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Comparative immunogenicity of outer membrane vesicles (OMVs) and outer membrane proteins (OMPs) of *Pasteurella multocida* capsular type a of pig

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Abstract
Swine Pasteurellosis has a great economic impact on pig husbandry. However, no commercial vaccine is available against swine pasteurellosis in India. An investigation was carried out to study the immunogenic potential of OMVs and OMPs of a pathogenic pig strain of *Pasteurella multocida* type A. The extracted OMVs revealed 12 distinct protein bands, while the OMPs of the same isolate exhibited 16 distinct bands. Nine of them were shared by both the fractions. The immunogenic potential of both the fractions revealed a significantly higher antibody response, conferred by OMV fraction than OMPs, throughout the entire period of immunization trial.

Keywords: *Pasteurella multocida*, outer membrane vesicles, outer membrane proteins, immunization, mice

Introduction
Swine pasteurellosis, caused by *Pasteurella multocida* is one of the important diseases of pig, causing heavy economic loss to the farmers. Among the capsular serogroups of *P. multocida*, type D of *P. multocida* is most commonly associated with progressive atrophic rhinitis (Eamens *et al*., 1988; Lariviere *et al*., 1992) [1, 2], while the pneumonic form is caused by both capsular types A and D (Djordjenic *et al*., 1998) [3]. The pathogenesis associated with this organism merges in terms of complex interaction of host specific factors and bacterial virulence factors like lipopolysaccharides (LPS), capsule, outer membrane proteins (OMPs), toxins, siderophores, sialidases, capsule *etc*. (Harper *et al.*, 2006; Fuller *et al.*, 2000) [4, 5].
Vaccination has emerged as the most attractive and effective approach in controlling swine pasteurellosis. Many works are being carried out to identify the most immunogenic component of *P. multocida*, in respect to the LPS-protein complex (Tsuji and Matsumoto, 1988) [6], purified lipopolysaccharide (Rhoades and Rimler, 1991) [7] and outer membrane proteins (Jain *et al.*, 2005) [8]. In spite of that, no suitable vaccine is yet to be commercially available against swine pasteurellosis.
Recently, the immunogenic and protective properties of Outer Membrane Vesicles (OMVs) have been widely tested and proven for several Gram-negative human pathogens. Being a naturally released antigen delivery vehicles, the OMVs are the heterogeneous mixture of surface antigens, including outer membrane proteins, periplasmic proteins, phospholipids and the lipopolysaccharide (LPS). The OMVs extracted from *Mannheimia haemolytica* and *P. multocida* was proved to have an immunogenic potential (Roier *et al*., 2013) [9].
Considering the heterogeneous immunogenic components, an attempt was made to study the immunogenic potential of outer membrane vesicles (OMVs) and its comparison with outer membrane proteins (OMPs), extracted from a pathogenic field isolate of *P. multocida* type A in mice model.

Materials and Method
***Pasteurella multocida* Strains:** A most pathogenic strain of *P. multocida* capsular type A was selected on the basis of mice pathogenicity trial, from a repository of 15 previously isolated field strains of swine pasteurellosis.

Extraction of outer membrane vesicles (OMVs) of *P. multocida*

The Outer Membrane Vesicles (OMVs) from the selected pathogenic strain of *P. multocida* capsular type A of pig were prepared as per the method described by Roier *et al.* (2012) [10], with slight modification in respect to the growth factors. Briefly, few pure colonies of *P. multocida* capsular type A were grown for overnight in brain heart infusion(BHI) broth (containing 0.5 % yeast extract). The broth culture was harvested by centrifugation at 5,000 x g for 10 min at 4ºC. The cell pellets were transferred to 500 ml BHI broth with 0.5 per cent yeast extract and grown for 13 hours at 37ºC in shaking water bath (120 rpm). Bacterial cells were pelleted by initial centrifugation at 6,400 x g for 10 min and the supernatant was further centrifuged at 16,000 x g for 6 min at 4ºC. The final supernatant was consecutively filtered through 0.45 µm and 0.2 µm pore size filters to ensure complete removal of bacterial cells, if any. The filtrate was finally subjected to ultracentrifugation at 1, 44,000 x g for 4 hours at 4ºC and the pellets were collected as OMVs. The OMVs pellet was re-suspended in 5 ml of PBS (pH 7.4) and stored at -20ºC for future use.

