MOLECULAR DIAGNOSIS OF HEMORRHAGIC SEPTICAEMIA IN A MURRAH BUFFALO AND ITS THERAPEUTIC MANAGEMENT WITH CEFTIOFUR SODIUM

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ABSTRACT

A 7 years old female Murrah buffalo was presented to the Referral Veterinary Polyclinic, Indian Veterinary Research Institute, Izatnagar with a history of high fever, anorexia and respiratory distress since 2 days. On clinical examination, high body temperature, congested conjunctival mucous membrane, open mouth breathing, tachypnoea, tachycardia and lymphadenopathy noticed. Clinical pathology revealed leukocytosis, neutrophilia with shift to left and blood sample was found to be negative for haemoprotozoan infection. Bacteriological culture of blood sample revealed mucoid dew drop colonies suggestive of *Pasteurella* spp. and on Gram's staining of bacterial culture, Gram-negative cocco-bacilli organisms were detected. Further, the results of *Pasteurella multocida* species specific-PCR (polymerase chain reaction), *Pasteurella multocida* multiplex capsular PCR typing and *Pasteurella multocida* serotype B specific PCR revealed that the isolate was of *Pasteurella multocida* serotype B: 2. ABST (Antibiotic sensitivity test) revealed that the organism was highly sensitive for antibiotic Ceftiofur. The animal was treated with Inj. Ceftiofur sodium (2.2 mg/kg, IM, SID) and other supportive treatment including anti-pyretics, anti-histamines, multivitamins, rumenotorics and probiotics for 5 days. The animal showed marked recovery after complete therapy.

Keywords: Murrah, buffalo, *Bubalus bubalis*, *Pasteurella multocida*, polymerase chain reaction, ceftiofur sodium

INTRODUCTION

Hemorrhagic septicaemia is also known as septicemic pasteurellosis or barbone disease; is an acute, fatal, septicemic disease of cattle and buffaloes caused by the gram-negative cocco-bacillus *Pasteurella multocida* (Tabatabaei *et al*., 2007). This disease is considered as the most economically important bacterial disease on the livestock industry in the tropical regions of the world, especially in African and Asian countries including India. The important clinical signs of acute infection are characterized by severe depression, pyrexia, submandibular oedema, dyspnea, recumbency and death (Horadagoda

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et al., 2001). Buffalo species are generally more susceptible to pasteurellosis than cattle and show more severe forms of disease with profound clinical signs (Radostits *et al*., 2009). In South and Southeast Asian countries, it causes severe economic losses and is ranked as the most important contagious disease of cattle and buffaloes (Benkirane and De Alwis, 2002). In cattle and buffaloes, morbidity and mortality due to hemorrhagic septicaemia has been recorded to be 95.25 and 21.19%, respectively (Khan *et al*., 2006). Case fatality approaches 100% if treatment is not followed at the initial stage of infection (De Alwis, 1992).

The disease can be diagnosed on the basis of clinical signs and symptoms, isolation and identification of the organism and their molecular characterisation. The present report depicts PCR based confirmatory diagnosis of acute case of *Pasteurella multocida* serotype B: 2 infection and its therapeutic management by using Ceftiofur sodium in a buffalo.

MATERIALS AND METHODS

Case History and Clinical Observation: A 7 years old female Murrah buffalo was presented to the Referral Veterinary Polyclinic, Indian Veterinary Research Institute, Izatnagar, Bareilly (India) with a history of fever, anorexia and respiratory distress since 2 days. Clinical examination of animal revealed high body temperature (105.1^{o}F), congested conjunctival mucus membrane, open mouth breathing, tachypnoea (62 per minute), tachycardia (90 beats per minute), lymphadenopathy of submandibular lymph nodes, dehydration (Figure 1). Blood samples were collected by jugular venipuncture. About 5 ml of blood sample was collected in a clean glass vial using EDTA as anticoagulant for haematological examination, haemoprotozoan examination and bacterial culture. A complete haematological examination was carried out as per standard method (Jain, 1986).

