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Characterization and Different Antigenic Preparations of *P. multocida* Along with their Quantitative and Qualitative Analysis

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ABSTRACT

Pasteurella multocida is a causative agent of a number of economically important diseases in livestock. Due to the fatal nature of the disease, there is urgent need for rapid diagnosis so that appropriate therapeutic and preventive measures could be undertaken. A study was designed to extract different antigens of *P. multocida* capsular type A and B which included whole bacterium, Capsular antigen, Outer membrane proteins (OMPs) and Lipopolysaccharides (LPS). Bacteria were grown on brain heart infusion broth and capsular antigen was separated by fractional precipitation with addition of polar organic solvents yielding capsular polysaccharide. OMPs were extracted by ultracentrifugation of the supernatant obtained from bacterial broth by addition of HEPES Buffer containing Sodium Lauryl Sarcosinate detergent and the detergent insoluble OMP enriched fractions were obtained. Whole cell antigen was obtained by centrifugation of sonicated *P. multocida* suspended in HEPES buffer. LPS was extracted by formalinized saline killing of bacteria followed by ultracentrifugation. Total protein concentration was found to be 8.97 mg/ml and 5.67 mg/ml for OMP capsular type A & OMP type B respectively, while for WCL it was 22.38 mg/ml& 26.89 mg/ml. Carbohydrate estimation of capsular type A & type B of LPS and capsular polysaccharide concentrations were estimated to be 188.32 µg/ml, 330.71 µg/ml and 4.08 mg/ml, 2.38 mg/ml respectively. OMPs and whole cell lysate extracted were subjected to discontinuous SDS-PAGE. Nine polypeptides of MW ranging from 15-100 kDa from OMPs preparation & fifteen polypeptides of MW ranging from 25-98 kDa from whole cell lysate were visualised.

Keywords: Therapeutic, lipopolysacchrides, ultracentrifugation, formalinized, polypeptides

Haemorrhagic septicaemia (HS) is a peracute disease of cattle and buffalo, caused by specific serotypes of the bacterial species *P. multocida* (Dawkins *et al.,* 1990). The gram negative Bacterium *P. multocida* has been classified into five capsular serogroups A, B, D, E and F by (Rhoades and Rimler, 1987) and (Heddleston *et al*., 1972) gave 16 somatic serotypes. Haemorrhagic septicaemia (HS), is caused by two serotypes of *P. multocida*, *viz* Asian serotype B:2 and African serotype E:2 (Carter-Heddleston) corresponding to 6:B and 6:E (Namioka-Carter). The major factors encountered in the pathogenesis of *Pasteurella* are polysaccharide capsule, endotoxins or lipopolysaccharides (LPS), outer membrane proteins (OMPs), fimbriae and adhesions, exotoxins, extra cellular enzymes and other factors that are still need to be investigated and elucidated. Studies utilising polyacrylamide gel electrophoresis (PAGE) have successfully classified various bacterial species and studies by Johnson *et al*. (1991) of protein and lipopolysaccharide (LPS) antigens of *P. Multocida* isolates have indicated a correlation between electrophoretic patterns and serotypic properties, although no protein(s) unique to HS were identified.

Many biochemical and serological methods have been used to study *P. multocida* which includes catalase, indole,

Table 1: The oligonucleotide sequences of primer pairs used in Multiplex CAP- PCR

Primers	Sequence (5'-3')	Gene Amplified	Amplicon size
CapA-F	TGCCAAAATCGCAGTCAG	hyaD-hyaC	1,044
CapA-R	TTGCCATCATTGTCAGTG		
CapB-F	CATTTATCCAAGCTCCACC	bcbD	760
CapB-R	GCCCGAGAGTTTCAATCC		
CapD-F	TTACAAAAGAAAGACTAGGAGCCC	dcbF	657
CapD-R	CATCTACCCACTCAACCATATCAG		
CapE-F	TCCGCAGAAAATTATTGACTC	ecbJ	511
CapE-R	GCTTGCTGCTTGATTTTGTC		
CapF-F	AATCGGAGAACGCAGAAATCAG	fcbD	851
CapF-R	TTCCGCCGTCAATTACTCTG		

oxidase and sugar fermentation tests. Due to limitations of these methods, more accurate methods to investigate HS are being used. In the present study, *P. multocida* isolates were confirmed by various biochemical methods along with the determination of their capsular type and these isolates were used to prepare antigens like Whole cell lysate, OMPs, LPS, capsular polysaccharide which were characterized using SDS-PAGE.

