

Development of OMP based indirect ELISA to gauge the antibody titers in bovines against *Pasteurella multocida*

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(Received 5 Mar 2015; revised version 27 Jun 2015; accepted 9 Aug 2015)

Summary

Pasteurella multocida (*P. multocida*) is an important pathogen of various domestic animals. The outer membrane proteins (OMPs) play a major role in pathogenesis and immunogenicity of *P. multocida*. The aim of the study was to develop indirect enzyme linked immuno sorbant assay (ELISA) based on OMPs to ascertain the antibody titers in animals post-infection or to gauge the potency of vaccine. The OMPs were extracted and purified from *P. multocida* P:52 (vaccine strain) and *P. multocida* B:2 isolated from natural outbreak of Haemorrhagic septicaemia (HS) and analyzed on SDS PAGE and through western blot. The OMPs profile of the vaccine strain and the isolate from the natural outbreak of HS were found to be similar. Optimization of various components viz. coating antigens, anti-species conjugate, etc. were carried out against both anti-*P. multocida* hyper immune and pre immune serum. Validation of OMP based indirect ELISA assay to measure immune response against *P. multocida* in bovine revealed 91% diagnostic sensitivity (DSN) and about 100% diagnostic specificity (DSP) at 25% cut off. OMP based indirect ELISA was found to be more specific, but less sensitive as compared to WCL based assay.

Key words: Diagnostic sensitivity, Diagnostic specificity, Indirect ELISA, Outer membrane proteins, *Pasteurella multocida*

Introduction

Pasteurellosis is an important infectious disease, especially considering its economic impact in South Asian countries including India. It is a gram-negative, facultative commensal of the upper respiratory tract of many animal species. However, under predisposing circumstances the organism is the etiological agent of a wide range of economically important diseases, including fowl cholera in poultry, Haemorrhagic septicaemia (HS) in cattle and buffalo, atrophic rhinitis in swine and snuffles in rabbits. The organism is also known to be the causative agent of pasteurellosis in American bison, yak, deer, elephants, camels, horses, elk and other wild animals (De Alwis, 1996). The pathogen consists of five capsular types A, B, D, E, and F and possible relationship exists between the capsular type and disease predilection (Boyce and Adler, 2001). HS, an acute septicaemic disease principally affecting cattle and buffalo 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). HS is considered an economically important disease in South East Asia (Verma and Jaiswal, 1998) including Indonesia, the Philippines, Thailand, Malaysia, the Middle East and Central and South Africa (Bain *et al.*, 1982). The disease has also been reported in American bison in 1967 and dairy cattle in 1969 in the

USA (Carter, 1982).

Among the major factors encountered in the pathogenesis of *Pasteurella* are the polysaccharide capsule, endotoxins or lipopolysaccharides (LPS), outer membrane proteins (OMPs), fimbria and adhesin, exotoxins, extra cellular enzymes and other factors that are still to be investigated and elucidated. The OMPs fulfill a number of roles that are critical for the bacterial cells, such as nutrient uptake, transport of molecules in and out of cell and interaction with the environment and host tissue (Hatfaludi *et al.*, 2010). The OMPs of gram-negative bacteria have a role in disease processes as it acts at an interface between the host and pathogen (Lin *et al.*, 2002). Thus OMP variation among the isolates may help in epidemiological survey by assessing their inter strain heterogeneity and can be used to assess intra species diversity (Davies *et al.*, 2003). OMPs of *P. multocida* play a significant role in the pathogenesis of pasteurellosis (Lubke *et al.*, 1994; Srivastava, 1998) and have been identified as potent immunogens (Lu *et al.*, 1991). The immunogenicity of selected OMPs of *P. multocida* was demonstrated in rabbits (Confer *et al.*, 2001), calves (Dabo *et al.*, 1997) and chickens (Zhang *et al.*, 1994). The protective role of OMPs against HS was reported by (Pati *et al.*, 1996). Various serological tests such as tube agglutination test, indirect hemagglutination, dot immunoblotting and enzyme linked immuno sorbant assay (ELISA) have been used to quantify

antibodies against *P. multocida*, employing an array of antigens such as OMP, whole-cell sonicates, formalin killed bacterial extracts, etc. There have been contradictory reports regarding the efficiency of antigens including their solubility, binding efficiency to solids matrices and homogeneity.

