Journal of Animal Research: v.8 n.1, p. 101-106. February 2018

DOI: 10.30954/2277-940X.2018.00150.16

Studies on Pathogenicity of *Pasteurella multocida* Serotype B: 2 in Mice

Neha Sahu[1], R.C. Ghosh[1*] and S.D. Hirpurkar[2]

[1]Department of Veterinary Pathology, College of Veterinary Science and Animal Husbandry, Chhattisgarh Kamdhenu Vishwavidyalaya, Anjora, Durg, Chhattisgarh, INDIA
[2]Department of Veterinary Microbiology, College of Veterinary Science and Animal Husbandry, Chhattisgarh Kamdhenu Vishwavidyalaya, Anjora, Durg, Chhattisgarh, INDIA

**Corresponding author: RC Ghosh; Email: rcghosh@rediffmail.com*

Received: 18 Nov., 2017 **Revised:** 20 Dec., 2017 **Accepted:** 22 Dec., 2017

ABSTRACT

In an experimental study, five field isolates of *Pasteurella multocida* recently isolated from natural sporadic cases from cattle were tested for their pathogenicity in mice. 0.2 ml of eighteen hours old broth cultures of each of the test isolates containing approximately 2.4×10^8 CFU/ml was inoculated into four mice (2 male and 2 female) by intra peritoneal route and two mice (1 male and 1 female) were kept as control and inoculated with Brain Heart Infusion (BHI) broth. All the mice died within 24 hours of inoculation. The presence of *P. multocida* in the dead mice was detected by smear examination followed by re-isolation of pure colonies on sheep blood agar (SBA) and was later confirmed by *P. multocida* specific polymerase chain reaction (PM-PCR). *P. multocida* was detected in all the dead mice. All the five isolates amplified *KMT1*gene and gave an amplified product of ~460 bp. Gross pathological changes comprised of mild congestion and haemorrhages in organs like lung, heart, kidneys, liver and spleen. Histopathological changes revealed congestion, haemorrhages along with fibrinous exudation in lung, haemorrhages, vascular congestion and varying degree of degenerative and necrotic changes in heart, sinusoidal congestion and hydropic degeneration in liver and congestion of intertubular capillaries in kidney.

Keywords: Pathogenicity, *Pasteurella multocida,* PCR, mice

Haemorrhagic septicaemia (HS) is an acute fatal septicaemic disease in cattle and buffalo, caused by strains of *P. multocida* especially serogroups B: 2 (Asian type) and E: 2 (African type) (Shivachandra *et al.,* 2011). *P. multocida* is a Gram-negative, nonmotile, coccobacillus known to affect a wide range of host species including wild and domestic animals. In cattle, natural infection occurs by inhalation or ingestion of *P. multocida* bacteria characterised by rapid disease progression with typical clinical signs of severe depression, pyrexia, submandibular oedema, dyspnoea, followed by recumbency and death (Horadagoda *et al.,* 2001). Asia and Africa are currently the global geographic regions in which HS occurs with the highest prevalence and has the greatest economic importance. It might be due to the pronounced changes in weather between seasons, including the monsoon, debility caused by seasonal scarcity of fodder and pressure of work in draught animals (Benkirane and De Alwis, 2002). The diagnosis of HS has been traditionally based on clinical signs, gross pathological lesions, and on the isolation of *P. multocida* from blood or bone marrow (Anon, 2012). Mouse is known to be very sensitive to *P. multocida* infection, hence intra-peritoneal (I.P) mice inoculation selectively enriches *P. multocida* (Laviere *et al*., 1993; Muhairwa *et al*., 2001) and is the choice not only for isolation from mixed bacterial population (Laviere *et al*., 1993) but also it could be used for the estimation and comparison of strain pathogenicity (Boyce, 2000). Ability to kill mice may depend on the virulence status of the *P. multocida* strain (Rutter, 1983). Nevertheless, mice represent the ideal tool to study HS as they are immunologically and genetically defined, easy to manipulate and inexpensive to maintain. Mouse inoculation is the most sensitive for surveillance and the detection of carrier animals (Christensen *et al*.,

2004). The phenotypic characterization of P. *multocida* by means of morphology, biochemical typing and serotyping are very much laborious and time consuming. The PCR based molecular diagnostic techniques are highly specific and have proved an alternative method of characterization to overcome the limitations of phenotyping (Ranjan *et al*., 2011). In the present communication pathogenic potential, molecular characterization by PCR and histopathological changes in different vital organs of mice following intra peritoneal inoculation of *P. multocida* B: 2 isolates recovered from cattle are described.