MATERIALS AND METHODS

Selection of *P. multocida* isolates and their confirmation

A total of two isolates with identity number 559 and 5043 of *P. multocida* earlier recovered from bovine nasal swabs and maintained in the Department of Veterinary Microbiology were used for the study. The organisms were revived on 5% defibrinated sheep blood agar at 37ºC for 24 h and colonies of *P. multocida* were subjected to biochemical tests that included IMVIC tests, sugar fermentation tests, catalase and oxidase test as described by (Muhairwa *et al*., 2001).

P. multocida specific Polymerase chain reaction (PM-PCR)

DNA was extracted from the overnight grown culture in Brain Heart infusion broth (BHI) by phenol-chloroform-isoamyl alcohol method Wilson *et al.* (1987). *P. multocida* polymerase chain reaction (PM-PCR) was carried out using species specific primers KMT1SP6 and KMT 1T7 designed by (Townsend *et al*., 1998) to amplify KMT1gene. The thermal cycle protocol was followed as per the method of (Townsend *et al*., 1998). The analysis of PCR product was carriedout in 1.5 per cent agarose gel stained with ethidium bromide (0.5 µg/ml). 1 kb DNA ladder (Biogene, USA) and appropriate controls were incorporated to rule out false positive and false negative results. The gel was viewed under UV light in Gel Documentation System (Gel Doc).

Multiplex PCR assay for Capsular type identification of the isolates

Capsule type was determined by Multiplex CAP-PCR using primers set specific for serogroups A, B, D, E and F as described by (Townsend *et al*., 2001); the sequences of which are given in table 1. The PCR was done in a final reaction volume of 25 µl in GeneAmp PCR System 9700 (LABINDIA, INDIA). Quantity and concentration of the various components used for Multiplex CAP-PCR was as per table 2. Multiplex CAP-PCR was performed using PCR protocol described by (Townsend *et al*., 2001) with minor modifications. The PCR products so obtained were electrophoresed and gel was stained with ethidium bromide (1µg/ ml).

Preparation of different antigens of *P. multocida*

For the extraction of whole cell protein, culture was prepared by inoculating the isolates on 5 % BA incubating overnight at 37°C. About 2-3 representative colonies were

selected and inoculated in 10 ml of BHI broth overnight in a shaking incubator. 1 ml of above culture was transferred to 500 ml of BHI broth for incubation (37C) and Pure culture obtained was centrifuged, the pellet were then suspended in NSS overnight at 4ºC, washed twice with normal saline and finally the pellet was suspended in 10 mM HEPES buffer (pH 7.4) along with10 µl of protease inhibitor cocktail set 111 (Calbiochem). The cell suspension was then subjected to 20 cycles with 70 % amplitude of sonication (Sonics VibraCell) on an ice bath for 30s each with 30s interval between each cycle for cooling. The sonicated material was then centrifuged at 5,000 rpm for 30 minutes at 4ºC. Supernatant containing whole cell protein was collected.

Outer membrane proteins of *P. multocida* isolates were extracted as per the procedure described by (Choi and Kim, 1991; Tomar*et al.*, 2002) with minor modifications. Bacterial cultures grown in BHI broth overnight were centrifuged and washed twice with PBS which were then subjected to sonication for lysis of cells. Intact cells and debris were removed by centrifugation and the supernatant containing the cell membranes were collected. The cell membranes from the above supernatants were pelleted out by ultracentrifugation (Sorvall RC 90) at 1, 00,000 *g* for 60 minutes at 4°C and then suspended in HEPES buffer containing 2% sodium N-lauryl sarcosinate incubating for 1 hour at 22°C. The detergent insoluble outer membrane protein enriched fractions were harvested by ultracentrifugation for 60 minutes at 4°C. The pellets were re-suspended in PBS and stored at -20°C.