RESULTS AND DISCUSSION

Haematological examination revealed severe leukocytosis (TLC-31,500/mm^3), neutrophilia (81%) with shift to left. The detailed haematological parameters before therapy and after therapy have been discussed (Table 1). Blood smear examination was found to be negative for haemoprotozoan or rickettsial infection. Blood sample was streaked on blood agar for bacterial culture, which showed non-hemolytic small glistening mucoid dew drop cream coloured colonies which is suggestive of pasteurellosis (Figure 2). On Gram's stainining of bacterial culture, presence of pink coloured Gram-negative cocobacilli organisms were noticed (Figure 3).

For further confirmation, the bacterial DNA (Deoxy-ribonucleic acid) was subjected to molecular detection by polymerase chain reaction. The reaction was carried out by using *Pasteurella multocida* species specific primers (Townsend *et al*., 1998) which amplify 460 bp product and cap primers (Brickell *et al*., 1998) which is expected to produce 760 bp product in serotype B. Genomic DNA from suspected *Pasteurella multocida* colonies was used as the template for PM-Multiplex PCR assay. The PCR reaction mixture consisted of a total 25 µl volume with each primer at a concentration of 3.2 picomoles, dNTPs at a concentration of 200 µM, 1xPCR buffer, 2 mM $MgCl_2$, 1U of *Taq* DNA polymerase and template DNA. The PCR amplification was

carried out with an initial denaturation at 95°C for 5 minutes, followed by 30 cycles of denaturation at 95°C for 30 seconds, annealing at 55°C for 30 seconds, extension at 72°C for 30 seconds and a final extension at 72°C for 5 minutes. A 5 µl amplified PCR product was mixed with 1 µl of 6x ready to use gel loading dye (MBI, Fermentas) and analyzed on 1.5% agarose gel containing 0.5 µg/ml ethidium bromide along with 100 bp DNA ladder (MBI-Fermentas) at 100 volts using 1x TAE electrophoresis buffer. The gels were visualized and photographed under UV-gel documentation system (Alpha Innotech Corp., USA).

The identification of *Pasteurella multocida* serotype B: 2 was carried out by using *Pasteurella multocida* serotype B: 2 specific primers (IPFWD and IPREV) which amplify 320 bp product (Brickell *et al*., 1998). The PCR amplification was carried out in 25 µl of total reaction volume using programmable thermal cycler. Best amplification of the desired product was obtained with 10 pico moles of primers, 1.6 mM of $MgCl_2$, 10 mM each of nucleotides and 1.5 units of *Taq* polymerase. The PCR amplification was carried out with an initial denaturation at 95°C for 5 minutes then 40 cycles of denaturation at 94°C for 1 minute, annealing at 53°C for 30 seconds, extension at 72°C for 45 seconds and the final extension at 72°C for 5 minutes. The PCR products were run along with 100 bp ladder in 1.5% (w/v) agarose gel in 0.5X Tris borate EDTA (TBE) buffer in horizontal submarine electrophoresis system and stained with ethidium bromide (0.5 µg/ml). The gel was examined on UV transilluminator and photographed by gel documentation system (Alpha Innotech Corp., USA). The results of PCR assay confirmed that the isolate was of *Pasteurella multocida* serotype B: 2 (Figure 4 and 5). Isolates were also subjected to *in-vitro* antibiotic susceptibility test (ABST) using different commonly used antibiotics: Ampicillin, Amoxycillin, Tetracyclin, Sulfadimidine, Gentamicin, Enroflaxacin, Streptomycin, Amikacin, Ciprofloxacin and Ceftiofur (Himedia, India) (Bauer *et al*., 1966). Antibiotic sensitivity test (ABST) revealed that the isolate was found to be resistant for commonly using antibiotics including Sulfadimidine, but, it was sensitive for Ceftiofur (Table 2). Based on the history, clinical signs and laboratory findings, it was confirmed as a case of *Pasteurella multocida* serotype B: 2 infection. The animal was treated with Inj. Ceftiofur sodium (Wofur, Vetoquinol) 2.2 mg/kg BW, IM, SID, Inj. Meloxicam (Melonex, Intas Pharmaceuticals) 0.5 mg/kg BW, IM, BID, Inj. Pheniramine maleate (Avilin Vet, MSD Animal Health) 0.5 mg/kg BW, IM, along with multivitamins, rumenotorics, and probiotics as supportive therapy. This treatment regimen was followed for 5 days and animal recovered completely after therapy.