Extraction of outer membrane proteins (OMPs) of *P. multocida*

The Outer Membrane Proteins (OMPs) were prepared by the protocol, previously described by Choi-Kim *et al.* (1991) [11] with slight modification as suggested by Kharb and Charan (2010) [12]. The selected *P. multocida* strain was grown in BHI broth (with 0.5% yeast extract) with iron restricted environment (150 µM 2, 2-Bipyridyl) at 37ºC overnight incubation with constant shaking. The growth was centrifuged at 5,000 x g at 4°C for 20 min and the pellets were carefully removed. The pellets were washed thrice with Phosphate Buffered Saline (PBS) solution and resuspended in 10 mM HEPES buffer (pH 7.4). The suspension was disrupted in a sonicator (Sonics, USA). Intact cells and large debris were removed by centrifugation at 5,000 x g at 4°C for 20 min. The supernatant was exposed to ultracentrifugation at 1,00,000 × g for 60 min at 4°C. The pellet containing total membrane was resuspended in 2 ml of 2 percent sodium lauryl sarcosinate (Sigma-Aldrich, USA) containing in 10 mM HEPES buffer (pH 7.4) and incubated at 22°C for 60 min. The detergent insoluble outer membrane enriched fraction was sedimented by further ultracentrifugation at 1, 00,000 x g for 60 min at 4ºC. The pellet was washed twice with sterile distilled water and the final pellet was resuspended in 0.1 M PBS (pH7.4) and was stored in -20°C till further use.

The protein concentration of both extracted OMVs and OMPs was estimated as per the method of Lowry *et al*. (1951) [13], before further study.

Protein profiling of OMVs and OMPs of *Pasteurella multocida*

The protein profile of OMVs and OMPs were analyzed by SDS-PAGE in 12.0 % gel, as per the method of Laemmli (1970). The samples were electrophoresed with a standard protein marker at 20 mA constant current until the bromophenol blue dye reached the bottom of the resolving gel (~120 mins). The stained gel was visualized by gel documentation system (Kodak, Germany).

Immunogenic potential of OMVs and OMPs in mice

Immunogenic potential of the extracted OMVs and OMPs of *P. multocida* capsular type A was evaluated in mice. Mice were immunized with respective vaccines prepared from extracted OMVs as well as OMPs.

Preparation of vaccines and immunization of mice

An adjuvanated vaccine preparation was formulated from the extracted OMPs, while another preparation was made from the OMVs extracts, without any adjuvant. The OMPs vaccines was prepared as per the method described by Joshi *et al.* (2013) [15] by incorporating Freund's Complete adjuvant with OMPs extracts of *P. multocida* capsular type A, to have a final protein concentration of 25 µg/ml. On the other hand, the protein concentration in the crude OMVs extracts of the same isolate was adjusted at 25 µg/ml to use as a vaccine without any adjuvant (Roier *et al*. 2012) [10]. Both the vaccine preparations were subjected for their sterility and safety, before immunization of animals.

Two groups of mice (Gr I and II) consisting of six animals in each group were selected for immunization trial. The mice of Gr I were immunized intra peritoneally with 0.2 ml (25µg/ml) of the OMVs of *P. multocida*, while the other group was injected intra muscularly with 0.2 ml (25 µg/ml) of adjuvanted OMPs vaccine. A separate control group of six mice (Gr III) was administered with sterilized PBS. The blood samples were collected from all the group of mice from the tail vein on the day before vaccination. The feed and water was withdrawn 12 hours before immunization.

