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Immunogenic potential of outer membrane vesicle (OMV) of *Pasteurella multocida* in pigs

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Abstract
Outer Membrane Vesicle (OMV) and bacterin were extracted from the most pathogenic porcine isolate of *Pasteurella multocida* capsular type A selected on the basis of pathogenicity trial in mice and vaccines prepared from the extracts were immunized in pigs. The comparative immunogenicity of the two vaccine preparations of *Pasteurella multocida* was determined by monitoring the corresponding serum antibody titres at different time intervals 0, 7, 21, 30, 45, 60, 75, 90 days post vaccination by indirect ELISA. The OMV extract vaccinated pigs showed a sharp increase in the ELISA antibody titre from the very first week (3.60±0.21) of immunization, reaching peak titre on the 60th day post vaccination (6.40±0.19) and started to decline on 90th day post vaccination. The pigs immunized with bacterin of the same strain of *Pasteurella multocida* showed serum antibody titre 3.20±0.34 on 7th day post-immunization, reaching peak titre on the 28th day (6.10±0.14) post-vaccination, thereby gradual decrease in the mean serum antibody titre (5.9±0.25) from the 60th day, till 90th day (5.70±0.23) post immunization. In both the vaccine preparations, the antibody titre showed gradual decreases up to 90th day post vaccination. The statistical analysis revealed no significant difference between the two vaccine preparations (OMVs and bacterin) based on mean serum antibody titre at different days post-immunization. The result of passive mouse protection test revealed that the 21st and 28th day post vaccination serum sample (OMV vaccine) and 28th day serum sample (bacterin vaccine) produced (100%) protection in mice.

Keywords: Outer membrane vesicle (OMV), *pasteurella multocida*, pigs, immunogenicity, ELISA, passive mouse protection test

Introduction
Pig farming has a special significance in the North Eastern Region India, as it plays an important role in uplifting the socio-economic status of sizeable proportion of the weaker section of the society in the region especially among the tribal communities. Though Pig farming is a profitable enterprise, they are prone to large number of infectious diseases like pneumonia, atrophic rhinitis, septicaemia, diarrhoea, swine fever etc. Among the various diseases, Swine pasteurellosis caused by *Pasteurella multocida* capsular types A and D is considered one of the most common diseases of pig encountered by the poor farmers causing heavy economic loss and has been reported in India and north eastern region of India (Verma, 1991 and Dev Sharma, 2001) [1, 2].

Swine pasteurellosis can be controlled by proper vaccination and good management practices (Sakano *et al.,* 1997; Liao *et al.*, 2006) [3, 4]. To control swine pasteurellosis different vaccines prepared from specific serotype of *P. multocida* can be used against the various forms of pasterurollosis. Different extracts of *Pasteurella* organism like saline and Potassium thiocyanate (KSCN) has also been tried as vaccines against pasteurellosis in animal (Sharma, 1995; Choubey, 2001) [6, 5]. Vaccine containing sub-cellular components of *P. multocida* has also been introduced (Ayalew *et al.,* 2013) [7]. Currently, the available vaccine against this disease consists of killed *P. multocida* B: 2 (P52) adjuvanted with alum or oil. One drawback of this vaccine is that its immune response is generally serotype-specific (Carpenter *et al.,* 1991) [8]; hence it is incapable of inducing adequate protective immunity against other types of *P. multocida*.

Outer membrane vesicles (OMVs) are the small spherical structures (approximately 10-300 nm) that are naturally released from the outer membrane (OM) of gram-negative bacteria.

Outer membrane vesicles (OMVs) naturally contain important surface antigens with outer membrane proteins, periplasmic proteins, phospholipids and the lipopolysaccharide (LPS). The immunogenic and protective properties of outer membrane vesicles (OMVs) have been widely tested and found effective against several Gram-negative human pathogens. In spite of reports on immunological potential of outer membrane vesicles (OMVs) of many of the Gram-negative human pathogen, no detail study has so far been made on outer membrane vesicles (OMVs) of *P. multocida*, particularly in India. However an attempt has been made to use sub-unit components (Ayalew *et al.*, 2013) [7] and Outer membrane vesicles (OMVs) (Roier *et al.*, 2013) [9] as vaccine candidates against *Mannheimia haemolytica* and *P. multocida* in animal. Being a naturally released antigen delivery vehicles with a heterogeneous mixture of diverse components, the *P. multocida* outer membrane vesicles (OMVs) are expected to be advantageous as a potential vaccine candidate over conventional vaccines as well as vaccines solely based on purified recombinant proteins. Keeping the above facts in view, the present study was undertaken with the following objectives:

1. To study the immunogenic potential of outer membrane vesicles (OMVs) of *Pasteurella multocida* in natural host.
2. To compare the immunogenic potential of outer membrane vesicle (OMV) with that of conventional (bacterin) vaccine in pig.