Haemorrhagic septicaemia is highly fatal and contagious bacterial disease of cattle and water buffalo. The *P. multocida* is classified into various serotypes (A, B, D, E and F). Haemorrhagic septicaemia in cattle and buffalo is caused by serotype B: 2 (Asia) and E: 2 (Africa). The Asian serotype B: 2 and the African serotype E: 2 (Carter and Heddleston system), corresponding to 6: B and 6: E (Namioka-carter system), are mainly responsible for the disease (Hopkins *et al*., 1998). The clinical manifestations of the typical disease caused by B: 2 or E: 2 strains include pyrexia, respiratory distress with mucopurulent nasal discharge, frothing from the mouth, recumbency and death (Figure 5).

In this report, the diagnosis of hemorrahagic septicaemia (septicemic pasteurellosis) was initially done by isolation and identification of the *Pasteurella* organisms and thereafter, the

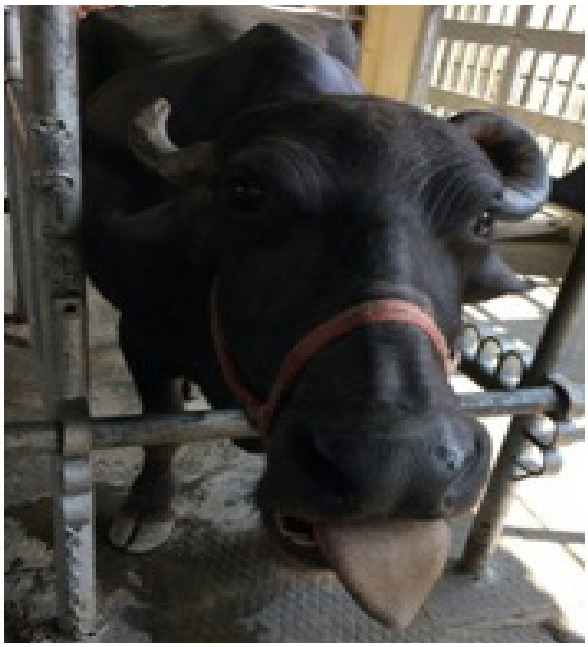

Figure 1. Animal showing the signs of respiratory distress and open mouth breathing.

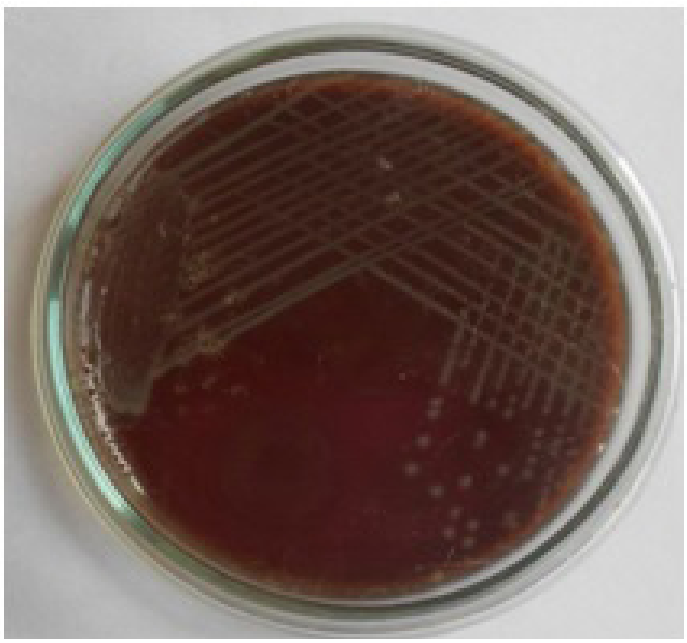

Figure 2. Mucoid dew-drop colonies of *Pasteurella* spp. on blood agar.

