

Research Article

**PCR AND ELECTRON MICROSCOPY BASED DIAGNOSIS OF
AN OUTBREAK OF HAEMORRHAGIC SEPTICEMIA IN
BUFFALO AND ITS CONTROL IN A FARM OF
WEST BENGAL, INDIA**

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ABSTRACT: An outbreak of Haemorrhagic Septicemia was investigated in Cattle Re-Settlement Project Farm, Ganganagar, North 24 Parganas, West Bengal, India in the month of December, 2013. Out of 102 nos. of buffalo, 25 animals were affected and 17 were died within 12 - 72 hours. The disease was diagnosed on the basis of history, clinical signs, post mortem findings, histopathological examination, bacteriological study, biochemical tests, biological test, PCR assay and finally by Electron microscopical examination. Antibiotic sensitivity test was done for the isolate. The ailing animals were successfully treated with antibiotic, analgesic and corticosteroid. The epidemic was finally controlled by therapeutic measures, immunization, restriction of movement of animals and proper disposal of carcasses.

Key words: Buffalo, Hemorrhagic Septicemia, Outbreak, PCR, Electron Microscopy, Diagnosis, Antibiogram, Immunization.

INTRODUCTION

Hemorrhagic Septicemia (HS) is an economically important bacterial disease of cattle and buffaloes (Chandrasekharan *et al.*, 1994) caused by Gram negative cocco-bacillary organism *Pasteurella multocida*. The disease is manifested by an acute and highly fatal septicemia principally in cattle and water buffaloes (Mitra *et al.*, 2013) and buffaloes are more susceptible than cattle (De Alwis 1990). It is reported for high morbidity and mortality

in unvaccinated population (Mondal *et. al.*, 2013). HS was found to cause highest mortality and the second highest morbidity in bovines in comparison to Anthrax, Black Quarter and FMD (Jindal *et al.*, 1996). HS in buffaloes is considered to be a major killer disease in Asia (Khera 1979). Occurrence of the disease has been reported throughout the year (Dutta *et al.*, 1990). The disease remains a significant obstacle to sustainable livestock production in most parts of topical Asia and Africa

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(Naz *et al.*, 2012). It is more common in the river villages and deltas of South-East Asia among buffaloes used in rice cultivation (Khan *et al.*, 2006). HS is widespread disease in India, occurs more frequently in poor animal husbandry conditions (Gajendragad *et al.*, 2012). The present communication recorded an outbreak of HS in buffaloes along with diagnose and control measures at Ganganagar Cattle Re-Settlement Project (CRS) Farm, North 24 Parganas, West Bengal, India in December, 2013.

MATERIALS AND METHODS

The outbreak spot was visited and the populations at risk, morbidity, mortality, clinical observation of ailing animals were recorded.

Samples: During post-mortem examination of dead animals, gross-pathological lesions were recorded. Tissue materials like lungs, liver, spleen, kidneys, heart, intestine and lymph nodes were collected in ice packed for bacteriological study. Peripheral blood smear prepared from ailing animals and heart blood smear from dead animal. Impression smears from liver, kidneys, spleen and lungs were taken for bacteriological examination. Giemsa and Methylene blue stain were used to demonstrate the micro-organisms in tissue impression and blood smears. Total 17 numbers of samples like nasal swabs, blood, bone marrow and tissue specimens (pieces of liver, spleen and lungs) were collected. The samples were further processed for isolation and identification of suspected pathogen by standard method (Carter 1984).

Isolation: Samples were inoculated on Nutrient agar, Blood agar (5% Sheep blood) and MacConkey's agar. Then smears prepared from representative colonies and microbes were

characterized by using Gram's staining and Leishman's staining method as followed by Naz *et al.*(2012).

Biochemical tests: Biochemical tests for all isolates were performed. Peptone water grown culture of each isolate was inoculated in 1% glucose, sucrose, sorbitol, mannitol, fructose, dulcitol, lactose, raticin, arabinose and maltose, incubated at 37°C for 72 hours. Indole, oxidase, catalase, urease production and nitrate reduction tests were carried out according to their standard bacteriological procedure (Carter 1984).