LPS antigen was prepared according to protocol described by Rebers *et al.* (1980) with slight modifications. Briefly, pure culture of *P. multocida* grown on 5% blood agar was inoculated on BHI Agar in 10 Roux bottles at 37ºC for 24 h. Then, the cells were washed off agar with 0.85 % sodium chloride solution containing 0.3 % formalin and the cell suspension obtained was stirred (170 rev/min) for 48 hours at 4˚C. The supernatant obtained after centrifugation was clarified by filtration through a 400-mesh nylon cloth and sequentially, filtrate was ultracentrifuged for 2 hours. Small gel-like pellets obtained were suspended in formalinized saline overnight at 4ºC. The suspension was again ultracentrifuged for 2 hours and repelleted three times by using formalinized saline. The remaining pellet was suspended in distilled water containing 0.1% formalin and 0.1% sodium acetate (pH 7.2) overnight at 4ºC for at least 3 days. The above cell suspension was centrifuged and slightly opalescent supernatant was dialysed for 3 days against distilled water served as antigen.

Capsular polysaccharide extraction was based on fractional precipitation from aqueous solution by addition of polar organic solvents as per as protocol used by (Penn and Nagy, 1976) so that least contamination with proteins would be detected. *P. multocida* of capsular type A and B were grown BA overnight and colonies obtained were cultured in BHI broth maintaining pH 7.2-7.4. Bacterial cells were harvested in NSS by centrifugation and the suspension obtained was heated at 60ºC for 1 hour and 0.2% (v/v) formalin was added.

Culture supernatant was obtained by centrifugation and then the Supernatant obtained was again centrifuged using Amicon Ultra-15 centrifugal filter devices (Merck Millipore Ltd.) to obtain concentrated culture supernatant to which 30 ml concentrated methanol and 0.4 gms of sodium acetate was added resulting in precipitation allowed to settle under gravity. Methanol supernatant obtained was filtered using Whatman 3 MM and Millipore 0.2 µm membrane, followed by addition of 60 ml concentrated acetone. The crude acetone precipitate (CAP) so formed were allowed to settle under gravity and re-suspended in water which were freeze dried giving partial purified crude capsular polysaccharide.

Crude acetone precipitate (CAP) was then ultracentrifuged by dissolving in saturated sodium acetate with 77 % phenol solution. Aqueous phase was dialysed against 0.1M $CaCl_2$ for 24 hours and pellet endotoxin was removed. Obtained supernatant was added ethanol and 1% saturated sodium acetate and again centrifuged that yielded Ethanol-purified precipitate which was dissolved in 0.85% saline served as antigen.

Estimation of protein and carbohydrate (polysaccharides) concentration

Protein concentration in OMP and whole cell lysate preparation was estimated by Quick start Bradford Protein Assay Kit (Biorad, USA). Standard curve was made with different dilution of Bovine serum albumin (2 mg/ml). The concentration of carbohydrates in LPS and Capsular antigens was estimated by phenol sulphuric acid method (Dubois *et al*., 1956) with glucose used as standard.

SDS PAGE of Antigens

Whole cell lysate and outer membrane protein (OMP) samples containing 15-20 µg/µl protein concentrations were mixed with equal volume of 2X sample buffer and then denatured by heating at 100ºC for 5 minutes in boiling water. Samples were then cooled and centrifuged at 10,000 rpm for 5 minutes.