MATERIALS AND METHODS

A total of 5 isolates of P. *multocida* B: 2 were isolated and identified from cattle of three districts *i.e.* Durg, Raipur and Dhamtari of Chhattisgarh State. Pathogenicity of each isolate was tested in 6-8 weeks old Swiss albino mice procured from the Department of Pharmacology and Toxicology, Post Graduate Institute of Veterinary and Animal sciences, Akola. A total of thirty mice, weighing 20-22 grams were used for this study. A loopful of *P. multocida* isolate cultures were inoculated into 4 ml of BHI broth and incubated for 18 hrs in a shaker incubator at 37° C. About 0.2 ml containing approximately $2.4x10^8$ CFU/ml of each isolate culture, was inoculated into four mice (2 male and 2 female) by intra peritoneal route and two mice (1 male and 1 female) were kept as control and inoculated with 0.2 ml of BHI broth. All the mice were kept under observation for 48 hours and mortality was recorded. Post-mortem of dead mice was conducted and gross changes in the vital organs were recorded. Blood smears were prepared from the heart blood of dead mice and stained with Giemsa's stain and simultaneously streaked on 5% SBA and McConkey's lactose agar (MLA) and incubated for 24 hours at 37° C for the re-isolation of *P. multocida.* The pieces of tissues from organs such as lung, liver, kidney, spleen and heart were collected aseptically for bacteriological examination as well as in 10% neutral buffered formalin for histopathological studies. The genomic DNA was extracted from the overnight culture using GeneiPure™ Bacterial DNA Purification Kit (MERCK Specialities, Mumbai) as per the manufacturer's guidelines. *P. multocida* polymerase chain reaction (PM-PCR) was carried out using species specific primers KMT1SP 6 and KMT1T7 as reported previously (Townsend *et al.,* 1998) to amplify *KMT1*gene (Table 1). Briefly, the PCR was a set at 25 µl reaction with 1 µl template DNA, 2X PCR Mastermix 12.5 µl, 1µl each of forward and reverse primers and 9.5µl of nuclease free water. The PCR reactions were carried out according to the programme: initial denaturation at 95°C for 5 min followed by 35 cycles at 95°C for 1 min for denaturation, annealing at 55°C for 1min and extension at 72°C for 1min and a final extension at 72°C for 10 min. The analysis of PCR product was carried out in 2% agarose gel stained with ethidium bromide (0.5µg/ml). 100 bp DNA ladder (Himedia, Mumbai) and appropriate controls were incorporated to rule out the false positive and false negative results. The amplicons were visualized as a single fluorescent band under UV light and documented by Gel documentation System (Bio Rad, UK). For histopathology, formalin fixed tissue samples were processed by routine paraffin embedding technique, sectioned at 4-5µ thickness and stained with hematoxylin and eosin following the procedure of Luna. Necessary approval from Institute Animal Ethics Committee was obtained for experimentation.

Table 1: Sequence of primer used to amplify *KMT1* gene

Primers	Sequence	Amplicon size
KMT1SP6	5'-ACTCGCTATT-TACCCAGTGG-3'	460bp
KMT1T7	5'-GCTGTAAACGAACTCGC-CAC-3'	

RESULTS AND DISCUSSION

All the mice injected with 0.2 ml of *P. multocida* culture broth died within 24 to 36 hrs following inoculation. After 24 hrs of incubation at 37° C, pure cultures of bacteria were obtained on blood agar, but on MLA no growth was seen. Re-isolated *P. multocida* cultures exhibited the same cultural and morphological features as that of the original test isolates and the smears revealed typical bipolar characteristics on Giemsa's staining. The isolated colonies on the SBA were small, glistening, mucoid, dewdrop like and non-haemolytic (Fig. 1) and showed Gram-negative bipolar, rods or cocco-bacilli on Gram staining (Fig. 2). All the isolates screened for *KMT1* gene by PM-PCR were found to give an amplified product of ~ 460 bp size (Fig. 3).