Histopathological examination: Tissue materials like lungs, liver, spleen, kidneys, heart, intestine and lymph nodes of dead animals were collected in 10% formalin for histopathological study and processed following standard method.

Pathogenicity test: Pathogenicity test of five isolates were carried out in six weeks old Swiss albino mice. A total of six mice were used for each isolate. Mice were inoculated intraperitoneally with 0.1 ml of inoculums. Control mice were injected with 0.1ml of sterile saline. All the mice were kept under observation and mortality was recorded. Blood smears prepared from heart blood of dead mice and stained with Giemsa stain. Resolution of *P. multocida* from heart blood of dead mice was carried out on sheep blood agar (Buxton and Fraser 1977). Pathogenicity test was performed as per method followed by Naz *et al.* (2012).

Antibiogram assay: Each isolate was tested for antibiotic sensitivity against 15 different antibiotics such as gentamicin, kanamycin, amikacin, tetracycline, doxycyclin, erythromycin, sulphadiazine, amoxycillin, ampicillin, chloramphenicol, enrofloxacin, norfloxacin, ciprofloxacin, ofloxacin and ceftiofur using the standard method of National

Committee for Clinical Laboratory Standards (NCCLS 1990).

***Pasteurella multocida* specific PCR assay:** PCR technology can be applied for rapid, sensitive and specific detection of *P. multocida* (Miffin and Blackall 2001, Townsend *et al.* (1998). In this study we have followed method described by Townsend *et al.* (1998) with some modifications.

A fraction of an isolated colony of the suspected organism was transferred directly into the PCR mixture. Template DNA was purified from pure colony by Quiagen DNA minikit (Germany), as per manufacturer protocol.

PCR conditions:

P. multocida specific Primer sequences (Townsend *et al.* 1998): :

KMT 1T 7 5'-ATC-CGC-TAT-TTA-CCC-AGT-GG-3'

KMT 1 SP6 5'-GCT-GTA-AAC-GAA-CTC-GCC-AC-3'

5µl of Template DNA was added to the PCR mixture (total volume of 50µl) containing Fermentas Hot start Mastermix 2X (25 µl/50µl reaction volume), 0.5µm of forward and reverse primer having sequence as mentioned below and sterile de-ionised nuclease free water (Fermentus).

Thermocycler parameters are as follows: Initial denaturation at 95°C for 5 minutes followed by 30 cycles of 95°C for 1 minute, 55°C for 1 minute, 72°C for 1 minute and final extension at 72°C for 7 minutes. Then 10µl of each sample mixed with 2µl of 6X Orange gel loading dye (Fermentus) along with 6µl of 100bp ladder (Fermentus) and electrophoresis was performed on 1% agarose gel in 1 X Tris-acetate EDTA buffer containing 50µg per ml of ethidium bromide solution (stock solution

10mg/ml, Fermentus) at 5V/cm for 1 hour. The PCR products were viewed by UV trans-illumination and the result was analysed by Quantity One gel analyzing software of gel-doc system (Biorad).

PCR was used only for the confirmatory diagnosis as the technique minimizes the time required by cumbersome biochemical tests. Although automated microbiological identification system gives confirmatory diagnosis within very short period but the technique requires very expensive equipments and costly reagents, whereas the conventional PCR technique as described here is considerable cheaper than the previous one.

In this PCR technique one positive control has been used using *P. multocida* reference strain (ATCC 12945) and one negative template control.

Electron-Microscopical Study:

Overnight broth culture of isolated organism taken from pure culture of blood agar media was used as test sample for this study. 5 microlitre sample was taken on carbon coated copper grids, wait for two minutes, wash with Tris MgCl₂ buffer, stain with 2% aqueous uranyl acetate and absorb the excess liquid with blotting paper. Now the grid is ready for EM viewing. This grid was examined at 30000X. This test was performed by FEI Transmission Electron Microscope

(Model No. TECNAI-12). This EM study was done as per the method followed by Jacques and Foiry (1987) and Nattawooti *et al.* (2011).