SDS-PAGE was performed using the discontinuous buffer system (Sambrook and Russell, 2001). The electrophoresis was carried out with 12 per cent resolving gel (1.5M Tris-HCl with pH 8.8, 10% SDS, 30% acrylamide + bisacrylamide (Merk) and 5% stacking gel (0.5M Tris-HCl pH 6.8, 10% SDS, 30% acryl bis) were prepared and polymerized chemically by addition of N, N, N',N' tertamethylenediamine (Sigma) to both the gel solutions along with 10% (w/v) ammonium persulphate (APS) (Sigma).

A 20 µl of the prepared samples were loaded into each well along with known molecular weight marker (MBI, Fermentas). Electrophoresis was done at a constant electric current (30mA) for stacking and 45mA during destacking. Gel was run until bromophenol blue reached the bottom, which took approximately 4 hours.

Staining and destaining of Gel

At the completion of electrophoresis, the gels were put into a container with staining solution containing 0.25% w\v Coomassie brilliant blue (CBR) R-250 dissolved in 50% (w\v) methanol 10% (v\v)acetic acid (Merck, Germany) in double distilled water. Gels were left in the staining solution for overnight on slow shaking and destained in methanol, acetic acid, and water with shaking until the bands became visible above the background.

RESULTS AND DISCUSSIONS

Bacteriological and biochemical characterization of *P.multocida* isolates

The *P. multocida* isolates with Lab Id number 559, 5043 of bovine origin from nasal mucosa of animals, yielded round, grayish, shiny and non-haemolytic colonies on BA at 37°C for 24 hours along with characteristic sweetish odour. No growth was seen on Mac Conkey's Agar even after incubation for 48 hours at 37°C. Gram staining of the smears revealed Gram negative coccobacilli organisms. These findings are similar to the findings of (Bergey *et al*., 1923); (Topley and Wilson.,1929; Little and Lyon, 1943; Mayer, 1958 and Anupama *et al*., 2003). The isolates gave positive results for the biochemical test like catalase, oxidase, and nitrate reduction whereas these were negative for urease production, sulphur reduction, motility, Methyl red (MR) and Voges-Proskauer (VP) tests. On TSI agar slants, acidic reaction was observed as indicated by yellow slant and yellow butt.

Similar findings have also been reported by (Shigidi and Mustafa, 1979; Chandrasekaran *et al*., 1981; Verma, 1991; Kumar *et al*., 1996 and Javia, 2004) but (Waltman and Horne, 1993) found variability amongst pasteurellae for oxidase production.

The selected isolates fermented glucose and sucrose and coincides with earlier findings of (Dao *et al*., 1973; Shigidi and Mustafa, 1979; Verma, 1991; Chawak*et al*., 2000 and Javia, 2004). The isolates fermented sorbitol with the production of acid and variable in maltose fermentation while none of isolates fermented lactose and salicin in the studies conducted by (Butt *et al*., 2003) which simulate with what was observed in this study.

Molecular characterization of *P. multocida* isolates

PCR based confirmation of *P. multocida* isolates

The isolates of were found to be positive for PM-PCR for a product of 460 bp, which is specific for PM-PCR. The primer pair KMT1SP6 and KMT1T7 amplified the KMT1 gene fragment from *P. multocida* that was electrophoresed to approximately 460 base pairs (Fig. 1). These findings confirmed the results obtained by (Townsend *et al*., 1998; Lee *et al.,* 2000; Townsend *et al.,* 2000 and Dutta *et al.,* 2001), who reported approximately 460 bp amplified product from all *P. multocida* isolates. Dutta *et al.* (2001) carried out PM-PCR using various serotypes of *P. multocida*. On using mixed bacterial culture lysate containing *P. multocida* they got similar results as obtained in pure culture. So it is concluded that the other bacterial contamination does not affect specificity of PM-PCR based identification of *P. multocida* isolates.