2. Materials and Methods

Ethical Approval

Ethical approval for the study was obtained from Institutional Animal Ethics Committee (IAEC), Assam Agricultural University (AAU), Khanapara campus vide approval No 770/ac/CPCSEA/FVSc/AAU/IAEC/15-16/311 dated 10.04.2015.

2.1 Bacterial strain

A total of nine capsular type A *P. multocida* isolated from clinical samples suggestive of swine pasteurellosis, were used for the study. The isolates were confirmed by PM-PCR as per the method recommended by Townsend *et al*. (1998) [10] and multiplex Cap-PCR as per the method described by Townsend *et al*. (2001) [11].

2. 2 Pathogenicity of *p. Multocida* Isolates

Pathogenicity of the isolates of *P. multocida* capsular Type-A were studied in mice following the method described by Curtis (1985) [12] with slight modification. The pathogenicity was interpreted on the basis of percentage of mortality instead of pathological lesions.

A total of 60 albino mice of the same age group (4-6 weeks) of either sex were divided in to ten groups (I to X), consisting of six animals in each group. All the mice were reared in the Laboratory animal house of Network Project on Haemorrhagic Septicaemia, Department of Microbiology, College of Veterinary Science, Assam Agricultural University, Khanapara, Guwahati, Assam under the same managemental condition. The animals were provided with clean water and antibiotic-free feed throughout the experiment. Group X was kept as uninfected control.

Mice (Grp I to IX) were injected intraperitoneally (i/p) with each of 0.1 ml of inoculum prepared from respective isolates of *P. multocida* type A containing 10^9 organisms per ml (Wijewardana, 1992) [13]. Mice of the control group (Gr. X) were injected with 0.1 ml sterile saline solution through the same route. The mortality rate was recorded at every 6 hrs interval for 72 hrs post-inoculation and postmortem changes in the dead mice were recorded. Re-isolation of the inoculated *P. multocida* was attempted from the internal samples, *viz.*, lung, liver and heart blood collected from mice died during pathogenicity trial. Reconfirmation of the organism isolated from dead mice as *P. multocida*, was done by pm-PCR and cap-PCR. Mice of the control group were sacrificed after 72 hrs and organ samples were collected for bacteriological examination.

2.3 Preparation of OMVs extract of *P. multocida type A*

The most pathogenic strain of *P. multocida* of capsular type A was subjected for extraction of Outer Membrane Vesicles (OMVs). OMVs of *P. multocida* were extracted as per the method described by Roier *et al.* (2013) [9] with slight modification in respect to the growth factors.

Pure colonies of *P. multocida* from blood agar plate were taken and grown in BHI broth (containing 0.5 % yeast extract) in 50 ml centrifuge tube by overnight incubation at 37 °C. The broth culture was harvested by centrifugation at 5000 x g for 10 min at 4°C. The cell pellets were transferred to 500 ml BHI broth with 0.5 per cent yeast extract and grown for 13 hours at 37°C 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 4 °C. 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, following filtration with 0.2 µm filter was tested for complete removal of viable bacteria by inoculating in to BHI agar plate. The filtrate was finally exposed to ultracentrifugation at 1, 44,000 x g for 4 hours at 4°C and the pellets were collected as OMVs and re-suspended in 5 ml of PBS (pH 7.4).

The protein concentration of OMVs extract was estimated as per the method of Lowry *et al.* (1951) [18] and expressed in milligram per milliliter (mg/ml). The Optical Density (O.D) values of the protein were determined by spectrophotometer (Systronics, India) at 660 nm wave length.

