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Molecular characterization of *Pasteurella multocida* serotype B:2 strain

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Abstract
The bacterium *Pasteurella multocida* is an opportunistic pathogen that is associated with the infections centering the animals such as bovines, swine, rabbits as well as poultry. It is widely known for its efficiency to cause Haemorrhagic Septicaemia, a complication characterized by high morbidity and mortality. In this study, *P. multocida* was cultured, isolated and was subjected to biochemical characterization for the purpose of identification. The DNA was then extracted from the cultured bacterium and the Molecular Characterization of *P. multocida* serotype B:2 strain was done by the employment of Polymerase chain reaction (PCR) targeting *ompH* using the specifically designed forward and reverse primers. The amplified products of desired gene were then visualized using 1% Agarose gel. The PCR yielded a 943 bp gene product. The molecular characterization of the bacterium *P. multocida* plays a very important role in demonstrating the efficiency of the *P. multocida* as a candidate for the production of vaccines.

Keywords: *Pasteurella multocida*, Polymerase chain reaction, serotype, Haemorrhagic septicaemia

Introduction
Pasteurella multocida is a gram negative coccobacilli and found as a commensal of respiratory tract of warm blooded animals. It causes diseases in animals (bovines, porcines, rabbits and poultry) immunosuppressed by stresses such as viral infections, heat, cold or humidity with aerosol transmission of infection between animals. Haemorrhagic Septicaemia (HS) is an acute, fatal septicaemic disease of cattle and buffaloes caused by the bacterium *Pasteurella multocida* and occur as a catastrophic epizootic in many Asian and African countries resulting in high mortality and morbidity. Serotypes B:2 and E:2 of *P. multocida* are associated with HS in cattle and buffaloes in Asia and Central Africa, respectively. In India, it is estimated to cause economic losses of more than 10 million rupees annually (Venkataramanan *et al.*, 2005) [23] thus it is responsible for maximum mortality of livestock in the country (National Animal Diseases Referral Expert System (NADRES) of the Project Directorate on Animal Disease Monitoring and Surveillance, 2012).

The pathogenic potential of *P. multocida* in vertebrate animals was recognized over a century ago and infections are broadly termed as Pasteurelloses. *P. multocida* infects a wide range of animal hosts causing specific infections that manifest differently. Indeed, *P. multocida* has a broad host range, but this peculiar property is poorly understood. Potential virulence factors of *P. multocida* have recently been reviewed (Hunt *et al*., 2000; Christensen and Bisgaard, 2000; Harper *et al*., 2006) [10, 4, 9] but no host-specific factors have been identified till yet. The pathogenesis of the disease is a result of complex interactions between host factors (species, age, immune status) and specific bacterial virulence factors which includes LPS, capsule, adhesin, outer membrane etc. (Boyce *et al*., 2012) [2].

The outer membrane proteins (OMPs) of Gram negative bacteria play essential roles in host-pathogen interactions and in disease processes. Outer membrane proteins of *P. multocida* play a significant role in the pathogenesis of pasteurellosis and have been identified as potent immunogens (Singh *et al.*, 2011) [19]. Outer membrane proteins of *P. multocida* have been recognized as immunodominant antigens and are thought to be responsible for cross protective immunity, since lipopolysaccharides (LPS) alone induces only partial protection against pasteurellosis in mice (Ryu and Kim, 2000) [17]. The immunogenicity of selected outer membrane proteins of *P. multocida* was demonstrated in rabbits, calves and chickens (Zhang *et al.*, 1994) [27].

The outer membrane proteins also have protective role against HS. The heat-modifiable and porin proteins are important classes of OMPs that are surface-exposed and exhibit molecular mass and antigenic variation. *P. multocida* expresses heat-modifiable (OmpA) and porin (OmpH) proteins on the cell surface.