Sera samples were collected from all the animals of vaccinated and control groups, at the day 7, 14, 21, 28, 60 and 90, and the antibody response following vaccination was measured against sonicated antigen by Indirect Enzyme Linked Immunosorbent Assay (I-ELISA), as described by Engvall and Pearlman (1971). The mean serum antibody titer were calculated and expressed in $\log_{10,}$ as per the method described by Joshi *et al.* (2013) [15]. The mean antibody titer at different time intervals were statistically analysed by using online software tools available at www.graphpad.com. for comparative evaluation of OMV and OMP extracts, in respect to their immunogenic potential.

Results and Discussion

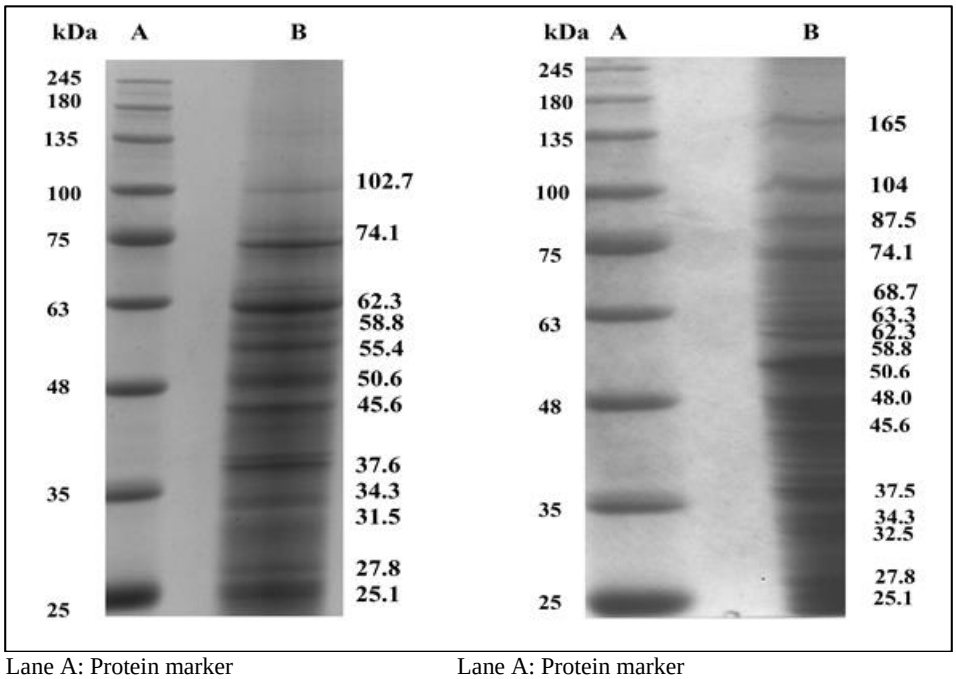
Protein Profiling of OMVs and OMPs by SDS-PAGE

OMVs and OMPs extracted from the same strain of *P. multocida* capsular type A of pig could reveal a protein concentration of 0.11mg/ml and 3.3 mg/ml, respectively. The extracted OMVs revealed presence of 12 distinct protein bands with molecular weight, ranging from 102.7 to 25.1 kDa. Seven of those protein bands of 74.1, 62.3, 55.4, 50.6, 45.6, 37.5 and 25.1 kDa were comparatively more distinct. A faint high mol. wt. protein band of 102.7 kDa size was also observed in the extracted OMVs. The characterizations of OMVs from various bacterial species in respect to the protein profile and their comparison with the bacterial fractions have long been used to identify the source of OMVs (Kulp and Kuehn, 2010). During an immunization trial in mice with OMVs of non-type able *Haemophilus influenzae*, Roier *et al.* (2012) [10] recorded 10 prominent protein bands within the range of 175.0 to 7.0 kDa size in the OMVs preparation. Among those visible bands, the most immunogenic proteins were found to be protective surface antigens D15 (80 kDa), Haem binding protein A (50.0 kDa), OMP1 (35.0 kDa), OMP2 (35.0 kDa), OMP5 (25.0 kDa) and OMP6 (15.0 kDa). Literature could not reveal much information, in respect to the protein profile of OMVs, specifically of *P. multocida.* However, Roier *et al*. (2013) [9] could demonstrate abundant no. of protein bands within the mol. wt., ranging from 17.0 to