2.4 Preparation of Bacterin of *P. multocida type A*

Formalin inactivated bacterin was prepared from the selected strains of *P. multocida* as per the method described by Dhanda *et al.* (1956) [14] with slight modification as proposed by Baxi (1966) [15]. The selected strains of *P. multocida* was grown in 50 ml of BHI broth for 18 hours at 37°C. Purity of the bacterial strains was tested and broth cultures were spread over a number of BHI agar plates. The plates were incubated at 37°C for 18 hours. Each plate was tested for purity and the growth was harvested by washing with sterile NSS and sterile glass beads. The bacterial suspensions were centrifuged at 3000 x g for 1 hour at 4°C. The sediment was washed thrice with sterile NSS. After the last centrifugation, bacterial pellet was resuspended in half of the initial volume with sterile NSS. The viable bacterial count was determined by pour plate method as described by Cruickshank *et al.* (1975) [16]. The concentration was finally adjusted to 10^9 organisms per ml of suspension (Wijewardana, 1992) [13].

2.5 Formulation of vaccines

Two different types of vaccines were formulated from the OMV extract and bacterin prepared from the most pathogenic porcine strain of *P. multocida*. OMV vaccine was prepared without any adjuvant. Bacterin vaccine was prepared by emulsifying the formalin inactivated bacterin containing 10^9 cells per ml with equal volume of Freunds incomplete Adjuvant (sigma). Stability of the emulsified preparations was examined at 4 °C for 24 hours.

Sterility of the antigens was tested by inoculating the preparations on blood agar and incubating at 37 °C for 48 hours. Duplicate samples were inoculated on Sabouraud's dextrose agar medium and incubated at 37 °C for 7-14 days.

Safety of the prepared antigens was evaluated in mice. Each preparation was injected at the rate of 0.2 ml subcutaneously to a group of three mice each as per the method of Wijewardana (1992) [13]. The mice were observed up to 3 days for any mortality or development of other abnormalities.

2.6 Immunization of Pigs

Immunogenic potential of OMVs and Bacterin extracted from the most pathogenic isolate of *P. multocida* capsular type A of pig origin was evaluated in Pig.

A total of 18 Piglets of same age, *i.e.* post weaning stage (45-50 days) of either sex were used in the present immunological trial of the prepared vaccines. Piglets were randomly divided into 3 equal groups (Group A, B and C) comprising of 6 (six) piglet in each group. Piglets were immunized with respective vaccines prepared from extracted OMVs and bacterin. The details of the immunogenic study are given in Table 1.

Table 1: Grouping of Pigs for Immunization with OMV and Bacterin Vaccines of *p. Multocida* type a.

Groups	Type of Vaccine	No. of piglets	Dose	Route of inoculation
A	OMV extract	6	3ml containing 3.3mg/ml	I/M
B	Bacterin vaccine	6	3ml containing 10^9 cells per ml	-do-
C	PBS	6	3ml	-do-

Blood samples were collected from the immunized piglets on the day of immunization (0 day) and at the intervals of 7, 21, 30, 45, 60, 75 and 90 days after vaccinations. Serum samples were separated and kept aseptically in sterile vials and stored at - 20 °C.

2.7 Immunological Assay

2.7.1 Preparation of Antigen

The sonicated antigen was prepared from a known pathogenic strain of *P. multocida* capsular type A of the repository, as per the method described by Manoharan and Jayprakasan (1995) [17]. Pure culture of *P. multocida* type A of porcine origin used for extraction of OMVs was obtained by growing in BHI broth supplemented with 0.5 per cent yeast extract. After 24 hrs of incubation at 37°C, the broth was centrifuged at 6000 rpm for 10 minutes and supernatant was discarded and pellet was washed three times with sterile Phosphate Buffered Saline (PBS) solution. Washed pellets were finally suspended in PBS. The cells of the bacterial suspension were disrupted by sonication for 10 cycles of 1 min. on time and 1 min, off time in an ice bath. The sonicated preparation was centrifuged at 8,000 rpm for 10 minutes at 4 °C and the supernatant was collected as antigen. Protein concentration of the antigen was determined as per the method of Lowry *et al.* (1951) [18] as described earlier.

2.7.2 Indirect Enzyme Linked Immunosorbent Assay (Indirect- ELISA)

The indirect ELISA was performed as per the method described by (Klaassen *et al.* (1985) [19]. The optimum concentrations of antigen, serum and conjugate were determined by checkerboard titration.