RESULT AND DISCUSSION

Total buffalo population of that area is nearly 3000. Epidemic occurred in shed no. 4, where 102 nos. of buffalo were present among which

25 were ill and 17 found died. The affected animals showed symptoms of fever (104° - 106° F), anorexia, congestion of mucous membrane, bloat, sneezing, salivation, severe respiratory distress, severe diarrhea, swelling near throat region and died within 12 - 72 hours. The symptoms corroborates with the findings of Mitra *et al.* (2013). Auscultation of lungs revealed abdominal lung sound with consolidation in some area. Total number of samples studied were 17.

Microscopical observation: Peripheral, heart blood smear and tissue impressions revealed presence of large number of Gram negative, bipolar cocco-bacilli organisms indistinguishable from *Pasteurella* sp.

Gross and Histopathological changes: Postmortem examination revealed congestion, haemorrhages and consolidation of lungs (Fig.1) and accumulation of increased amount of straw coloured fluid along with fibrinous exudates in the abdominal and thoracic cavity. Intestinal contents were blood stained and mesenteric lymph nodes were found enlarged and haemorrhagic. Edema was observed in subcutaneous tissue and also in thorax, tongue and neck. Exudate was found in pleural cavity pericardial sac. Enlargement of bronchial and medio-sternal lymph nodes were found with marble appearance of lungs due to thickening of septa. Grossly, heart exhibited congestion and petichial haemorrhages on epicardium and endocardium. These observations corroborate with the findings of Mitra *et al.* (2013) and Mondal *et al.* (2013).

Histopathological examination showed grey hepatization of lungs (Fig.2), Congestion, haemorrhages and necrosis with severe neutrophilic infiltration in pleura and intertubular septa. Alveoli were packed with serosanguineous mass and bronchioles revealed

degeneration in the lining epithelial cells. Severe congestion and haemorrhages were observed in cortex and medulla of lymph nodes, epicardium and myocardium of heart and mucosa and serosa of intestine. Other organs like liver, kidneys and spleen showed degenerative and vascular changes of toxemia. The gross and histopathological observations corroborate with the findings of previous worker (Jindal *et al.* 1996).

Morphology and cultural character: : The organisms grow aerobically at 37° C. In blood agar media profuse growth occurs without any haemolysis (Fig. 3). Ten bacteriological isolates were recovered from the samples. All the isolates exhibit smooth, glistening, translucent colonies on nutrient agar, failed to grow on McConkey's agar and produce non-haemolytic dew-drop like colonies on sheep blood agar. Gram's stained and Leishman stained smears from all isolates revealed microscopically Gram negative bipolar cocco-baccili, short ovoid rods with a tendency to bipolar staining.

Biochemical tests: The organism showed the following biochemical characteristics produce indole and H_2S , reduce nitrate, no growth in McConkey's agar, no haemolysis on blood agar, no urease production, no liquifaction of gelatin, no motility at 22° C, production of acid without gas in glucose, sucrose, galactose, fructose and mannitol.

Pathogenicity test : All the field isolates killed mice within 6 to 24 hours post - inoculation. Leishman stained smears prepared from heart blood of dead mice revealed bipolar organisms. From heart blood of mice colonies representative of *P. multocida* were isolated on sheep blood agar.

