas of coagulation necrosis typical of pneumonic pasteurellosis (Praveena *et al.* 2014). In calves also cranial lobes of lungs were consistently

affected in experimental *Pm* A infection (Dowling *et al.* 2002, Praveena *et al.* 2014). Serotype D:1 (Al -Haddawi *et al.* 2001) and F (Jaglic *et al.* 2011) in rabbit produced similar lesions in respiratory tract. This is probably the first study of *Pm* B:2 experimental infection in rabbit. Intra nasal route employed in present study is highly relevant to natural aerosol *Pm* infection. *Pasteurella multocida* B:2 infection evade all upper respiratory tract defences in very short time and produced fatal diseases in rabbits. *Pasteurella multocida* B:2 produced typical clinical sign and pyrexia in all infected animals within 24 hours PI, moderate haemorrhagic pneumonia and first mortality in 36 hours, marked fatal bronchopneumonia with 83.33 percent mortality in 108 hours. In present study characteristic subcutaneous edema with serosanguinous fluid was not seen in rabbits of challenged group.

Resident pulmonary intravascular macrophages of the host play important role in lung lesions in HS. As reported by Warner *et al.* (1988), circulating endotoxin activated pulmonary intravascular macrophages may then lead to influx of other inflammatory cells and cascading injury. In contract experimentally immunosuppressed rabbits showed diffuse pulmonary haemorrhages without the characteristic inflammatory lesions after experimental *Pm* F infection (Jaglic *et al.* 2008). In present study, alveolar oedema and haemorrhages present at 36 hours may have been an endothelial inflammatory response affecting capillaries and post-capillary venules due to cytokines released by activated macrophages and/or recruited heterophils. Toxins produced by *Pm* B:2 alone or in combination with products of inflammatory cells induced necrosis of blood vessels walls in lungs of rabbits that died after 36 hours. Injured blood vessels wall and/or diapedesis may be responsible for marked edema and haemorrhages in alveoli. Necrosis of the endothelium together with infiltration of heterophils into the walls of the vessels seen after experimental *Mannheimia haemolytica* infection was reported by (Jericho 1989). Bacterial toxins also lead to leukocytes necrosis and production of oat cells characterised by streaming pattern of condensed chromatin material.

Pasteurella multocida induced central nervous system lesions observed in the present study are not completely understood. *Pasteurella multocida* meningitis is a rare clinical occurrence, rarely reported in humans (Green *et al.* 2002). In the present study, meningitis was observed in three experimentally infected rabbits. Kpodekon (1983) also recorded meningitis, encephalitis and/or otitis in rabbits experimentally infected with *Pm* through infraorbital nerve, nebulisation, or intravenous route. Irrespective of route of infection, neuritis or perineuritis of trigeminal nerve was developed in most infected rabbits. Nerve lesions provoked neural lymph stagnation which induced retrograde centripetal circulation of lymph up to the brain leading to meningitis as opined by Kpodekon (1983).

Direct blood smear prepared from blood showed the presence of bipolar organism in three animals after 24 h PI. Impression smear prepared from lung showed typical bipolar organism (Fig. 10). Other organs, viz. liver, spleen, heart and bone marrow also showed bipolar organisms in smears. No bipolar organism was seen in blood smear or impression smear from rabbits of control group.

The challenged animals in this study developed septicemia as evidenced by the presence and re-isolation of the challenge organism from a variety of tissues and emboli seen in capillaries of brain. Presence of *Pm* B:2 was confirmed in bone marrow, heart, spleen, liver and lung by PCR. Positive samples yielded 460 bp (Fig. 11) and 760 bp amplicon on 2 % Agarose gel in species specific and serotype B:2 specific PCR respectively. Lungs of all the six animals found positive for *Pm* by species specific PCR. Bone marrow (5 rabbits), heart (5 rabbits), liver (4 rabbits), spleen (5 rabbits), kidney (3 rabbits) and brain (1 rabbit) sample were *Pm* positive out of samples collected from six animals of challenged group. All these positive samples were also found positive by serotype specific PCR indicating the type B:2. No amplification was seen with

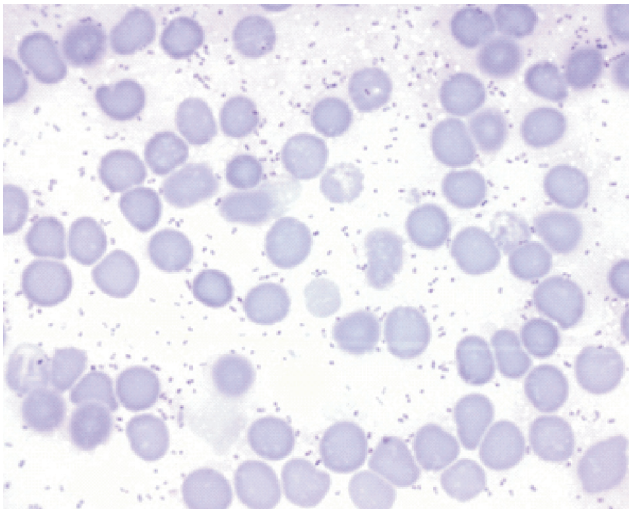


Fig. 10. Impression smear of lung showing bipolar organisms. (Field stain 1000×).

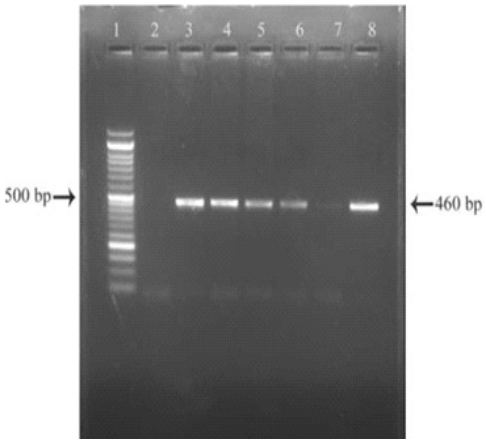


Fig. 11. Species specific PCR using KMT1SP6 and KMT1T7 Primer: Lane 1, Ladder; Lane 2, Negative control; Lane 3, Lung; Lane 4, Liver; Lane 5, Heart; Lane 6, Spleen; Lane 7, Kidney; Lane 8, Bone marrow.

the blood, tissues or culture obtained from control animal. Molecular detection of *Pm* by PCR was also reported by several workers. Shivachandra *et al.* (2004) successfully applied PCR on tissue samples from birds died of fowl cholera. Singh *et al.* (2010) also detected *Pm* from morbid tissues viz. liver, spleen and kidney collected in different preservative from mice infected with *Pm*.

In conclusion, intra-nasal inoculation of rabbit with *Pasteurella multocida* B:2 isolated from buffalo died of HS resulted in to marked inflammation in respiratory tract leading to congestion, hemorrhages, edema, infiltration of inflammatory cells with fibrin deposition resulting in to fibrinosuppurative bronchopneumonia. Further more studies are required to elucidate pathogenesis of meningitis caused by experimental infection of *Pasteurella multocida* B:2 infection in rabbits.

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