In the present study OMP based indirect ELISA has been developed to investigate the immune status of the bovine by assessing the humoral antibodies specific to *P. multocida* B:2 and follow the course of infection as well as to gauge the efficiency of vaccination.

Materials and Methods

Pasteurella multocida strains

Lyophilized standard strain of *P. multocida* P:52 was procured from Indian Veterinary Research Institute, Izatnagar, Bareilly, India. The culture was revived by dissolving it in brain heart infusion (BHI) broth (Merck, Germany) and then inoculating on the 5% sheep blood agar (BA) (Hi-Media, Mumbai) and incubating at 37°C for 24 h. *Pasteurella multocida* B:2 was also isolated from a natural outbreak of HS in buffaloes. Isolation was done by culturing blood sample on 5% BA and on MacConkey Lactose Agar (MLA). For confirmation of the isolates *P. multocida* specific polymerase chain reaction (PCR) method was employed (Townsend *et al.*, 2001).

Extraction of outer membrane proteins (OMPs)

Outer membrane proteins from *P. multocida* B:2 strain were extracted (Choi-Kim *et al.*, 1991; Tomer *et al.*, 2002). Pure culture of *P. multocida* P:52 was grown in 250 ml of BHI in one liter flask by incubating it overnight in shaking incubator at 200 rpm at 37°C. The culture was centrifuged at 5,000 rpm for 20 min at 4°C. The supernatant was discarded and the pellet was used for extraction of OMPs. The culture pellets were obtained by centrifugation at 5,000 rpm for 20 min and washed twice in PBS and then resuspended in 10 mM HEPES buffer (pH = 7.4) (Sigma Life science, USA). 10 µL of protease inhibitor cocktail set 111 (Calbiochem, Germany) was added to mixture. The cell suspension was placed on ice then subjected to 25 cycles of each cycle of 30 s with 70% amplitude of sonication (Sonics Vibra Cell), with 30 s interval for cooling between each cycle.

Whole cells and debris were pelleted out by centrifugation in “3K15 centrifuge” (Sigma, USA) at 5,000 rpm for 20 min at 4°C and the supernatants containing the cell membranes were collected. The cell membranes from the above supernatants were pelleted out by ultra-centrifugation (Sorvall RC 90) at 100,000 g for 60 min at 4°C. The pellets were suspended in HEPES buffer containing 2% sodium N-lauryl sarcosinate (Fluka Analytical, Switzerland) and incubated for 1 h at 22°C. The “detergent insoluble OMPs enriched fractions” were pelleted out by ultracentrifugation at 100,000 g for 60 min at 4°C. The pellets were resuspended in PBS and stored at -20°C.

Protein concentration in OMP preparation was measured with Quick Start Bradford Protein Assay kit (Biorad, USA) in a microtiter plate. Standard curve was prepared with different dilution of bovine serum albumin (2 mg/ml) provided in kit. 5 µL of protein dilution or test sample and 250 µL of Bradford reagent were added, mixed and allowed to develop color. Optical density (OD) of color developed was measured at 595 nm wave length using microtitre plate reader (Thermolab System, UK) within 1 h of development of color. The concentration was determined by standard curve.

