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Research Article

CHARACTERIZATION OF PASTEURELLA MULTOCIDA B: 2 BY CONVENTIONAL METHODS AND BY PCR AND THEIR ANTIBIOGRAM

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ABSTRACT

A total of nine strains of *Pasteurella multocida* subspecies *multocida* were isolated from buffaloes suspected for haemorrhagic septicaemia. All the nine isolates were sensitive to chloramphenicol, cefotaxime ceftriaxone, enrofloxacin, erythromycin, gentamicin, pefloxacin, ofloxacin and oxytetracycline. Six isolates exhibited resistance to ampicillin, amoxycillin and streptomycin. Multiplex PCR using the 2 primer sets i.e. KMT1SP6-KMT1T7 and KTSP61-KTT72 produced 2 amplification products of size 460 bp and 590 bp, respectively in all the isolates. PCR was applied directly on the samples without genomic DNA extraction.

Key words: Antibiogram, *Pasteurella multocida*, PCR

Pasteurella multocida is an important respiratory pathogen responsible for causing many disease syndromes in various species of livestock. Haemorrhagic septicemia (HS) is an acute, septicemic disease principally affecting cattle and buffaloes throughout India particularly in high humid, tropical and subtropical zones. The occurrence of disease is higher in buffaloes as compared to cattle and the incidence rate, mortality and case fatality rate are greater in the young calves as compared to the adult in both cattle and buffaloes (Khan *et al.*, 2006). The organisms are responsible for huge economic losses in India due to high morbidity and mortality in different species of animals (Singh *et al.*, 1996). Inspite of the extensive control measures, the epidemics of HS are still being reported from different regions of the country throughout the year (Jindal *et al.*, 2002). Therefore there is a need to find out geographical distribution of the disease in different parts of the country with their serological and molecular characterization.

The present study describes the detection of *Pasteurella multocida* B: 2 from apparently healthy and HS suspected animals by conventional methods and PCR directly on the samples without extracting genomic DNA alongwith their antibiotic sensitivity pattern.

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MATERIALS AND METHODS

A total of 150 samples collected from apparently healthy and diseased/dead cattle and buffaloes from July 2007 to July 2008, comprised of nasal swabs, peripheral blood from live animals (apparently healthy and diseased) and heart blood, nasopharyngeal and tracheal swabs from dead animals. These samples were inoculated on brain heart infusion agar containing 5% defibrinated sheep blood and incubated at 37°C for 24 h. The suspected colonies were picked up and pure cultures were obtained. The organisms were identified on the basis of their cultural, morphological and biochemical characteristics as described by Quinn *et al.* (1994).

Pathogenecity was tested by inoculating 0.2 ml of 18 h broth culture of the isolates (4.0 x 10^9 CFU/ml) intraperetoneally in 3 weeks old Swiss albino mice and observing them for signs of ill health or mortality within 24 h. Upon death, smears were made from heart blood and stained with methylene blue to observe the bipolar organisms. Simultaneously, heart blood was streaked on blood agar plates for reisolation of the organism. Antibiotic sensitivity of the isolates was done by disc diffusion method (Bauer *et al.*, 1966) using different antibiotic discs.

The isolates were confirmed by polymerase chain

reaction (PCR) using *P. multocida* (PM) species specific (KMT1T7, KMT1SP6) and *P. multocida* (B: 2) (HS) type specific primers (KTSP61, KTT72) developed by Townsend *et al.* (1998). Multiplex PCR was carried out using both the primer sets simultaneously. All the150 samples were subjected directly to PCR without genomic DNA extraction and using Taq PCR corekit (Qiagen) in Hybaid OmnR thermocycler. Nasal, nasopharyngeal and tracheal swabs were initially incubated in BHI broth at 37°C for 6 h and bacterial cultures were then centrifuged (4000 rpm, 20 min). Supernatant was discarded and the pellet was washed twice in phosphate buffer saline (PBS) and finally suspended in 50 µl PBS to be used as sample for PCR. Blood was centrifuged (3000 rpm, 5 min) and plasma was separated. Plasma was again centrifuged at high speed (8000 rpm, 10 min), supernatant was discarded and the pellet was washed twice in phosphate buffer saline (PBS) and finally suspended in 50 µl PBS to be used as sample for PCR. Touch PCR was applied on single bacterial colony of the isolate grown on blood agar plate that was picked up with a sterile tip and mixed in the reaction mixture for amplification.