175.0 kDa. They could also record three proteins, OmpA (37.0 kDa), OmpH (32.0 kDa), and P6 (15.0 to 16.0 kDa) as the major immunogenic proteins of *P. multocida* in the OMVs, in comparison to nine immunogenic protein in OMVs of *M. haemolytica.*

In the present study, the OMP fraction, extracted from the same *P. multocida* isolate exhibited 16 distinct protein bands, ranging from165.0 to 25.1 kDa size. Four of them (74.1, 50.6, 48.0 and 37.5 kDa) were found to be comparatively more intense. The OMPs extract of *P. multocida* could also reveal three distinct high mol. wt. proteins (87.5, 104.0 and 165.0 kDa). In a similar study, Snips *et al.* (1988) also reported a high mol. wt. protein in the OMPs fraction, extracted from an avian strain of *P. multocida.* Lu *et al.* (1991) could also observe detection of protein bands within the range of 64.5 kDa and 23 kDa size in the OMP fraction of *P. multocida*. Among the different protein fractions of OMPs of *P. multocida* serotype A: 3, protein of 34.0 kDa size was reported to be the major protein (Choi-Kim *et al*., 1991) [11], while Zhang *et al*. (1994) [20] found 35.5 kDa protein band to be the major immunogenic protein of *P. multocida* OMPs. Iron-restricted environment was recorded to be suitable for expression of high mol. wt. proteins in the OMPs extracts of *P. multocida* (Chawak *et al.*, 2001) [21]. Similar type of observation on 20 polypeptide bands (16 to 90 kDa) was recorded by Tomer *et al.* (2002) [22], during the protein profiling of OMPs extracts of the haemorrhagic septicaemia vaccine strain (P_{52}). Based on the band thickness and intensity, they reported three polypeptides of 31, 33 and 37 kDa size to be the major OMPs. During their study, expression of OMPs of 33 kDa size, exhibited by the vaccine strain could not be recorded in other field strains of *P. multocida.* Their study revealed strain variability among the vaccine strain and field strain, in respect to their protein profile.

The present SDS-PAGE study exhibited nine protein bands (74.1, 62.3, 58.8, 50.6, 45.6, 37.5, 34.3, 27.8 and 25.1 kDa), found to be shared by both the OMP and OMV fractions of the same strain of *P. multocida.* This was in agreement with the findings of Roier *et al.* (2013) [9], who could also observe similar protein sharing between the respective OMVs and OMPs preparations of *P. multocida* and *M. haemolytica*. They also opined that among the proteins shared by both OMVs and OMPs, ompH protein, corresponding to 37.0 kDa might play a significant role in immunogenicity. Based on the present observations on protein profile of OMVs and OMPs extracted from a single strain of *P. multocida*, it can be ascertained that certain proteins of the OMPs constitute a part of OMVs. However, to draw a conclusive remark on common immunogenic proteins in both OMVs and OMPs extracts of *P. multocida*, a further study is required in respect to different growth conditions associated with expression of OMVs and OMPs and also required to identify most immunogenic proteins in OMVs and OMPs in the process of vaccine development against swine pasteurellosis.



Lane A: Protein marker
Lane B: OMVs Extract

Lane A: Protein marker
Lane B: OMPs Extract

Fig 1: SDS-PAGE analysis of protein profiles of outer membrane vesicles (OMVs) and outer membrane proteins (OMPs) of *P. multocida* type A of pig origin