Each well of the ELISA plate (Nunc, Polysorp) was coated with sonicated antigen of 2 (µg/ml protein in PBS (pH 7.4) and the plate was incubated at 4°C overnight. The overnight incubated plates were washed thrice with PBST (0.05% Tween 20 in PBS, pH 7.4) and blocked with 100 µl per well of blocking buffer with one hour incubation at 37°C. Wells were washed three times with PBS-T following incubation. Antigen coated wells were charged with 100 µl of 2-fold serial dilution of test serum samples in blocking buffer (5% Lactalbumin Hydrolysate in PBS-T). The initial dilution was stalled with 1:100. The plates were incubated at 37°C for 1 hour and then washed three times with PBS-T. A volume of 100 µl diluted (1: 2000) conjugated anti-mouse IgG (Sigma Aldrich, USA) was added to each well and incubated for one hour at 37°C, followed by washing with PBS-T. After washing, bound conjugate was visualized by adding 100 µl of 0PD/H_20_2 substrate solution (Bangalore Genei, India) per well for 15 minutes in dark, at room temperature. The reaction was stopped by adding 100 µl of H2SO4 solution to each well. The optical density (O.D) of each well was measured at 450 nm wave length in an ELISA reader (Bio Rad, Model PR 4100). Antigen control and antibody control were taken in each plate for calculation. The mean serum antibody titre was expressed in $\log_{10}$ scale. Corrected optical density (OD) value [OD of the test sample - OD of the negative sample ≥ 0.1 in the highest dilution of the serum was considered as the titre.

2.8 Passive Mouse Protection trial in mice

Passive Mouse Protection trial in mice was done as per method described by David *et al.* (1976) [29].

Three groups (I, II and III) of mice consisting of six mice in each group were taken for passive mouse protective trial for different days *viz.*, 0^{th}, 7^{th}, 14^{th}, 21^{st}, 28^{th}, 60^{th} and 90^{th} post vaccination serum samples against OMVs and bacterin vaccines respectively. Group III was considered as the control group for different days post vaccination serum samples. All the animals of group I were inoculated with 0.2 ml of serum of immunized OMV preparation through intramuscular (i/m) route. Similarly, the animals of group II were inoculated with 0.2 ml of serum of immunized bacterin preparation through intramuscular (i/m) route. The details of the trial are given in Table 2.

The animals were challenged with 0.2ml *P. multocida* crude bacteria at the concentration of $1x10^7$ organisms per ml after 24hrs of serum inoculation and observed up to 72hrs for protection. Re-isolation of the inoculated *P. multocida* was attempted from the internal samples, *viz.*, lung, liver and heart blood collected from mice died during the protection trial.

2.9 Statistical Analysis

The mean antibody titre in all the vaccinated groups of animals, as detected by indirect-ELISA, were calculated and statistically analyzed as per the method described by Joshi *et al.* (2013) [21]. The antibody responses were statistically analyzed by using online software tools available at www.graphpad.com for comparative evaluation in respect to the immunogenic potential of OMVs and bacterin extracted from the most pathogenic strain of *P. multocida* capsular type A of pig origin.

Table 2: Grouping of Mice for Passive Mouse Protection Trial

Days of collection	Group	No of mice inoculated	Antiserum	Dose (ml)	Route	Challenged dose (ml)*	Challenged route
0th	I	6	OMV	0.2	i/m	0.2	i/p
	II	6	Bacterin	0.2	i/m	0.2	i/p
	III	6	Control	0.2	i/m	0.2	i/p
7th	I	6	OMV	0.2	i/m	0.2	i/p
	II	6	Bacterin	0.2	i/m	0.2	i/p
	III	6	Control	0.2	i/m	0.2	i/p
14th	I	6	OMV	0.2	i/m	0.2	i/p
	II	6	Bacterin	0.2	i/m	0.2	i/p
	III	6	Control	0.2	i/m	0.2	i/p
21st	I	6	OMV	0.2	i/m	0.2	i/p
	II	6	Bacterin	0.2	i/m	0.2	i/p
	III	6	Control	0.2	i/m	0.2	i/p
28th	I	6	OMV	0.2	i/m	0.2	i/p
	II	6	Bacterin	0.2	i/m	0.2	i/p
	III	6	Control	0.2	i/m	0.2	i/p
60th	I	6	OMV	0.2	i/m	0.2	i/p
	II	6	Bacterin	0.2	i/m	0.2	i/p
	III	6	Control	0.2	i/m	0.2	i/p
90th	I	6	OMV	0.2	i/m	0.2	i/p
	II	6	Bacterin	0.2	i/m	0.2	i/p
	III	6	Control	0.2	i/m	0.2	i/p

***** 1×10^7 organisms per ml

3. Results and Discussion

All the nine isolates showed an amplified product of 460 base pair (bp) by Pm-PCR which is similar in finding by Dutta *et al.* (2001) and Capsular type A *hyaD-hyaC* gene with an amplified product of 1044 bp. Tang *et al.* (2009) [24]. (Fig 1).