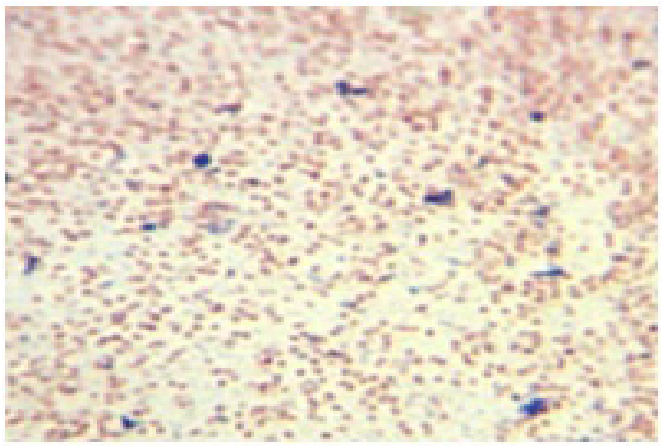

Figure 3. Demonstration of Gram-negative pink coloured cocobacilli organisms stained with Gram's stain (1000x).

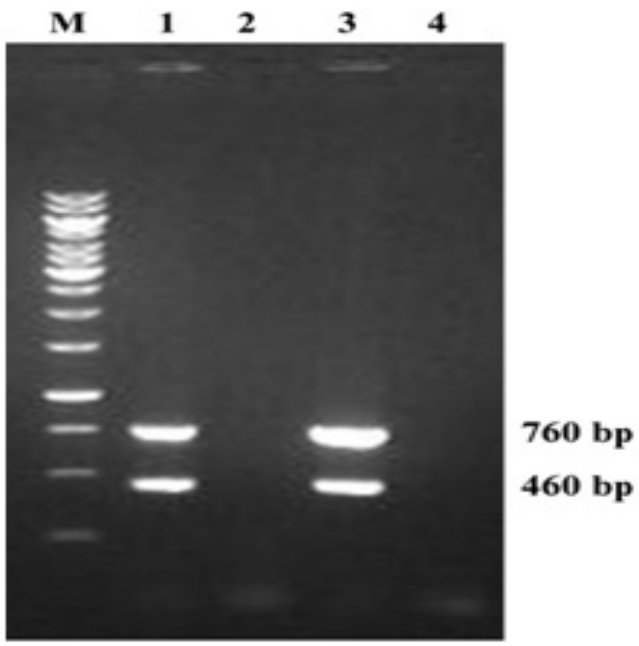

Figure 4. Agarose gel electrophoresis of the PCR products of *Pasteurella multocida* species specific PCR (PM-PCR) and Multiplex capsular PCR.
Lane M: 100-bp DNA ladder
Lane 1: Positive control (product size 760 bp and 460 bp)
Lane 2: Negative control (no template)
Lane 3: Positive sample
Lane 4: Negative sample