The cycling parameters used for all kinds of samples were same i.e. initial denaturation at 95°C for 4 min, 30 cycles of denaturation at 95°C for 4 min, primer annealing at 55°C for 1 min and extension at 72°C for 1 min and a final extension step at 72°C for 6 min. After the PCR amplification reaction, the amplified products were subjected to agarose gel electrophoresis using 1.5% agarose in tris-borate-EDTA (TBE) buffer containing ethidium bromide (0.5µg/ml) and visualized on UV transilluminator at 260 nm wavelength.

RESULTS AND DISCUSSION

Nine isolates of *P. multocida* B: 2 obtained from diseased or dead buffaloes (Table), showed typical characters of *P. multocida*. The cultures showed small, circular, glistening and dew drops like colonies on blood agar. They were non-hemolytic and failed to grow on MacConkey's lactose agar. All the isolates appeared as bipolar, non-motile gram-negative coccobacilli that were catalase and oxidase positive, produced indole, reduced nitrates but lacked urease activity and were negative for methyl red, Voges Proskauer, ONPG (Ortho-Nitrophenyl-β-Galacto-pyranosidase), citrate and lysine utilization, phenylalanine deamination, H_2S production and esculin hydrolysis test. All the isolates utilized ornithine except isolate LDH-74. All the isolates produced acid from xylose, glucose, saccharose, sucrose, galactose, mannitol, mannose, and sorbitol but did not utilize lactose, dulcitol, maltose, trehalose, inositol and arabinose. Except isolate LDH 74, all the isolates produced acid from adonitol, rhamnose, cellobiose, malbiose and raffinose. Similar results were also reported by Arora *et al.* (2005). All the isolates were pathogenic for mice, as 0.2 ml of the 18 h broth culture injected i/p killed the mice within 24 h. The organisms were recovered from the heart blood of the mice. The methylene blue stained smears made from heart blood of the dead mice revealed typical bipolar organisms. Antibiogram revealed that all the isolates were sensitive to chloramphenicol, enrofloxacin, gentamicin, ofloxacin and pefloxacin whereas seven of the isolates were sensitive to oxytetracycline and six to cefotaxime, ceftriaxone and erythromycin. Six isolates exhibited resistance to ampicillin, amoxicillin and streptomycin. Similar results were reported by Arora *et al.* (2005) and Sharma *et al.* (2007).

The results of PCR amplification exhibited the presence of 2 amplified products of 460 bp and 590 bp. The presence of 460 bp band indicates that all the isolates are *P. multocida*, whereas 590 bp band signifies the presence of B: 2, B: 2, 5 or B: 5 serotype (Fig). So multiplex PCR using both *P. multocida* species specific and HS type specific primers holds promise for an efficient and quick diagnosis of animal

Table

Isolation of *P. multocida* B: 2 from various samples

Sample	Number of samples	Number of samples positive for *P. multocida* B: 2
Nasal swabs	102	2
Peripheral blood	32	5
Nasopharyngeal swabs	8	0
Heart blood	5	2
Tracheal swabs	3	0
Total	150	9

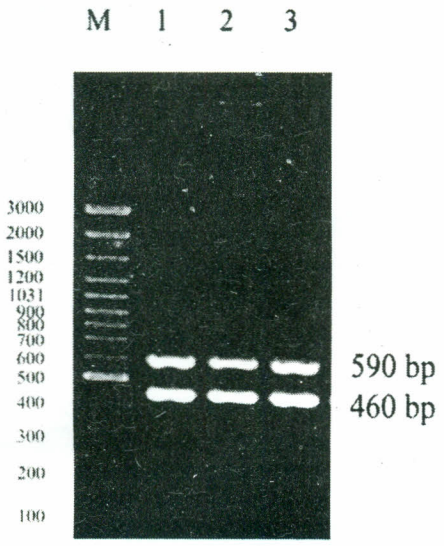

Fig. Agarose gel showing PCR products of *P. multocida* B: 2 isolates.
M: 100 bp DNA ladder, Lane 1: LDH 68, Lane 2: LDH 69, Lane 3: LDH 70.

pasteurellosis. Similar amplifications were observed when the PCR was applied directly on nasal swabs, nasopharyngeal swabs, tracheal swabs, blood or bacterial colony thereby implying that the diagnosis can be attempted directly on the samples without the need to culture the organism or extract the genomic DNA. Also the use of HS-specific PCR will obviate the need for complex serotyping procedures and the use of animals for antiserum production. Similar observations were also given by Townsend *et al.* (1998) and Dutta *et al.* (2001). PCR applied directly on samples further reduces the time for the diagnosis of the disease in contrast to detection by conventional methods. Similar observations were also given by Shivashankara *et al.* (2001).

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