OMP of *P. multocida* have been implicated as protective immunogen. *P. multocida* expresses both heat modifiable protein (OmpA) and porin (OmpH). OmpH possessed both specific and cross-reacting epitopes which are abundantly expressed on the bacterial surface, OmpH is a surface-exposed conserved immunodominant porin that is detected in 100% of bovine isolates and it has been viewed as a potential vaccine candidate (Dabo *et al.*, 2008) [6]. It contains a high proportion of antiparallel β-chains, giving it a barrel shape. Work has been carried out on the prospect of using OmpH as a subunit vaccine in native and recombinant forms.

Material and Methods

Revival of *P. multocida* serotype B:2 culture

The ampoule containing culture of *P. multocida* serotype B:2 (strain P52) was streaked directly on streaked on blood agar plate for isolation of a single colony and incubated overnight at 37 °C. The colony characters were observed on blood agar plate.

Pathogenicity test of the organism

Approximately 0.5 ml of 10^{-5} dilution of 18 hrs. old culture of P52 was injected intraperitoneally into three healthy mice. All of them died within 36-38 hrs of inoculation. The organisms were reisolated in pure culture from heart blood and spleen of the dead animals.

Characterization of Culture

The purity and identity of the culture was tested by morphological, cultural and biochemical examinations. The biochemical tests were carried out as per the standard protocols described by Cowan and Steel (1970) [5].

Morphological characterization

Slides were prepared by picking single colony and the air dried heat fixed smear of the culture was then stained with Gram's stain. The blood smear from heart blood of experimentally infected mice stained by Leishman stain. The smears were observed to study the morphology of bacteria under oil immersion microscope.

Cultural characterization

The isolate was streaked onto blood agar and MacConkey agar plate and incubated overnight at 37 °C.

Biochemical characterization

The biochemical tests such as indole, methyl red, voges-proskauer, catalase, oxidase and nitrate reduction test were performed with *P. multocida* isolate. Then, the isolate was also subjected to sugar fermentation test using glucose, fructose, galactose, maltose, mannitol, sucrose, salicin, raffinose, inositol and rhamnose.

Molecular Characterization

Polymerase chain reaction (PCR)

For confirmation of *P. multocida* B:2, HS specific PCR assay was carried out following the extraction of genomic DNA from *P. multocida* serotype B:2.

Isolation of genomic DNA of *P. multocida*

P. multocida serotype B:2 genomic DNA was isolated following the method of Wilson (1987) with a slight modification. About 2.0 ml of 16 hrs. broth overnight grown culture (single colony inoculated in brain heart infusion broth) was inoculated in 50 ml of BHI broth and subjected to incubation at 37 °C under constant shaking for 18 hrs. Bacterial pellet was obtained by centrifugation at 8,000 rpm for 10 min at 4 °C. The pellet was washed twice in PBS. The pellet was then suspended in lysis buffer containing 2.0 ml TE buffer (10 mM Tris-HCl, pH 8.0 and 1 mM EDTA, pH 8.0), 300 µl SDS (10% w/v) and 5µl of proteinase K (20 mg/ml) followed by rapid pipetting and kept at 37 °C for one hr. The lysate was treated with 500 µl of 5 M NaCl and 100 µl cetyl trimethyl ammonium bromide (CTAB) (10% w/v in 0.7 M NaCl) and tubes were kept at 68 °C in a water bath for 10 min. Equal volumes of chloroform: isoamyl alcohol (24:1, v/v) were added in each tube, mixed gently and then centrifuged at 10,000 rpm for 10 min. The supernatant was collected in a separate tube. Bacterial DNA was precipitated by adding 1/10 volume of ammonium acetate (7.5 M) and double the volume with chilled absolute ethanol. The tubes were kept at -20 °C for one hr. and the DNA was spooled out with sterile micropipette tip. Then, it was dissolved in 500 µl sterile triple glass distilled water containing RNaseA (20 µg/ml) by incubating at 37 °C for one hr. Equal volume of phenol: chloroform: isoamyl alcohol (25:24:1 v/v/v) was added in each tube, mixed gently and centrifuged at 10,000 rpm for 10 min. The upper aqueous layer was collected into another fresh microcentrifuge tube and DNA was precipitated as mentioned earlier. The DNA pellet was obtained by centrifugation at 13,000 rpm for 20 min at 4 °C. Pellet was washed with 70% ethanol, dried and re-suspended in 100 µl of TE buffer and finally stored at -20 °C in small aliquots. Isolated bacterial DNA was checked for purity and quantified in Nanodrop spectrophotometer and stored at -20 °C for further use.