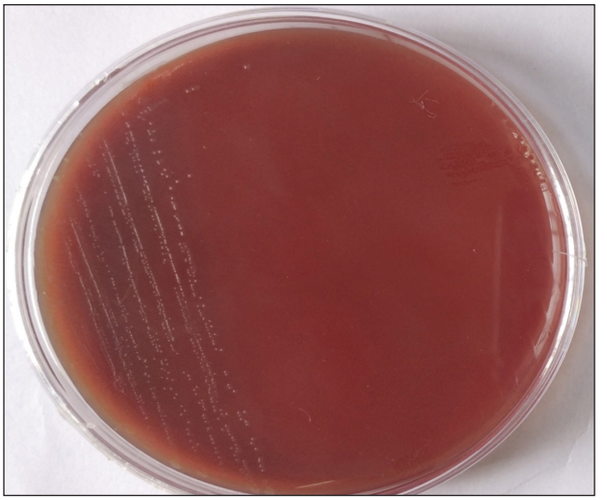

Fig. 1: Showing non- haemolytic, smooth muciod, dew drop like colonies of *Pasteurella multocida* on SBA

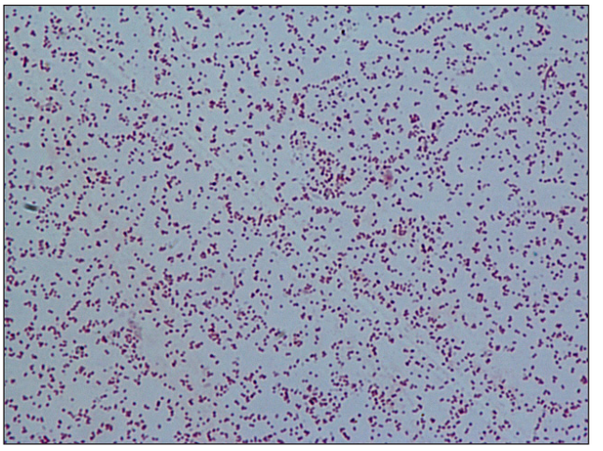

Fig. 2: Microphotograph showing Gram negative bipolar cocco bacillary rods confirming *Pasteurella multocida*. Gram's stain × 100

Grossly, organs like lung, heart, liver, spleen and kidney of the experimentally inoculated mice showed mild congestion and haemorrhages at necropsy. Histopathological studies were carried out to study the microscopic alterations induced by the organisms in the tissue. Histopathologically, lungs revealed congestion and haemorrhages along with fibrinous exudation. Mononuclear cellular infiltration was noted in the bronchioles (Fig. 4). Heart tissue revealed severe haemorrhages, vascular congestion and varying degrees of degenerative and necrotic changes (Fig. 5, 6). Liver showed sinusoidal congestion and hydropic degeneration (Fig. 7). Kidney showed congestion of intertubular capillaries (Fig. 8). Sections of spleen revealed severe haemorrhages and depletion of lymphocytes from Malphigian corpuscles (Fig. 9). All the mice in control group remained healthy throughout the experimental period and did not develop any signs or symptoms of pasteurellosis and survived.

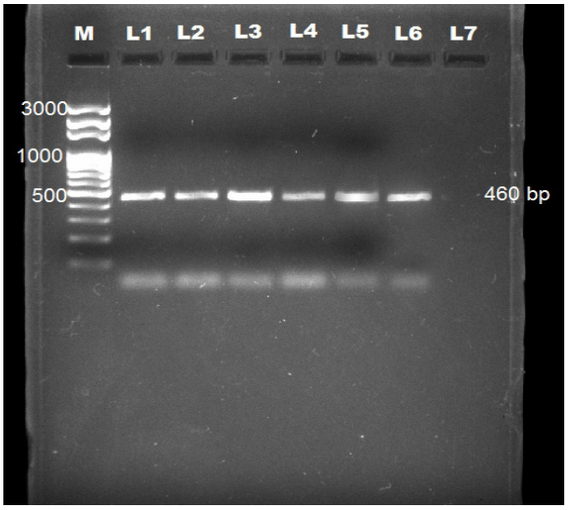

Fig. 3: Amplification of KMT1 gene (460bp) of *Pasteurella multocida* Lane M : 100 bp DNA ladder. Lane 1-5 : Re-isolated cultures of *Pasteurella multocida* from mice; Lane 6 : Field isolate of *Pasteurella multocida;* Lane 7 : Negative control.