SDS-PAGE and western blot analysis of OMPs of *P. multocida*

SDS-PAGE was performed according to Sambrook and Russel (2001), using the discontinuous buffer system. The electrophoresis was carried out with 10% and 12% resolving gel. Protein samples were mixed with equal volume of 2 X sample buffer, vortexed and denatured by heating at 100°C for 5 min or at 60°C for 10 min to find out heat modifiable nature of OMPs. Protein sample was then cooled and centrifuged at 10,000 rpm for 5 min. Gel was stained with coomassie brilliant blue (CBR-250V) stain with shaking for 1 h and then destained for 2 h using destaining solution. Western blot analysis was performed according to the procedure explained by Hudson and Hay (1991) with suitable modifications. OMPs were first separated on 12% resolving gel by SDS-PAGE. Then separated proteins from the gel were transferred onto 0.45 µm nitrocellulose membrane sheet (Whatman) by using Hoefer miniVE electrotransfer unit. For western blot study polyclonal hyper immune sera raised in rabbits was used. This was done to assess the immunogenicity of OMPs.

Development of an immunoassay to measure immune response against *Pasteurella multocida*

Two types of antigen comprised of outer membrane proteins of *P. multocida* and whole cell lysate were used. The antiserum raised in calf against *P. multocida* P:52 was procured from Division of Bacteriology, IVRI, Izatnagar, Bareilly (UP), India. Antiserum was checked for its positivity by agar gel precipitation test (AGPT) using OMPs and whole cell lysate (WCL) as antigen.

Standardization of indirect ELISA assay

For indirect ELISA, titration of different reagents was done using Checker Board Titration (CBT) as per Crowther (2001) with minor modifications.

Validation of indirect ELISA to measure immune response against *P. multocida* in bovine

Validation of the indirect ELISA was carried out to determine the appropriate cut off point that would serve as threshold between positive and negative samples. For this, 120 bovine serum samples from cattle that had previously been vaccinated (VS) with low saponified HS vaccine were used. In addition to these, 36 serum samples were also collected from calves with no history

of vaccination against HS or occurrence of HS like disease in them as well as in their dams. These were designated as non-diseased and non-vaccinated (NDNV) animals. These NDNV animals were also ruled out for the presence of *P. multocida* by attempting isolation of the bacterium from nasal swabs on 5% sheep Blood Agar (BA) and also using PM-PCR on these nasal swabs (Townsend *et al.*, 2001). The serum samples collected were analyzed with indirect ELISA by using both antigens (OMPs and WCL) simultaneously as per method of Takada-Iwao *et al.* (2007) with some modifications. Briefly, wells of microtitre plates were coated overnight at 4°C with optimized concentration of respective antigens (OMPs and WCL) in 100 µL coating buffer. Appropriate samples like test sera, positive and negative control were included. After incubation, wells were emptied and washed with 200 µL PBST 0.05% by keeping washing buffer for 5 min in wells. This process was repeated three times. All wells of both plates were loaded with 100 µL of blocking buffer (3% skimmed milk in PBS). Plates were incubated at 37°C for 2 h. Wells were washed thrice with PBST 0.05%. All microtitre plates included positive control (calf polyclonal anti *P. multocida* P:52 serum (CPPS)), negative control (NDNVS) and test sera. 100 µL of each serum sample diluted at 1:400 with (PBS + 1% skimmed milk) was added in duplicate wells. All 156 test sera samples were analyzed simultaneously with OMPs and WCL. Plates were incubated at 37°C for 2 h. Plates were washed thrice with PBST. 100 µL of rabbit anti-bovine IgG ALP conjugate in 1:2000 (diluted in PBS) dilution was added to each well of both plates. Plates were incubated at 37°C for 2 h. Plates were washed thrice with PBST 0.05%. Substrate used in this study was pNPP and prepared by adding 1 mg per ml of carbonate-bi-carbonate buffer (pH = 9.6). 100 µL of substrate was added to each well. Then plates were incubated at room temperature in dark for 30 min. Reaction was stopped by adding 50 µL of 3M NaOH solution. Absorbance of color developed was measured at 405 nm wave length using microtitre plate reader of (ThermoLab System, UK). At the same time, 76 sera samples were tested by using their different dilutions.