Estimation of DNA

All the DNA samples were dissolved in appropriate quantity of TE buffer and O.D. was measured at 260 nm and 280 nm and then DNA concentration was estimated with the following formula as:

DNA concentration (µg/ ml) = OD260 x Dilution factor x 50
Purity = OD at 260 nm / OD at 280 nm

P. multocida serotype B:2 specific PCR assay

The identification of *P. multocida* serotype B:2 was carried out by using *P. multocida* species specific PCR, HS-causing type-B-specific PCR assay (Townsend *et al*., 1998; Townsend *et al*., 2001) [21, 22] with respective primers as mentioned in adjoining table. PCR reaction was performed in 0.2 ml PCR tubes. The contents were mixed thoroughly and given short spin to settle down the reaction components. The tubes were then placed in a thermocycler. The PCR amplification was carried out with an initial denaturation at 94 °C for 5 min, followed by 30 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min, extension at 72 °C for 1 min and a final extension at 72 °C for 10 min.

Primer	Primer sequence	Orientation (5'-…….-3'
KTT 72	AGGCTCGTTTGGATTATGAAG	Forward
KTSP 61	ATCCGCTAACACACTCTC	Reverse

PCR Reaction mix: 25 µL

Forward	2.0 µl
Reverse	2.0 µl
DNA template	2.0 µl
DreamTaq™ Green PCR Master mix*	12.5 µl
Nuclease free water	6.5 µl
Total	25 µl

The set up and running conditions were used as described below:

Steps	Temperature	Time	No. of cycles
Initial denaturation	94 °C	05 min	01
Denaturation	94 °C	01 min	30
Annealing	55 °C	01 min	
Extension	72 °C	01 min	
Final extension	72 °C	10 min	01
Hold	04 °C	∞	

Agar gel electrophoresis of PCR products

1 % agarose was prepared in 1X TBE buffer and boiled in a microwave oven. It was allowed to cool to 50 °C and ethidium bromide was added (stock, 10 mg/ml) @ 0.5 µg/ml of gel volume. Molten gel (40 ml) was poured into horizontal agar gel electrophoresis apparatus and gel comb was inserted. Then, gel was allowed to solidify at room temperature for 20 min. Electrophoretic tank was filled with 1X TBE buffer and then gel was immersed in buffer. Samples were loaded with 100 bp molecular weight DNA ladder marker. Electrophoresis was carried out for 30 min at a constant voltage of 80 V. Gel was visualized under ultraviolet (UV) light using gel documentation system.

PCR Based Amplification of outer membrane protein H (*ompH)* gene

The *ompH*-specific primer *OmpH*-F and *OmpH*-R were used as per Joshi *et al.* (2013)

Primer	Primer sequence	Orientation (5'-…….-3'
ompH F	TCAGGATCCCAGCAACAGTTTACAATCAAGA	Forward
ompH R	CTACCCGGGTTAGAAGTGTACGCGTAAACCA	Reverse

PCR Reaction mix: 25 µL

Forward	2.0 µl
Reverse	2.0 µl
DNA template	2.0 µl
DreamTaq™ Green PCR Master mix	12.5 µl
NFW	6.5 µl
Total	25 µl

Total volume was maintained with sterilized ultra pure water.

PCR amplification was performed using the following cyclic conditions

Steps	Temperature	Time	No. of cycles
Initial denaturation	94 ºC	05 min	01
Denaturation	94 ºC	01 min	30
Annealing	62 ºC	01 min	
Extension	72 ºC	01 min	
Final extension	72 ºC	10 min	01
Hold	04 ºC	∞	

Electrophoresis and determination of PCR product

Ten µl of amplified product was loaded on to 1% agarose gel in 1X TBE buffer and sample was run at 5 volts/cm for one hr. Gel was observed under ultraviolet light to visualize the bands. The size of amplified DNA was determined by comparing with standard molecular weight marker and were photographed by gel documentation system.