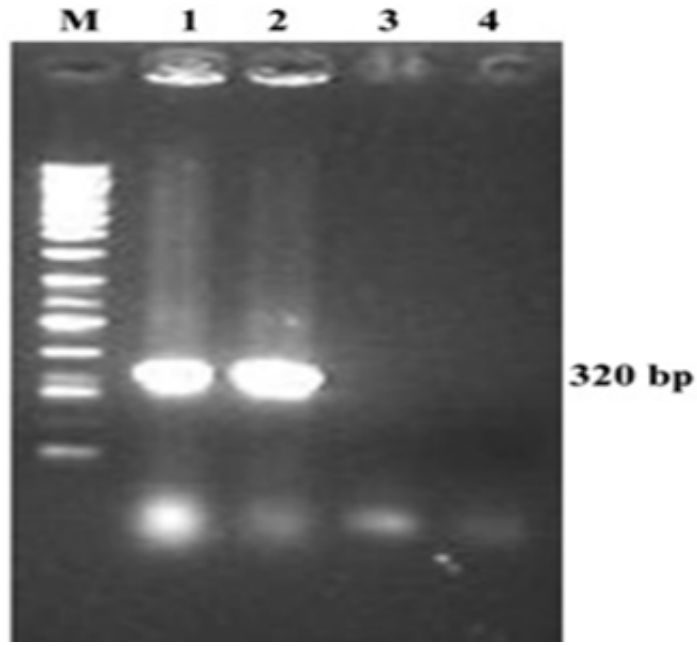

Figure 5. Agarose gel electrophoresis of the PCR products *Pasteurella multocida* serotype B: 2 specific primers.
Lane M: 100 bp DNA ladder
Lane 1: Positive control (product size 320 bp)
Lane 2: Positive sample
Lane 3: Negative control (no template)
Lane 4: Negative sample

Table 1. Changes in haematological parameters of the animal before and after therapy.

Parameters	Before therapy	After therapy
Haemoglobin (g%)	10.65	10.61
PCV (%)	31.02	30.83
TEC (10^6/mm^3)	6.33	6.01
TLC (/mm^3)	31,500	8856
Neutrophils (%)	81	48
Lymphocytes (%)	16	46
Eosinophils (%)	2	1
Basophils (%)	0	1
Monocytes (%)	1	4
Platelet count (lacs/mm^3)	2.49	3.29

Table 2. Antibiotic susceptibilities of the isolates, S-Susceptible, I-Intermediate, R-Resistant.

Antimicrobial agent	S	I	R
Ampicillin			R
Amoxicillin			R
Tetracyclin		I	
Sulfadimidine		I	
Gentamicin			R
Enrofloxacin		I	
Streptomycin			R
Amikacin		I	
Ciprofloxacin		I	
Ceftiofur	S		

involvement of *Pasteurella multocida* serotype B: 2 was identified by using PCR techniques like *Pasteurella multocida* species specific-PCR, *Pasteurella multocida* multiplex capsular PCR and *Pasteurella multocida* serotype B specific PCR. Pasteurellosis can be diagnosed by isolation and identification of organism in culture media or blood smear examination and can be confirmed by using various diagnostic techniques like rapid slide agglutination test, indirect haemagglutination test, ribotyping, restriction endonuclease analysis, colony hybridization assay, species specific PCR, multiplex PCR (Ranjan *et al*., 2011). Ifmlin and Blackall (2001) reported that PCR technology can be applied for rapid, sensitive and specific detection of *P. Multocida*. The *P. multocida* PCRs can be used to identify all subspecies of *P. multocida* (Townsend *et al*., 1998; Ifmlin and Blackall, 2001). Multiplex capsular PCR typing system is highly specific to identify the *P. multocida* (Ranjan *et al*., 2011). The rapidity and high specificity of two of the *P. multocida*-specific assays will provide optimal efficiency without the need for additional hybridisation (Townsend *et al*., 1998; Ifmlin and Blackall, 2001).

Antibiotics like Penicillin, Amoxicillin, Cephalothin, Ceftiofur, Cefquinome, Streptomycin, Gentamicin, Spectinomycin, Florfenicol, Tetracycline, Sulfonamides, Trimethoprim/Sulfamethoxazole, Erythromycin, Tilmicosin, Enrofloxacin and Norfloxacin can be effectively used against *P. multocida* infection (OIE, 2008). Antimicrobial susceptibility testing (AST) or ABST (Antibiotic sensitivity test) is particularly necessary for *P. multocida* as it may show resistance to commonly used antimicrobial agents (Kehrenberg *et al*., 2001). In this particular case, the antibiotic resistance was noticed for commonly using antibiotics including Sulfadimidine, but, it was found to be sensitive for Ceftiofur. Inj. Ceftiofur 2.2 mg/kg, BW, IM, SID is effective against *Pasteurella multocida* infection and can be used as antibiotic of choice in case of cattle and buffaloes (Radostits *et al*., 2009). Disease can be effectively controlled by vaccination. Bacterins, aluminium hydroxide gel vaccine, alum-precipitated vaccine (APV) and oil-adjuvanted vaccine (OAV) are commonly used as