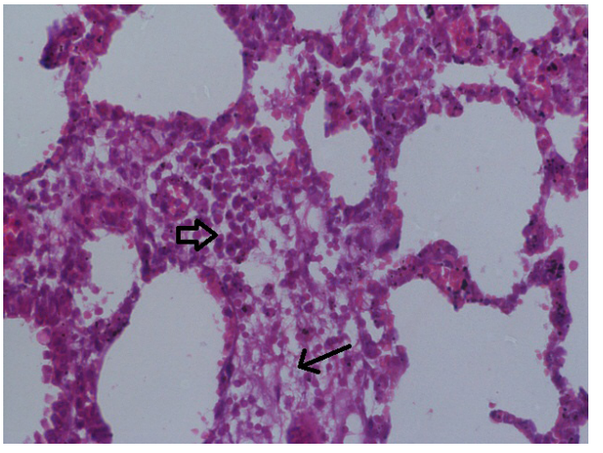

Fig. 4: Microphotograph of lung showing fibrinous exudation (thin arrow) and mononuclear cellular infiltration (thick arrow). H & E × 400

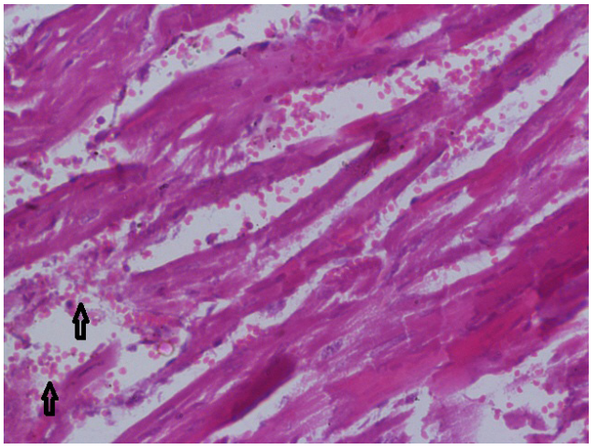

Fig. 5: Microphotograph showing myocardial haemorrhages. H & E × 400

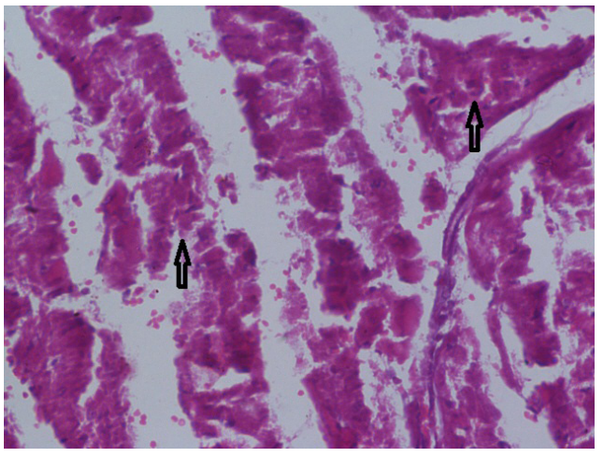

Fig. 6: Microphotograph showing severe degenerative and necrotic changes in myofibers. H & E × 400

HS continues to be a threat to livestock. Vaccines developed so far are unable to completely control and eradicate the disease. Outbreaks or cases of HS have been recently reported in India (Hedau *et al*., 2017; Basheer Ahamad *et al*., 2005; Mitra *et al*., 2013; Kumar *et al*., 2006).

All the experimentally inoculated mice death within 24-36 hrs indicated that they were exposed to infective doses of highly virulent *P. multocida* which overcomes innate immunity, rapidly replicates and disseminates from the site of inoculation to the internal organs that cause bacteremia, septicemia, septic shock and the death of infected mice (Woolcock and Collins, 1976).

These observations support the results of the previous studies which explained that endotoxemia of gram-negative organism can initiate septic shock. The process began with the proliferation of microorganism at the site of infection and invaded the blood stream directly or might have proliferated locally and released various toxins in the blood stream, which in turn stimulates the release of plasma precursors or cells (monocyte, macrophages, endothelial cells, neutrophils and others) of endogenous mediators of sepsis leading to multiple organ failure, disseminate intravascular coagulation and death (Kumarm *et al*., 2007).