Purification of amplified products

Amplified products were analyzed on 1% agarose gel and eluted from gel using QIA quick gel extraction kit (Qiagen) as per the manufacturer's recommendations. Band of interest from gel was excised with a clean, sharp scalpel. Three volumes of buffer QG was added and mixture was incubated at 50 ºC for 10 min until the gel slice has been completely dissolved. DNA/agarose solution was applied to a minelute column assembled in 2 ml collection tube and centrifuged at 10,000 rpm for 10 minutes. Flow through was discarded and 750 µl of PE buffer was added to the column and centrifuged at 10,000 rpm for 10 min. Column was centrifuged for an additional one min at 13,000 rpm. Column was placed in 1.5 ml microcentrifuge tube and 30 µl of EB (10mM Tris-Cl, pH 8.5) was used for elution of DNA.

Result and Discussion

Revival of *P. multocida* serotype B:2 (P52) culture

Culture of *P. multocida* P52 was revived on Brain Heart Infusion (BHI) broth and blood agar. Characteristic non-haemolytic colonies were obtained which were gram-negative coccobacilli.

Pathogenicity test of the organism

Mice were inoculated intraperitoneally with 0.5ml of 10^{-5} dilution of 18hrs. old broth culture of *P. multocida* P52 and they were found dead within 24-48 hours. The re-isolated colonies showed characteristic of *P. multocida.*

Characterization of Culture

P. multocida produced small, smooth, circular, glistening and dew drop like colonies without haemolysis on blood agar (BA) plate on incubation at 37 ºC for 24 hrs. The colonies were found to be watery, discrete and translucent with a characteristics odour of the culture.

Morphological characterization

On microscopic examination of stained smears, the isolate was found to be Gram-negative cocco-bacilli, arranged singly or in pairs. The bipolar reaction exhibited by the isolates following methylene blue staining was an indication of *P. multocida*. No motility was observed in stab culture in semisolid agar at 37 °C.

Cultural characterization

There was no growth on MacConkey's lactose agar (MLA).

Biochemical characterization

Biochemical characterization revealed that *P. multocida* fermented glucose, fructose, galactose, maltose, mannitol and sucrose but salicin, raffinose, inositol and rhamnose were not fermented. Indole production along with catalase and oxidase production was also shown by the purified organism.

Rajkhowa *et al.* (2012) [16] and El-Jakee *et al.* (2016) [8] studied cultural, morphological and biochemical characteristics of *P. multocida*. The organism is Gram-negative short ovoid rod with bipolar staining characteristics, non-haemolytic, aerobic to facultative anaerobic and produces indole, oxidase, catalase, ferment carbohydrates with slight gas production and failed to grow on MacConkey agar. Similar type of studies were also conducted by Verma (1991) [24] and De Alwis (1996) [7]. Chawak *et al.* (2000) [3] reported the biochemical characterization of *P. multocida* of avian origin and they were uniform in fermentation of fructose, mannitol, sucrose and glucose. Yadav *et al.* (2016) [26] isolated *P. multocida* B:2 were from buffaloes and cattle. The isolates were found genetically distinct from standard *Pasteurella multocida* strain P52 (Vaccine strain of India).Two isolates shared same profile while all other isolates shown different profiles. This study provides a clear evidence of presence of more than one isolate in single outbreak of HS and also provide indication of high genetic variation among field isolates of *Pasteurella* sp. and may be the reason of vaccine failure and outbreaks. Likewise studies conducted by Jogi and Shakya (2013) [11], also revealed that the field strain is different from the vaccine strain which may be a possible reason for outbreaks.

Molecular Characterization

Isolation of genomic DNA of *P. multocida*

The genomic DNA of *P. multocida* P52 was isolated and its concentration was found to be 540 ng/µl. The A260: A280 ratio of the extracted DNA from overnight culture of *P. multocida* P52 was 1.8 indicating the high purity of DNA without RNA and protein contamination.