CAP- PCR for capsular type identification

The multiplex capsular-PCR provides a rapid and highly specific alternative to conventional capsular serotyping. It is highly specific for strains genetically capable of producing a serogroup-specific capsule. Among the isolates identified as *P. multocida;* A and B capsular serogroups were found by capsular-PCR typing. The genomic DNA extracted by phenol-choloform-isoamyl method from bacterial isolates was subjected to CAP-PCR with the capsule-specific primers pairs (CAPA, CAPB, CAPD, CAPE, and CAPF) which were unique for each of 5 serogroups. Primers amplified *hyaD-hyaC, bcbD* genes which were electrophoresed at 1,044 bp and 760 bp (Plate 4.4). Thus, the isolates were typed as capsular type A and B.

Table 2: Quantity and concentration of various components used in Multiplex CAP- PCR

Sr. No.	Components	Quantity
1.	10 X PCR buffer (Biogene, USA)	2.5 µl
2.	MgC_{l2} (50mM) (MBIFermentas)	1.5µl
3.	dNTPs (25mM) (MBIFermentas)	1.0 µl
4.	CapA-F	1.25µl
5.	CapA-R	1.25 µl
6.	CapB-F	1.25 µl
7.	CapB-R	1.25 µl
8.	CapD-F	1.25 µl
9.	CapD-R	1.25 µl
10.	CapE-F	1.25 µl
11.	CapE-R	1.25 µl
12.	CapF-F	1.25 µl
13.	CapF-R	1.25 µl
14.	*Taq* DNA polymerase (1U/ µl) (Biogene, USA)	0.5 µl
15.	Template DNA	3.0 µl
16.	Distilled water	4.0 µl

These findings confirmed the results obtained by (Townsend *et al*., 2001; Zsuzsanna *et al*., 2007; Jabbari *et al*., 2006) who reported 1,044 and 760 bp amplified product for capsular type A and B of *P. multocida.*

Quantitative and qualitative analysis of antigens of *P. multocida*

Quantification of antigens

Protein concentrations of antigens was estimated by Bradford method while carbohydrates (polysaccharides) by phenol-sulphuric acid method and concentrations obtained have been shown in Table 3. The highest protein yield was obtained for WCL followed by OMP and capsular antigen. Bradford method can estimate as low as 10µg of protein present in a sample while phenol sulphuric acid estimates mainly polysaccharides in a given sample.

Table 3: Different concentrations of extracted antigens of *P. multocida*

Lab Id	Capsular type	WCL (mg/ml)	OMP (mg/ml)	LPS (µg/ml)	Capsular polysaccharide (mg/ml)
5043	A	22.38	8.97	188.32	4.08
559	B	26.89	5.62	330.71	2.38

Qualitative analysis of the prepared antigens

WCL and OMPs antigens were assessed by discontinuous SDS-PAGE. Samples were prepared by solublizing them at 100ºC for 5 minutes and were electrophoresed on 12% and 10% gel. Molecular weights of whole cell lysate and outer membrane proteins were analyzed and determined by using a high molecular weight standard marker having 7 protein bands from 6.5 kDa – 97.4 kDa. Whole cell lysate of both serotypes showed a range of bands between 25 kDa to 98 kDa. About 15 bands of molecular weight 74, 68, 65, 63, 60, 56, 54, 50, 49, 42, 36, 33, 24 and 21 kDa were observed in WCL. Outer membrane proteins showed 9 bands between range of 15 kDa to 100 kDa. The bands detected were 100, 81, 50, 48, 44, 40, 34, 26, 16 kDa. Out of these OMPs, major proteins with thick and intensely stained bands were with molecular weight 100kDa, 26kDa. In WCL, most intensely stained bands were 74kDa, 47kDa, 42kDa and 36kDa which were considered as major proteins.