Serum antibody titer in immunized mice

The indirect ELISA revealed a very low level of mean antibody titer (0.064 ± 0.00) in the mice (Gr I), kept for vaccination with OMVs of *P. multocida* type A, while the other two groups (Gr. II and III) showed 0.062 ± 0.00 mean serum antibody titer on the day of pre-immunization (0 day) against *P. multocida* capsular type A. Following vaccination with the crude OMVs, mice of Gr I showed a sharp increase in the mean serum antibody titer on 7th day (1.991±0.00). The increasing trend of mean serum antibody titer continued till it reached its peak on 21st day (3.069±0.02) and was maintained till 28th day (3.061±0.00) of post-immunization. The increase in mean serum antibody titer from 0 days to the day of peak titer (21st day) was found to be statistically significant (P<0.01). However, the slight fall in ELISA titer between 21st and 28th day of post immunization was statistical insignificant, while a significant fall in the mean serum antibody titer was observed till 90th day (3.019 ±0.00) post-vaccination (Table 1 and Figure 2). A similar trend of significant increase in the mean serum antibody titer (P<0.01)

could also be detected in the group of mice (Gr-II) on the day 7 (2.388±0.001) of post immunization with oil adjuvanated vaccine prepared from OMPs. of *P. multocida* type A. The inclined pattern of the serum antibody titer was maintained until the titer reached the peak on the 60th day (2.878±0.00) post-vaccination. Although, no significant increase in antibody titer could be observed between 7th and 14th day of vaccination, the increase of mean ELISA titer between the 14th day and the day of peak titer was found to be significantly (P<0.01). From the 60th day, there was a gradual decrease in the mean serum antibody titer till 90th day (2.857 ± 0.00) of immunization, which was statistically significant (P<0.05). The preimmunization mean serum antibody titer (0.062±0.00), observed in the control group (Gr III) was almost static during the entire experimental period, which was not statistically different at any of the time intervals during the study.

There was an apparent difference between the two vaccine preparations (OMPs and OMVs) of the same isolates, in respect to the mean antibody titer reflected at different days of post vaccination and the day of peak titer, However, the statistical analysis could reveal a significant difference (P<0.01) between both the vaccine preparations, during the entire period of study. This was an indication for the OMVs as a comparatively superior immunogen to OMPs.

A pronounced induction of IgM titer of at least 10-fold, after immunization with OMVs derived from non-type able *H. influenzae* or *V. cholerae* was reported by Schild *et al*. (2009) [24] and Roier *et al.* (2012) [10]. The present observation in respect to the immunogenic potential of *P. multocida* OMVs was in agreement with findings of Roier *et al.* (2013) [9], who also recorded the median IgM antibody titer in mice, following immunization with *P. multocida* OMVs. However, in contrast to the present observation, they could see a mild increase that peaked on day 28 followed by slight decline. In their study, median IgA and IgG1 antibody titer to *P. multocida* of the immunization groups was found to be increased in contrast to IgM. They observed the highest level at the endpoint of the experiment on day 39.

Contrary to the present observation on immunogenic potential of OMPs, Pati *et al.* (1996) [25] could record highest mean $\log_{10}$ antibody titer on day 21 post-vaccination with OMPs vaccine of *P. multocida* B:2. Successful use of OMPs of *P. multocida* as immunogenic components was also previously reported by various workers. Fuller *et al.* (2000) [5] reported the OMPs extracted from strains of *P. multocida* capsular type A and D of pig origin to be immunogenic. Both the iron regulated OMPs and the normal OMPs of *P. multocida* B:2 were reported to be immunogenic for mice (Kharb and Charan, 2010) [12]. They could record that both preparations provoked significant antibody response in immunized mice. However, antibody response was found to be higher following immunization with iron regulated OMPs than that elicited by normal OMPs.

No information could be traced out from available literature in respect to the comparative evaluation of those two immunogens. However, the present observation in respect to the OMVs of *P. multocida* as a better immunogen over the OMPs might be due to the fact that OMVs are the mixture of OMPs, lipopolysaccharide, periplasmic protein and phospholipids that make the OMVs a robust immunogen.