prophylactic agents (OIE, 2008).

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REFERENCES

Bauer, A.W., W.M.M. Kirby, J.C. Sherris and M. Tarek. 1966. Antibiotic susceptibility testing by a standardized single disk method. *Am. J. Clin. Pathol*., **45**: 493-496.

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

Brickell, S.K., L.M. Thomas, K.A. Long, M. Panaccio and P.R. Widders. 1998. Development of a PCR test based on a gene region associated with the pathogenicity of *Pasteurella multocida* serotype B: 2, the causal agent of haemorrhagic septicaemia in Asia. *Vet. Microbiol*., **59**: 295-307.

De Alwis, M.C.L. 1992. Haemorrhagic septicaemia-A general review. *Brit. Vet. J*., **148**: 99-112.

Hopkins, B.A., T.H.M. Huang and L.D. Olson. 1998. Differentiating turkey post vaccination isolants of *Pasteurella multocida* using arbitrarily primed polymerase chain reaction. *Avian Dis*., **42**: 265-274.

Horadagoda, N.U., J.C. Hodgson, G.M. Moon, T.G. Wijewardana and P.D. Eckersall. 2001. Role of endotoxin in the pathogenesis of haemorrhagic septicaemia in the buffalo. *Microb. Pathogenesis*, **3**: 171-178.

Ifmlin, J.K. and P.J. Blackall. 2001. Development of a 23S rRNA-based PCR assay for the identification of *Pasteurella multocida*. *Lett. Appl. Microbiol*., **33**: 216-221.

Jain, N.C. 1986. *Schalm's Veterinary Haematology*, 4th ed. Lea and Febiger, Philadelphia, USA. p. 1168.

Kehrenberg, C., G. Schulze-Tanzil, J.L. Martel, E.C. Dancla and S. Schwarz. 2001. Antimicrobial resistance in *Pasteurella* and *Mannheimia*: Epidemiology and genetic basis. *Vet. Res*., **32**: 323-339.

Khan, A., U. Saddique, R. Ahmad, H. Khan, Y. Mohammad and M. Zubair. 2006. Sero-surveillance of hemorrhagic septicemia in cattle and buffaloes in district Malakand, North-West Frontier province. *J. Anim. Vet. Adv*., **5**: 912-915.

OIE. 2008. *Terrestrial Manual*. Haemorrhagic Septicaemia, p. 739-751.

Radostits, O.M., C.C. Gay, K.W. Hinchcliff and P.D. Constable. 2009. *Veterinary Medicine: A Textbook of the Diseases of Cattle, Horses, Sheep, Pigs, and Goats*. Pasteurellosis. Saunders Elsevier, Edinburgh, London, New York, Oxford, Philadelphia, St. Louis, Sydney, Toronto, USA. p. 921-947.

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

Tabatabaei, M., G.M. Jula, A.R. Jabbari and M. Esmailzadeh. 2007. Vaccine efficacy in cattle against hemorrhagic septicemia with live attenuated aroA mutant of *Pasteurella multocida* B: 2 strain. *Journal of Cell and Animal Biology*, **1**: 62-65.

Townsend, K.M., A.J. Frost, C.W. Lee, J.M.

Papadimitriou and H.J. Dawkins. 1998. Development of PCR assays for species- and type-specific identification of *Pasteurella multocida* isolates. *J. Clin. Microbiol*., **36**: 1096-1100.