Antibiotic sensitivity test : Among the 15 antibiotics tested the isolates were highly sensitive to ceftiofur (88.50%), moderately



Fig.1: Congestion, hemorrhages and consolidation of lungs

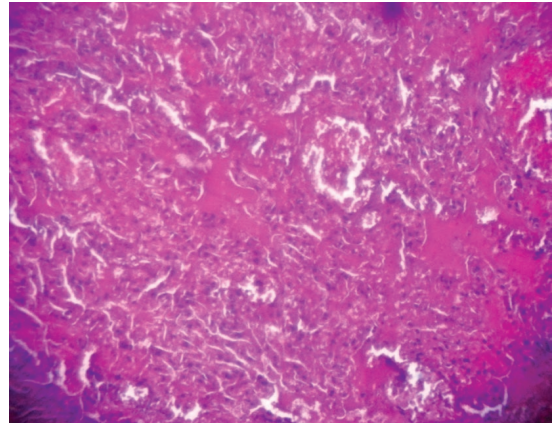


Fig.2: Lung tissues showed grey hepatization



Fig. 3: *P. multocida* colonies in blood agar media

sensitive to enrofloxacin (60.30%), norfloxacin (55.25%), ciprofloxacin (46.50%), ofloxacin (60.50%), gentamicin (47.80%), amikacin (58.50%), and less sensitive to kanamycin (35.50%), tetracycline (30%), doxycyclin (28.50%), erythromycin (27.60%), and resistant to sulphadiazine (17.50%), amoxicillin (15%), ampicillin(18.50%), chloramphenicol (20%).

PCR Assay: During this test, 620 base pair

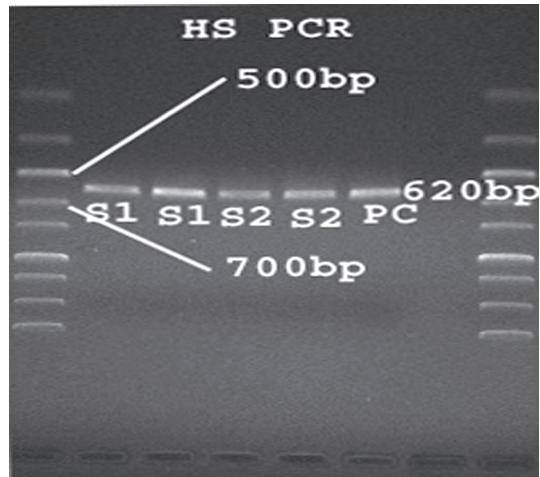


Fig.4 : PCR test result of *P. multocida*

band correlates the findings of Townsend *et al.*, 1998a Accurate laboratory detection of *P. multocida* depends on the isolation and identification of suspected bacterial colonies. Numerous studies for diagnosis of *P. multocida* have been carried out with variable results. The phenotypic identification systems by means of morphology, biochemical typing, stereotyping etc. are very much laborious and time

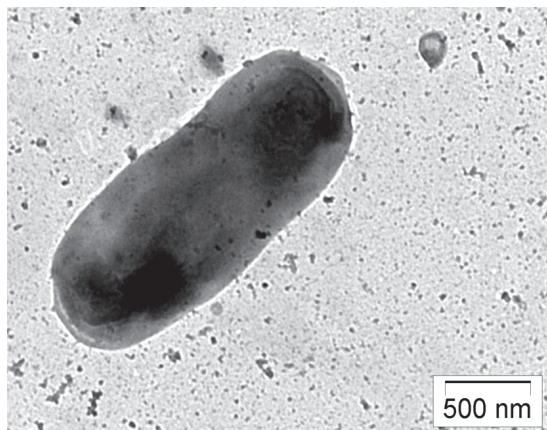


Fig.5: Electron Micrograph showing negatively stained *P. multocida* organism.

consuming. So, we have employed PCR technique capable of identifying B cultures with any combination of somatic antigen by the amplification of a 620bp fragment with the KT SP61 primers (Townsend *et al.*, 1998). The ability of the PCR assays described in this study can provide rapid diagnosis of the HS causing serotype has the potential to reform HS diagnosis in the State of West Bengal.

Electron Microscopy: The negative contrast Electron Microscopic study of *Pasteurella multocida* organism revealed a thick and regular capsule with an extra protein covering on both the end and appearance of a typical bipolar characteristic bacterial particles.