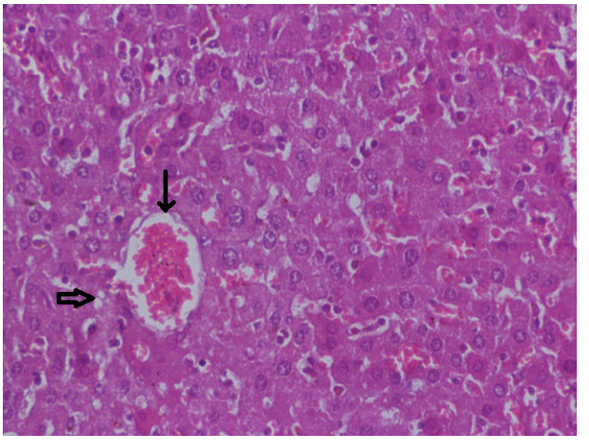

Fig. 7: Microphotograph of liver showing congestion (thin arrow) and hydropic degeneration (thick arrow). H & E × 400.

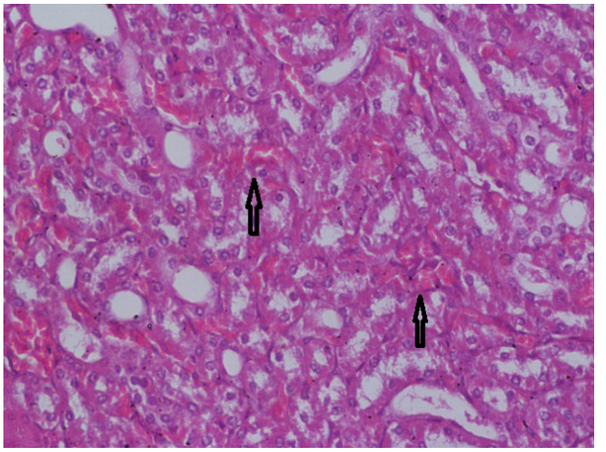

Fig. 8: Microphotograph of kidney showing congestion in intertubular capillaries. H & E × 400

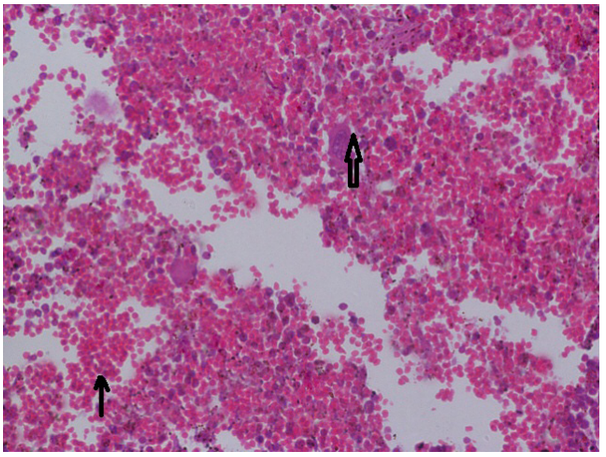

Fig. 9: Microphotograph showing haemorrhages (thin arrow) and depletion of lymphocytes from Malphigian corpuscles (thick arrow) in spleen. H & E × 400

All the isolates of *P. multocida* re-isolated from mice exhibited typical, cultural and morphological characteristic features as that of the original test isolates recovered from the clinical cases. Similar to our findings, Naz *et al.* (2012) reported that *P. multocida* could be isolated from the heart blood of the experimentally infected mice isolated from buffaloes. Also, an experimental study conducted in Saudi Arabia showed that different organs were demonstrated to be predilection sites for *P. multocida*, which include lung, heart, trachea, spleen, liver, pharynx and oedematous fluid in the neck and nasal cavity (Al-Humam *et al.,* 2004).

In the present study, the field isolates *P. multocida* from cattle were identified as serotype B: 2 and the same serotype have also been described as pathogenic from bovines (Waffa *et al.,* 2014). According to them the isolates were pathogenic to mice both on intraperitoneal and subcutaneous injection. However, in the present study, the isolate was adjudged as pathogenic only by intraperitoneal infection. In agreement to the current study, *P. multocida* serotype A:1 was proved pathogenic to mice and was reisolated from heart blood after conducting pathogenicity test by injecting 0.2ml of 18-hr broth culture (Kapoor *et al.,* 2004; Dutta *et al.,* 2001). Further, *P. multocida* of sheep origin was not virulent for the laboratory animals (Upadhaya *et al.,* 1972).