Data processing

Intensity of color development was measured in the form of optical density at 405 nm wave length using microtitre plate reader. OD values so obtained were converted into percent positivity (PP) using formula:

$$PP = (\text{OD value of test sera} / \text{OD value of positive control}) \times 100$$

These PP-values were then used to calculate the DSN and DSP of indirect ELISA.

DSN = Positive cases among vaccinated serum samples (TP)/total number of serum samples tested from vaccinated animals

Diagnostic specificity was calculated by testing serum samples from NDNV animals.

DSP = Negative cases among (NDNV) serum samples/total No.

of NDNV serum samples tested

DSN and DSP for indirect ELISA were calculated at 18, 20, 25% cutoff values. Three cut off points of 18%, 20% and 25% based on PP were selected to differentiate between positive and negative serum. These cut off values were selected according to Crowther (2001) with some modifications.

The overall efficiency (Ef) of a diagnostic test, defined as the percentage of cases correctly classified as diseased or healthy, is estimated using the following relationship:

$$\text{Percentage Ef} = \frac{\text{True positive samples} + \text{true negative samples}}{\text{total number of samples tested}} \times 100$$

Results

Isolation and confirmation of *P. multocida*

Pasteurella multocida P:52 isolate procured from IVRI, Izatnagar and *P. multocida* recovered from heart blood from a natural outbreak of HS were confirmed by culturing them on 5% BA and MLA. On BA, round, grayish, shiny and non-hemolytic colonies were observed; whereas no growth was observed on MLA. *Pasteurella multocida* P:52 and *P. multocida* isolated from natural outbreak of HS were confirmed by *P. multocida* specific PCR and produced 460 bp amplicons.

Extraction and purification of OMPs

OMPs were extracted from *P. multocida* P:52 (standard strain) and *P. multocida* B:2 strains from an outbreak of HS by sarcosyl method and upon quantification were consistently found to be around 6 mg/L of 18 h old culture.

SDS PAGE of OMPs

Extracted OMPs were analyzed by SDS PAGE. Samples were prepared by solubilizing them at different temperatures, i.e. 60°C (Fig. 1) and 100°C. OMPs solubilized at 100°C were electrophoresed on SDS PAGE 10% and 12% (Figs. 2 and 3).

The OMPs preparation solubilized at 60°C (Fig. 1) exhibited a total of nine protein bands clearly observed with approximate molecular weight of 16, 20, 30, 34, 37, 41, 51, 55, 87 kDa. Out of these OMPs, major proteins with thick and intensely stained bands were of molecular weight of 16 kDa, 30 kDa and 41 kDa. The SDS profile of OMPs solubilized at 100°C run on 10% and 12% (Figs. 2 and 3) revealed major OMPs of 34 kDa and 37.5 kDa along with minor OMPs of approximate molecular weight of 16, 25, 30 and 52 kDa. The OMP pattern was also compared with WCL (Fig. 3). Multiple intensely stained bands ranging from 24 kDa to 50 kDa in case of WCL were observed. Protein bands with less intensity included 16, 26, 32 and 37 kDa.

Isolation and PM-PCR for detection of *P. multocida* from NDNV animals

The nasal swabs which were streaked on sheep BA did not yield any *P. multocida* isolate and on employing

the PM-PCR, none of the samples showed *P. multocida* specific amplicons.