In clinical cases, diagnosis of HS is made on the basis of herd history, clinical signs, gross pathological lesions, morphology, cultural and biochemical characters of organisms, biological tests, histo-pathological changes. However confirmation of sensitive clinical diagnosis needs isolation and identification of the organism from morbid samples, PCR assay and Electron Microscopy. In the present study

cultural, morphological, biochemical characteristics of all the isolates recovered from morbid materials were in accordance with those of *P. multocida* (Dutta *et al.*, 1990, Kumar *et al.*, 2009, Mitra *et al.*, 2013).

Treatment and Control: The affected animals were treated with (i) Inj. Ceftiofur sodium @ 1gm I/M for 5 days, (ii) Inj. Meloxicam @20ml I/M for 5 days, (iii) Inj. Prednisolone @10ml I/M for 5 days (in tapering doses). The buffaloes with early stage of disease responded to the treatment.

The total herd (1100 nos.) was vaccinated with HS broth vaccine (manufactured by IAH&VB, Kolkata) @5 ml S/C at brisket region. The control measures include proper vaccination, disposal of carcasses by deep burial with lime and bleaching powder, restricted animal movement, segregation of the affected animals, cleaning and disinfection of the contaminated premises by burring and application of disinfectants.

Long bones from the suspected carcasses of HS were found to be the sample of choice for isolation of *P. multocida*. Isolation of *P. multocida* made from wide range of organs such as kidney, heart, liver, brain, tonsils, and lymph nodes from experimentally infected calves (OIE 1992, Singh *et al.*, 2007). From the liver and blood samples, recovery of the causative organisms was poor. Gross pathological changes might have inactivated the causative agents in these samples (Naz *et al.*, 2012).

All the field isolates of *P. multocida* was found pathogenic for mice and killed mice within 6-24 hours post-inoculation. In the BALB/C mice, when experimentally infected through I/P route, an over whelming septicemia was observed within 30 hours post-infection

(Ramdani *et al.*, 1990).

Ten pure isolates of *P. multocida* were tested for their sensitivity against different antibiotics available for the treatment of bacterial infection in animals. All the isolates were found highly sensitive (90%) to ceftiofur sodium, ceftriaxone, ciprofloxacin and enrofloxacin. Yoslumia *et al.*, (2008) and Kumar *et al.*, (2009) also found enrofloxacin the most effective antibiotic against *P. multocida*.

HS is one of most important diseases of bovines in Asian and Middle Eastern country. Many states of India were marked as high risk zones. Gajendragad *et al.* (2012) reported eleven outbreaks of HS in West Bengal. The morbidity and mortality rate were 1.18% and 0.54% respectively. HS outbreak in buffalo was reported in Murshidabad District of West Bengal by Mitra *et al.* (2013). Incidence of HS in buffalo by *P. multocida* type B was reported by Naz *et al.* (2012) in Pakistan, Biswas *et al.* (2004) in India. About 26 outbreaks have been recorded in Punjab state from 1989 to 1990 (Saini *et al.*, 1991). Outbreaks of HS was recorded in Srilanka (De Alwis and Vipulasiri 1980) Zimbabwe (Lane *et al.*, 1992), South Asia, the Middle East and Africa (FAO 1991), Aurangabad, Behar, India (Kumar *et al.*, 2011).

In conclusion, all the field isolates were similar in cultural, morphological, biochemical and serological characteristics and can be used for development of HS vaccine to control the disease. The local isolates of HS showed increasing level of resistance to the antibiotics that were extensively used in field for treatment of the disease. It is therefore recommended to monitor the antibiotic sensitivity of *P. multocida* from time to time in future to design the effective regimen for treatment of the disease.

This study suggested restriction of animal movement, disposal of carcasses properly by deep burial method with lime powder and spray formalin and other potent disinfectant in the sheds and annual vaccination programme against HS for effective control of the disease.

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