All the pure cultures recovered from heart blood were also confirmed by species specific PM- PCR assay as *P. multocida*. The result of the present study concludes that PM-PCR assay could be used to identify *P. multocida* (Townsend *et al.,* 1998).

The gross and histopathological changes were in agreement with other researchers who mentioned that gross pathology of pasteurellosis in mice was characterized by petechial haemorrhages similar to that observed in cattle and buffalo with HS (Ramdani *et al.,* 1990; Kapoor *et al.,* 2004; Patel, 2004).

CONCLUSION

All the parameters examined suggest a role for the mouse as a tool to study HS and are sufficiently encouraging to warrant further evaluation and comparison of the disease of livestock with infections in mice. The similarity of HS in cattle to the disease in mice infected with the *P. multocida* is highly indicative of the usefulness of mice as a model for understanding pathogenesis of bovine pastrurellosis.

ACKNOWLEDGEMENTS

The authors are thankful to Dean, College of Veterinary Science and Animal Husbandry, Chhattisgarh Kamdhenu Vishwavidyalaya, Anjora, Durg, Chhattisgarh, for providing necessary facilities to carry out the research work. The authors also thankfully acknowledge the help received from RKVY funded project on "Strengthening of Veterinary Pathology Laboratory for Diagnosis of Diseases in Livestock and Poultry and ICAR grant".

REFERENCES

Al-Humam, N.A., Al-Dughaym, A.M., Mohammed, G.E., Housawi, F.M. and Gameel, A.A. 2004. Study on the isolation and pathogenicity of *Pasteurella multocida* Type A in calves in Saudi Arabia. *Paks. J. Biol. Sci.* **7:** 460-463.

Anon. 2012. Haemorrhagic septicaemia. OIE terrestrial manual 2008, 1– 13 (Chapter 2.4.12). The World Organisation for Animal Health (OIE), Paris. *http://www.oie.int/.*

Basheer Ahamad, D., Sood, S., Soodan, J.S., Agrawal, R., Sharma, A.K., Sabiha, A., Kadari, M., and Patil, R.D. 2005. An outbreak of per acute haemorrhagic septicaemia in buffaloes of Jammu region (India): A Pathological Study. *Indian J. Vet. Pathol.*, **29**: 78-81.

Benkirane, A. and DeAlwis M.C.L. 2002. Haemorrhagic septicaemia, its significance, prevention and control in Asia. *Vet. Med. Czech.,* **47:** 234–240.

Boyce, J.D. and Adler, B. 2000. The capsule is a virulence determinant in the pathogenesis of *Pasteurella multocida* M1404 (B: 2). *Infect. Immun.,* **68**: 3463-3468.

Christensen, H., Angen, O., Olsen, J.E., Bisgaard, M. 2004. Revised description and classification of atypical isolates of *Pasteurella multocida* from bovine lungs based on genotypic characterization to include variants previously classified as biovar 2 of *Pasteurella canis* and *Pasteurella avium*. *Microbiol.,* **150**:1757–1767.

Dutta, T.K., Singh, V.P. and Kumar, A.A. 2001. Rapid and specific diagnosis of animal pasteurellosis by using PCR assay. *Indian J. Comp. Microbiol. Immunol. Infect. Dis.,* **22:**43-46.

Hedau, M., Kurkure, N.V., Jangir, B. L., Chopde, N.A., Kalorey, D.R. and Bhandarkar, A.G. 2017. Pathological diagnosis of an outbreak of pasteurellosis in buffaloes of Vidharbha region in India. *Indian J. Vet. Pathol.,* **41**: 38-40.

Horadagoda, N.U., Hodgson, J.C., Moon, G.M., Wijewardana, T.G. and Eckersall, P.D. 2001. Role of endotoxin in the pathogenesis of haemorrhagic septicaemia in the buffalo. *Microb*. *Pathog*., **30**: 171-178.

Kapoor, V., Katoch, R.C., Sharma, M. and Asrani, R.K. 2004. Pathogenicity test of *P. multocida* in mice A:1. *Indian J. Anim. Sci.,* **74:**495-496.