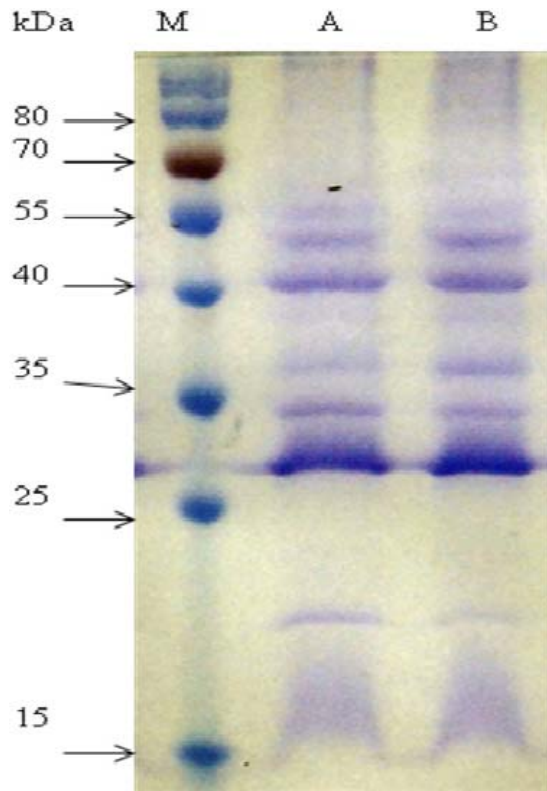


Fig. 1: SDS PAGE analysis of OMPs solublized at 60°C. M: Protein marker. Lane A: OMPs of *P. multocida* P:52, and Lane B: OMPs of *P. multocida* from outbreak of HS

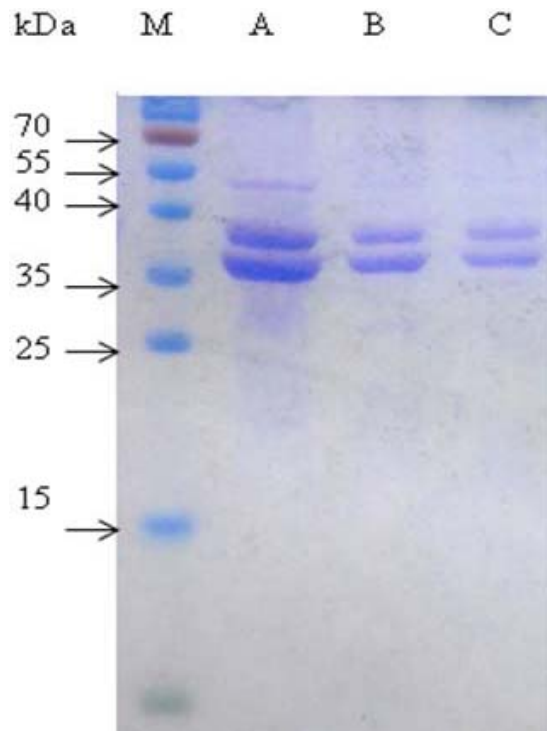


Fig. 2: SDS PAGE (10%) analysis of OMPs solublized at 100°C. M: Protein marker, Lane A: OMPs from *P. multocida* P:52, and Lanes B and C: OMPs of *P. multocida* from outbreak of HS

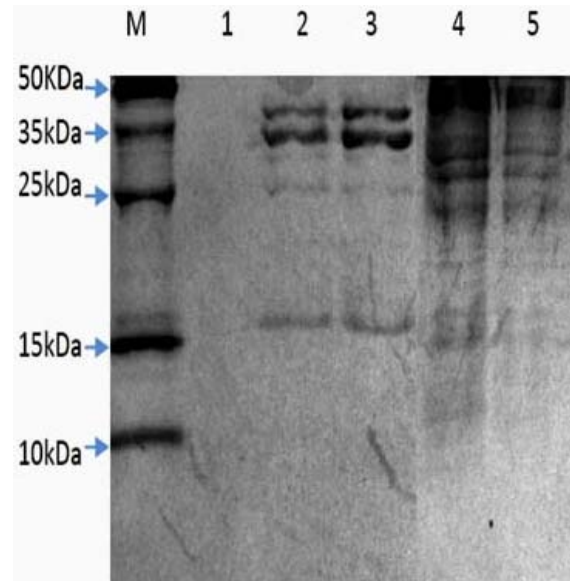


Fig. 3: SDS PAGE (12%) analysis of OMPs and WCL solublized at 100°C. M: Protein marker, Lane 1: Blank, Lanes 2 and 3: OMPs of *P. multocida* P:52 and *P. multocida* field isolate, and Lanes 4 and 5: WCL of *P. multocida* P:52 and *P. multocida* isolated from outbreak of HS