If there is protein or phenol contamination, O.D. 260/280 will be significantly less than 1.0 (Sambrook *et al.*, 1989) [18]. The yield of genomic DNA was 1.8 confirms the purity of isolated DNA sample. Agarose gel electrophoresis of the isolated DNA revealed that DNA was relatively intact and without RNA.

P. multocida serotype B:2 specific PCR assay

Pasteurella multocida-PCR assay using KTT 72 and KTSP 61 primers amplified a PCR product of 620 bp specific to *P. multocida* P52. Five µl of amplified product was analyzed by electrophoresis in 1% agarose gel stained with ethidium bromide. The size of amplicon was compared with DNA ladder and found to be of approximately 620 bp (Fig 01).

Kasten *et al*. (1997) [13] and Liu *et al*. (2004) [15] found that PCR is an alternative to bacteriological culture for identifying the species *P. multocida.* HS-specific and species-specific PCR analysis of *P. multocida* vaccinal strains were demonstrated useful in distinguishing hemorrhagic septicemia-causing type B strains. Waheedullah *et al.* (2009) [25] stated that PCR analysis carried out for HS causing strain conformation by using primer pairs KTT72 and KTSP61 showed that only HS causing strains were amplified. It was also observed that PCR amplification performed directly on bacterial colonies or cultures was an extremely rapid, sensitive method of *P. multocida* identification. The observations made by Kasten *et al*. (1997) [13] Liu *et al*. (2004) [15] and Waheedullah *et al*. (2009) [25] were totally in agreement with our results in amplification of *P. multocida* P52 vaccinal strain.

PCR amplification of outer membrane protein H (*ompH)* gene of *P. multocida* P52

The *ompH* gene of *P. multocida* P52 was amplified by PCR using specific forward OmpH-F and reverse OmpH-R primers in 25 µl reaction mixture. Amplification of the *ompH* gene of *P. multocida* P52 yielded the expected product of 942 bp in 1% agarose gel (Fig.02)

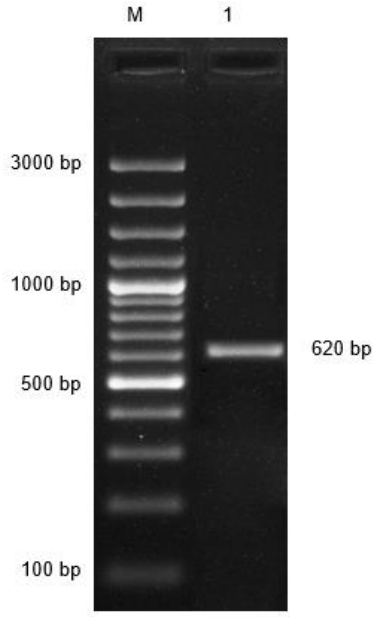

Fig 1: Serotype B:2 specific PCR of *P. multocida* (P52) Lane M : DNA marker 100bp Lane 1 : Amplified product at 620bp

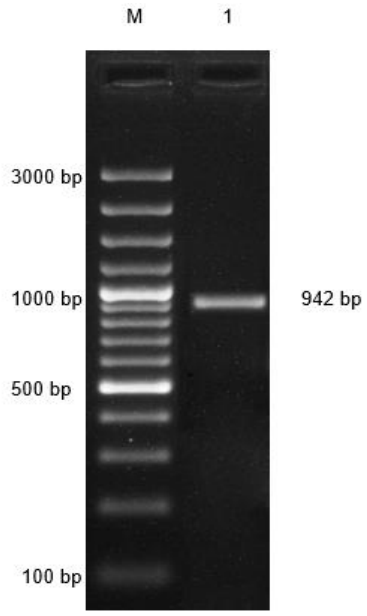

Fig 2: Amplification of *ompH* gene of *P. multocida* Lane M : DNA Ladder 100bp Lane 1 : Amplified product at 942bp

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