Table 1: Mean serum antibody ($\log_{10}$) of mice following immunization with OMVs and OMPs preparation of *P. multocida* at different days post-immunization

Preparation	Mean ± SE log base 10 titer at different days of post-immunization						
	0 day	7th day	14th day	21st day	28th day	60th day	90th day
OMVs	$0.064^{A}{}_{a} \pm 0.00$	$1.991^{B}{}_{c} \pm 0.00$	$2.449^{C}{}_{c} \pm 0.00$	$3.069^{D}{}_{c} \pm 0.02$	$3.061^{D}{}_{c} \pm 0.00$	$3.020^{E}{}_{c} \pm 0.00$	$3.019_{c}{}^{E} \pm 0.00$
OMPs	$0.062^{A}{}_{a} \pm 0.00$	$2.388^{B}{}_{b} \pm 0.001$	$2.397^{B}{}_{b} \pm 0.00$	$2.439^{C}{}_{b} \pm 0.001$	$2.765^{D}{}_{b} \pm 0.002$	$2.878^{E}{}_{b} \pm 0.00$	$2.857^{F}{}_{b} \pm 0.00$
Control	$0.062^{A}{}_{a} \pm 0.00$	$0.048^{A}{}_{a} \pm 0.00$	$0.056^{A}{}_{a} \pm 0.05$	$0.060^{A}{}_{a} \pm 0.007$	$0.046^{A}{}_{a} \pm 0.001$	$0.064^{A}{}_{a} \pm 0.004$	$0.054^{A}{}_{a} \pm 0.007$

Means in a row bearing different superscripts and means in a column bearing different subscripts differed significantly.

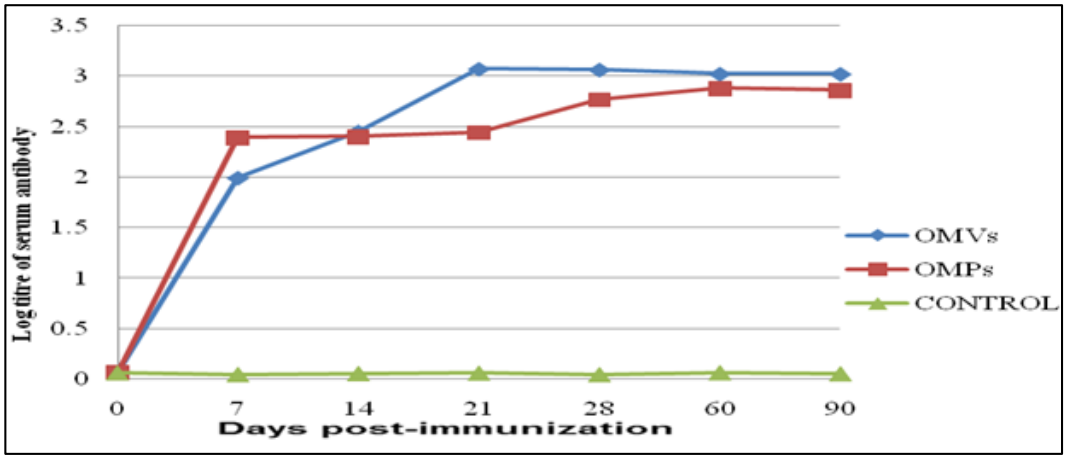


Fig 2: Graphical representation of mean (±se) serum antbody titer ($\log_{10}$ value) in mice at different days following immunization with OMVs and OMPs-based vaccine preparations

Conclusion

The extracted OMVs and OMPs from a same strain of pathogenic *P. multocida* capsular type A of pig origin were found to be consisted of variable no. of proteins with different molecular sizes. Growth under the influence of iron chelating agents in the environment could favour the expression of high molecular weight protein in both OMVs (102.7 kDa) and OMPs (104.0 and 165.0 kDa). Sharing of nine protein bands by both the fractions of the same isolate was an indication for presence of a part of OMP in the OMV fraction. Both the OMV and OMP fractions of the same isolate of *P. multocida* were found to immunogenic for mice, with a peak antibody

titer on 21days and 60th days of vaccination, respectively. Immunogenic potential of the OMVs fraction was found to be superior and statistically significant (P<0.01) to that of OMPs of *P. mutocida.*

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