Demonstration of immunodominant OMPs

The most antigenic component in both isolates appeared with approximate MW of 37.5 kDa when samples were solublized at 100°C (Fig. 4). On the other hand, when samples were prepared at 60°C, three major immunodominant polypeptide bands with approximate MW of 18, 30 and 42 kDa along with three minor immunodominant polypeptide bands with MW of 51, 38 and 35 kDa were observed (Fig. 5).

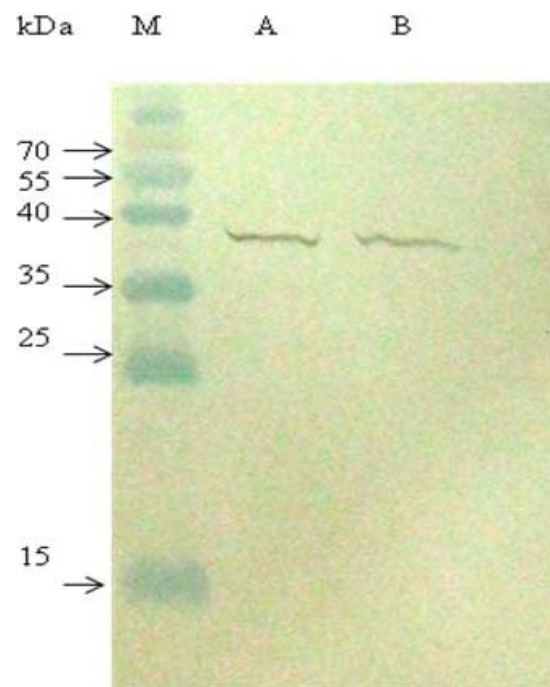


Fig. 4: Western blot analysis of OMPs solublized at 100°C. M: Protein marker, Lane A: OMPs of *P. multocida* P:52, and Lane B: OMPs of *P. multocida* from outbreak of HS

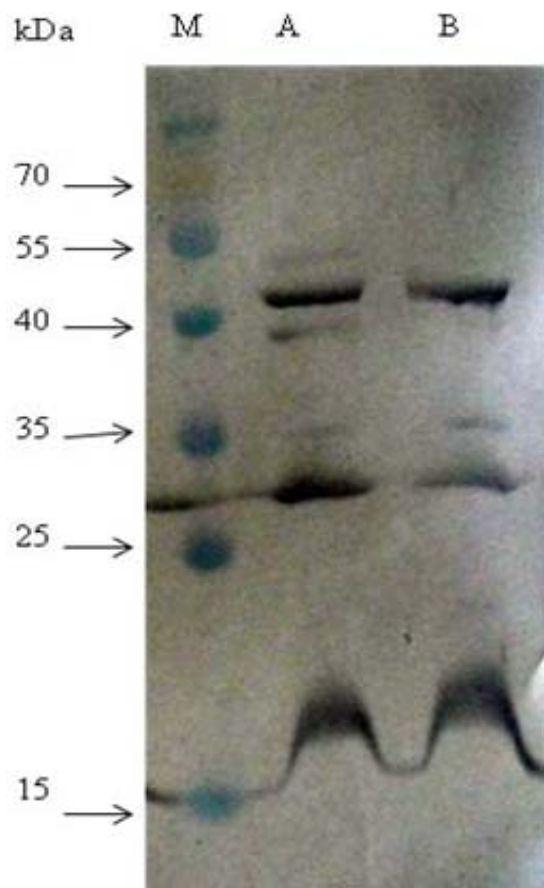


Fig. 5: Western blot analysis of OMPs solubilised at 60°C. M: Protein marker. Lane A: OMPs of *P. multocida* P:52, and Lane B: OMPs of *P. multocida* from outbreak of HS