Multiple intensely stained protein bands were observed in whole cell lysate but thicker and less numbers of bands were observed in purified OMPs preparation (Plate 3). Similar, work for *P. multocida* has been carried out by Wasnik (1998) who observed 13 protein fractions in OMP with molecular weight ranging from 16 to 93 kDa. Previous studies reported that pattern of OMPs on SDS-PAGE depends upon many factors including growth media, availability of iron and phase of growth at which

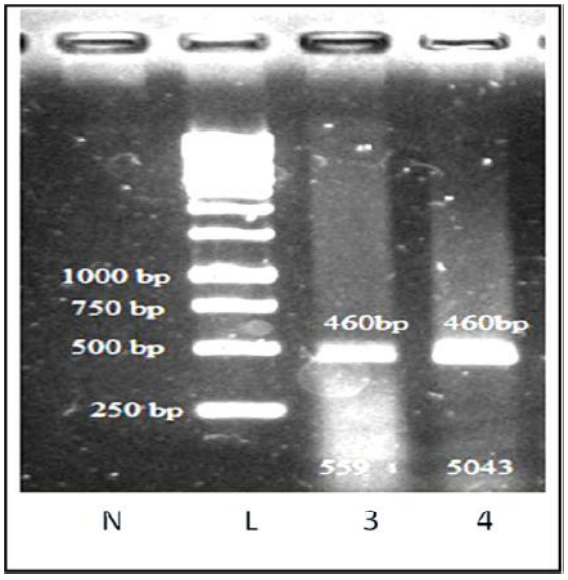

Fig. 1: PM-PCR- Lanes N-Negative control, L- Ladder (1kb), 3-4 *P. multocida* specific gene amplicon

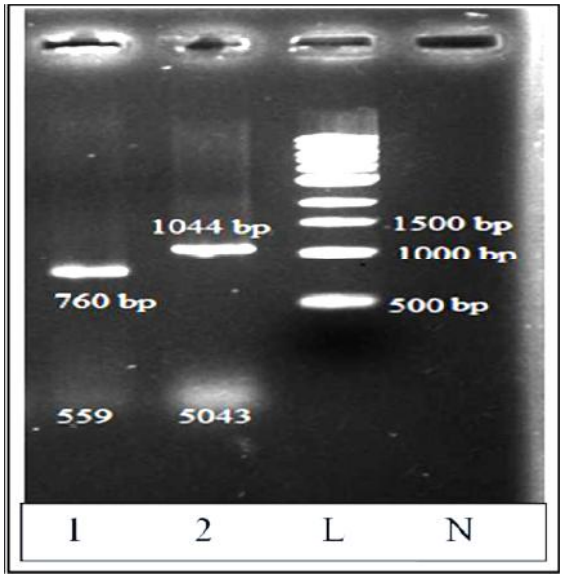

Fig. 2: CAP-PCR Lane1- capsular type B (559), Lane 2- capsular type A (5043), Lane L-Ladder (1kb), Lane N- Negative control

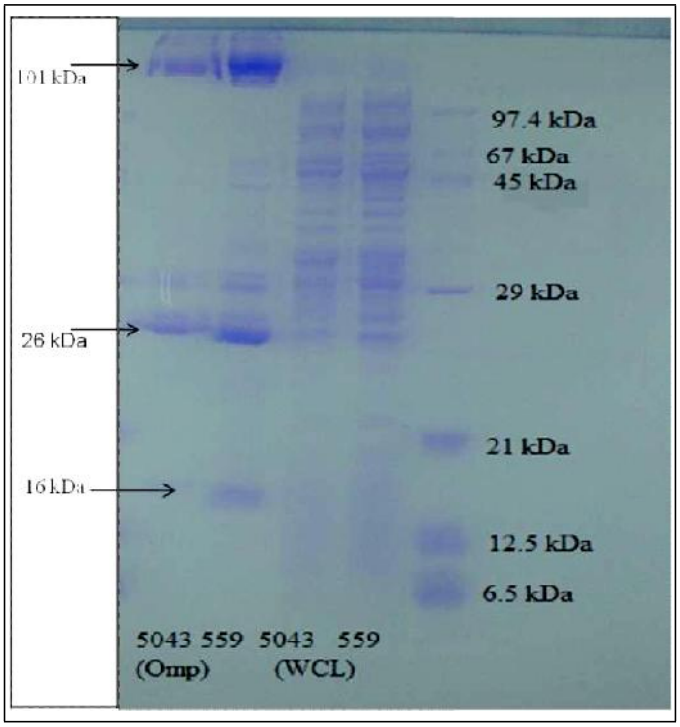

Fig. 3: SDS-PAGE. Lane 1- OMP capsular type A, Lane 2- OMP capsular type B, Lane 3- WCL capsular type A, Lane 4 – WCL capsular type B and Lane L- ladder

cultures had been harvested for OMPs preparation. The results from this experiment demonstrate that the protocol used for extraction of antigens from *P. multocida* worked optimally.