Kumar, A.A., Harbola, P.C., Rimier, R.B. and Kumar, P.N. 1996. Studies on *Pasteurella mullocida* isolates of animal and avian origin from India. *Indian J. Compar. Microbiol. Immuno. Infect. Dis.,* **17**: 120-24.

Kumar, H., Sharma, S., Mahajan, V., Verma, S., Arora, A.K., Kaur, P. and Sandhu, K.S. 2006. Pathology and PCR based confirmation of haemorrhagic septicemia outbreaks in bovines. *Indian J. Vet. Pathol.,* **30**: 5–8.

Kumarm, V., Abbas, A. K, Fausto, N. and Mitchel, R. N. 2007. "Robbins Basic Pathology". 8th Ed. Saunders, Philadelphia. Published by Library of Congress. USA. pp. 102-103.

Laviere, S., Leblanc, L., Mittal, K.R. and Martineau, G.P. 1993. Comparison of isolation methods for the recovery of *Bordetella bronchiseptica* and *Pasteurella multocida* from the nasal cavities of piglets. *J. Clin. Microbiol.,* **31**: 364-367.

Luna, A. G. 1968. Manual of histological staining methods of the Armed Forced Institute of Pathology, 3rd ed. McGraw Hill book Co, London: 124-125.

Mitra, J., Chowdhury, M. and Bhattacharya, C. 2013. Outbreak of Hemorrhagic Septicemia in Free range buffalo and cattle grazing at Riverside grassland in Murshidabad district, West bengal, India. *Explor. Anim. Med. Res.,* **3**:178-182**.**

Muhairwa, A.P., Christensen, J.P. and Bisgaard, M. 2001. Relationships among Pasteurellaceae isolated from free ranging chickens and their animal contacts as determined by quantitative phenotyping, ribotyping and REA-typing. *Vet. Microbiol*., **78**: 119–137.

Naz, S., Hanif, A., Maqbool, A., Ahmed, S. and Muhammand, K. 2012. Isolation, characterization and monitoring of antibiotic resistance in *Pasteurella multocida* isolates from buffalo (*Bubalus bubalis*) herds around Lahore. *J. Ani. Plant Sci*., **22**(3): 242-245.

Patel, H.K. 2004. Biochemical characterization, antimicrobial sensitivity, PCR based detection and mouse pathogenicity of *Pasteurella multocida* field isolates. Unpublished M.V.Sc. thesis submitted to the Anand Agricultural University, Anand.

Ramdani, H.J.S., Dawkins, R.B., Johnson, T.L., Spencer and Adler, B. 1990. *Pasteurella multocida* infections in mice with reference to Haemorrhagic Septicaemia in cattle and buffalo. *Immuno. Cell. Biol.,* **68**:57-61.

Ranjan, R., Panda, S.K., Acharya, A.P., Singh, A.P. and Gupta, M.K. 2011. Molecular diagnosis of haemorrhagic septicaemia - A Review. *Vet. World,* **4**: 189-192.

Rutter, J.M. 1983. Virulence of Pasteurella multocida in atrophic rhinitis of gnotobiotic pigs infected with Bordetella bronchiseptica. *Res. Vet. Sci.,* **34**: 287–295.

Shivachandra, S.B., Viswas, K.N. and Kumar A.A. 2011. A review of Hemorrhagic Septicemia in cattle and buffalo. *Anim. Health Res. Rev*., **12**: 67-82.

Townsend, K.M., Frost, A.J., Lee, C.W., Papadimitriou, J.M. and Dawkins, H.J.S. 1998. Development of PCR assay for species and type specific identification of *P. multocida* isolates. *J. Clin. Microbiol.,* **36:** 1096-1100.

Upadhaya, A.S, Dayalu, K., Murthy, B.S.K. and Nalljiah, R.D. 1972. Isolation of *Pasteurella multocida* Carter's type B in sheep. *Mysore J. Agri. Sci.,* **6**: 480-85.

Waffa, A., Ahmed Al-Gebouri, N.M. and Al-Maaly, N. M. 2014. Study the pathogenicity of *Pasteurella multocida* in mice. *Bas. J. Vet. Res.,* **1**:312-323.

Woolcock, J.B. anf Collins, F.M. 1976. Immune mechanism in *Pasteurella* multocida-in mice. *Infect. Immun.,* **13**: 949-958.