Standardization of indirect ELISA for detection of *P. multocida* antibodies in bovine

Titration of antigen (OMPs and WCL) with calf polyclonal anti *P. multocida* P:52 serum (CPPS) and non-diseased non vaccinated calf serum (NDNVS) suggested that OMP concentration of 78 to 156 ng was enough to bind the whole surface of well without much wastage. Thus 100 ng OMPs was taken as a concentration to be used in indirect ELISA. For WCL as antigen, 400 ng per well was found enough to bind whole

surface of well.

The rabbit anti-bovine IgG conjugated with ALP was optimized at dilution 1:1600 in the assay as this dilution gave acceptable titration curve against a range of different serum dilutions.

The OD values were converted to percent positivity (PP) based on the OD value of positive control. These PP-values were used to calculate DSN and DSP of indirect ELISA.

Validation of indirect ELISA for detection of antibodies against *P. multocida* in bovine

Based on the PP-values three cut off values were selected to differentiate between positive and negative serum as per Crowther (2001) with some modifications. The DSN, DSP and overall Ef of indirect ELISA using OMPs and WCL as an antigen are depicted in Table 1.

Discussion

The study was aimed to develop an indirect ELISA based on OMPs, with the help of those antibodies against *P. multocida* that could be detected in bovines in a variety of situations viz. after natural or post-challenge infection or to gauge the efficiency of vaccine; and to screen the carrier animals that may act as a potential source of spread of infection to susceptible animals. Isolation of the microorganism is considered a gold standard for disease diagnosis and the same holds true even for diseases caused by *P. multocida*, however, its association with nasal tracts of healthy animals makes microbial isolation inconspicuous from a diagnosis standpoint. *Pasteurella multocida* could inhabit its niche without mounting any immune response except during infection when the animal is either under stress or immunosuppressed. The antibodies would also be generated post-vaccination. *Pasteurella multocida* infection is usually acute or peracute in nature leading to death within hours of infection. In some cases, however, animals survive the infection and become healthy. During the course of infection or at post-mortem, isolation of *P. multocida* in pure culture from systemic organs, blood and nasal secretions is considered as

Table 1: Sensitivity (DSN), specificity (DSP) and overall efficiency (Ef) of indirect ELISA using OMPs and WCL as antigens

Cut-off %	Antigen	NVNDS #	VA *	Sensitivity % (DSN)	Specificity % (DSP)	Efficiency % (Ef)
18	OMP	32(-) 4(+)	114(+) 6(-)	95	88.8	93.5
	WCL	26(-) 10(+)	116(+) 4(-)	96.6	72.7	92.3
20	OMP	32(-) 4(+)	112(+) 8(-)	93.3	88.8	92.3
	WCL	30(-) 6(+)	114(+) 6(-)	95	83.3	92.3
25	OMP	36(-) 0(+)	110(+) 10(-)	91.6	100	93.5
	WCL	34(-) 2(+)	112(+) 8(-)	93.3	94.4	93.5

Non diseased non vaccinated animals, and * Vaccinated animals. Also (-) and (+) indicates negative and positive results in ELISA

confirmatory. Given these facts, the purpose of indirect ELISA was therefore *per se* not for the diagnosis of *P. multocida* infection but only to monitor antibodies under various conditions as mentioned elsewhere in this manuscript.

Regarding the development of OMP based immune assay, extracts of OMPs from *P. multocida* P:52 and *P. multocida* serotype B:2, isolated from a natural outbreak of HS in buffalo were compared to see whether there were any differences in OMPs profile of standard vaccine strain and local pathogenic isolate. This was done so as to make an informed decision on the use of OMPs as a coating antigen in indirect ELISA.