Knights *et al*. (1990) suggested that organisms at different *in vitro* passage level express proteins with different molecular weights. Many studies have been carried out on OMPs pattern of *P. multocida;* however workers were only able to reach a consensus on few membrane proteins. A significant variation has been observed in MW, number of polypeptides band and thickness of polypeptide bands for OMPs and WCL. Ullah *et al.* (2008) studied that the electrophoretic profiles of whole cells preparations appeared to be homogeneous, regardless of animal species from which they were originally obtained and observed about 12 protein bands in complex whole cell profile of *P. multocida*. Truscott and Hirsh (1988) demonstrated an outer membrane protein with anti-phagocytic activity from *P. multocida* of avian origin, and concluded that 50 kDa protein band was common in whole cell and OMPs of *P. multocida*.

CONCLUSION

A total of two *P. multocida* isolates of bovine origin having lab identification no. 5043 and 559 maintained in the Department were selected and grown on 5% defibrinated blood agar medium along with characterisation and confirmation by conventional bacteriological, biochemical methods as well as molecular methods. Growth on blood agar was observed without haemolysis and no growth was seen on Mac Conkey's Lactose agar (MLA) even after incubation for 48 hours at 37°C for both the isolates. The isolates gave positive results for the biochemical tests like catalase, oxidase and nitrate reduction, however, were negative for urease production, sulphur reduction, motility, MR and VP tests. In triple sugar iron agar slants, both the isolates produced acid slant and acid butt reaction. The isolates were then subjected to PM-PCR after DNA extraction and found positive as the primer pair KMT1SP6 and KMT1T7 amplified KMT1 gene fragment of *P. multocida* that was electrophoresed to approximately 460 bp. CAP-PCR of the isolates was done to identify capsular type using capsule specific primers pair and these primers amplified hyaD-hyaC, bcbD genes. Thus, the isolates were typed as *P. multocida* capsular type A (Lab Id 5043) and *P. multocida* capsular type B (Lab Id 559) which were electrophoresed at 1,044 bp and 760 bp.

Theseisolates were then used for the preparation of antigens which included WCL, OMPs, LPS, CPS. WCL was prepared by growing pure culture of isolates in BHI broth overnight and bacterial pellet was obtained by centrifugation and then suspended in HEPES buffer. This suspension was then subjected to sonication leading to bacterial cell lysis and thus whole cell proteins were obtained by collecting supernatant after centrifugation. OMPs were extracted by ultracentrifugation of the above collected supernatant and resuspending the pellet in N-lauryl sarcosinate in which the detergent insoluble OMPs enriched fractions were harvested. Capsular antigen was prepared by fractional precipitation with addition of polar organic solvents that yielded high molecular weight capsular polysaccharide. LPS was extracted by series of ultra-centrifugation steps of the bacterial suspension obtained from roux bottles in order to remove endotoxin. Then, these antigens were analysed qualitatively and quantitatively. The concentrations of the protein antigens (OMPs, WCL) were estimated by Bradford method and carbohydrate antigens (LPS, CPS) were estimated by phenol sulphuric acid method. The highest protein yield was obtained for WCL followed by OMPs, CPS and then LPS. Qualitative analysis of antigens was done by SDS-PAGE which showed reduction in multiple intensely stained bands for OMPs as compared to WCL meaning enrichment for OMPs. Fifteen polypeptide bands of molecular weight ranging from 25-98 kDa of WCL and about nine polypeptide bands of molecular weight ranging from 15-100 kDa of OMP were visualized.

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