The OMPs profile of *P. multocida* P:52 strain and *P. multocida* serotype B:2 isolated from the natural outbreak of HS was studied simultaneously and no apparent differences in two isolates were observed. Wasnik (1998) measuring the similar attributes had concluded that OMPs of *P. multocida* (B:2) strain P:52 exhibited 13 protein fractions with MW ranging from 16 to 93 kDa. The author also reported the heat modifiable nature of OMPs which was also evidenced in our observations. However, previous studies have reported the apparent modifications may be due to many factors including growth media, availability of iron and phase of growth.

In present study, five OMPs with approximate MW of 16, 30, 34, 37.5 and 41 kDa were considered to be major OMPs based on the intensity of stain and band thickness. Among these, 34 kDa and 37 kDa seem to correspond with the work published by Pati *et al.* (1996) who concluded 30 kDa and 37 kDa sized proteins to be two predominant OMPs of strain P:52. Our analysis also align to a study by Tomer *et al.* (2002) who considered three OMPs with MW of 31, 33 and 38 kDa of P:52 strain to be major OMPs.

Using antiserum raised in rabbit against *P. multocida* P:52 strain it was revealed that the immunogenicity produced by protein fractions of both *P. multocida* P:52 (standard strain) and *P. multocida* B:2 strain at sizes of 37.5 kDa when electrophoresed at 100°C and 18, 30, 42, 51, 38 and 35 at 60°C with 18, 30 and 42 were major immunogens. Pati *et al.* (1996), using rabbit anti *P. multocida* serum have previously suggested that proteins with MW of 44, 37 and 30 kDa were the major immunogen in OMPs derived from P:52, a vaccine strain of *P. multocida* B:2. Studies of Tomer *et al.* (2002) have also established OMP of 37 kDa as a major immunodominant protein in isolates from HS. These workers further suggested that the 37 kDa OMP could be diagnostically useful as antigen in enzyme immunoassay for serological detection of *P. multocida* infection. Sthitmatee *et al.* (2013) has highlighted the prospect of a 39 kDa (OMP-H) protein of *P. multocida* strain P-1059 to be used in cross protection. Similar studies conducted by Kumar *et al.* (2013) have established the cross-protective nature of a 87 kDa OMP of *P. multocida* serogroup B:2 strain P52 which produced protection against B:2 and A:1. Our study also corroborates the finding of the study by Saeed *et al.* (2009) who reported

OMP of MW 16, 26, 30, 37 and 50 kDa were recognized by hyper-immune calf sera, among these, proteins of 37 and 50 kDa were the major immunodominant OMPs.

The diagnostic sensitivity of indirect ELISA decreased as the cut-off point to differentiate between positive and negative sera samples was increased when using OMPs and WCL (Table 1) as antigens. However, diagnostic specificity increased as the cut off value was increased although there was not much variation in overall efficiency of the test. In this assay, 91.6% diagnostic sensitivity and approximately 100% diagnostic specificity and 93% overall efficiency was obtained at PP cutoff of 25%.

The comparison of OMP based indirect ELISA with WCL based indirect ELISA on the basis of DSN and DSP was done. It was observed that DSN of both tests was comparable although WCL assay was found slightly more sensitive than OMPs assay. This may be because of the presence of a number of cross reactive antigens including LPS and non specific proteins present in WCL preparation that may have reacted with non specific antibodies present in sera samples tested. The important aspect that is yet to be accomplished about indirect ELISA is to test the sera samples against related OMP based antigen of related pathogens like *Haemophilus* in particular and *E. coli*, *Shigella* in general to check for any cross reactivity and specificity. It is important as OmpA of *P. multocida* P:52 has 65% identity with OmpA of *Haemophilus influenzae* (Swissprot: P38368.1), 47% with OmpA of *E. coli* (Swissprot: P0A9111) and 45% with OmpA of *Shigella dysenteriae* (Swissprot: Q8Z750.4) at amino acid level. Not a complete gene but a specific portion of *ompA* gene can be cloned and expressed so that recombinant protein would act as specific antigen for development of diagnostic assay.

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