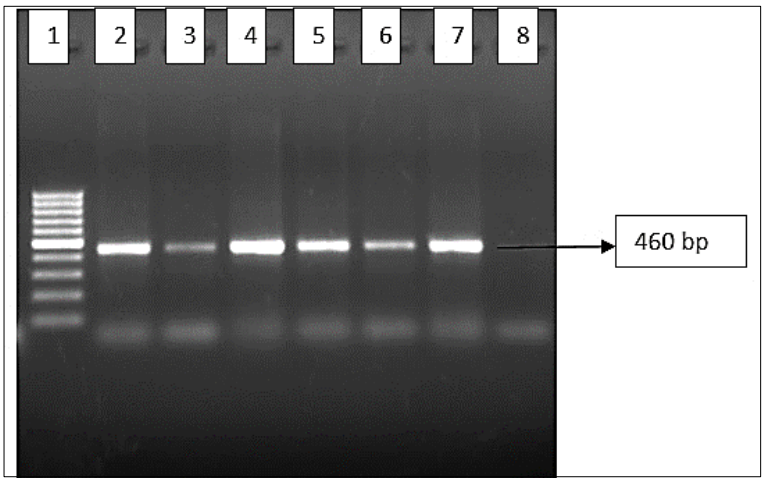

Fig 1: PM-PCR for detection of *KMT1* gene (460 bp) of *P. multocida*, Lane 2 to 6: positive for *kmt* gene; Lane 7: Positive control; Lane 8: Negative control and Lane 1:100bp DNA Ladder

3.1 Pathogenicity trial of *P. multocida* in mice

During the study, a total of 7 (77.77%) isolates were found to be pathogenic causing death of mice at different time intervals (Table 3). Out of the 7 pathogenic isolates of *P. multocida* subjected to pathogenicity trial, two (KP10 and KP14) were found to be highly pathogenic causing 100 per cent mortality within 48 hr of post inoculation. The other three strains (SP12, IP15 and IP26) were found to cause 83.33 per cent mortality in mice. IP4 produced 66.66 percent mortality while KP22 produced 50 percent mortality. No death was recorded in the mice injected with isolates SP20 and IP 32 as well as the control group.

In the present study, (28.33%) mice died within 24hr post-inoculation, whereas, (16.66%) died within 24-48hr and (11.6%) died within 72hr post-inoculation respectively.

Among the two *P. multocida* isolates that produced (100%) mortality in inoculated mice, one strain (KP10) was found to be the most pathogenic causing 100 percent mortality within 24 to 48 hours of inoculation. The other isolate (KP14) was found to cause death of inoculated mice at different time periods (0 to 48 hrs.)

Table 3: Pathogenicity of *Pasteurella Multocida* Type A Isolates in Mice

Group	Isolate	Serotype	Dose (ml)*	Route	No. of mice inoculated	No. of mice died within hrs			Total no. died
						24	48	72	
I	SP12	A	0.1	i/p	6	2	3	-	5(83.33)
II	SP20	A	0.1	i/p	6	-	-	-	0 (0.00)
III	IP4	A	0.1	i/p	6	-	2	2	4(66.66)
IV	IP15	A	0.1	i/p	6	1	2	2	5(83.33)
V	IP26	A	0.1	i/p	6	5	-	-	5(83.33)
VI	IP32	A	0.1	i/p	6	-	-	-	0 (0.00)
VII	KP10	A	0.1	i/p	6	-	6	-	6(100.00)
VIII	KP14	A	0.1	i/p	6	3	3	-	6(100.00)
IX	KP22	A	0.1	i/p	6	-	-	3	3(50.00)
X	Control	-	0.1**	i/p	6	-	-	-	0 (0.00)
						17 (28.33)	10 (16.66)	7 (11.66)	33 (55.00)

*0.1 ml inoculums containing 10^9 numbers of organisms
**0.1 ml of sterile NSS

Figures in parenthesis indicate percentages.
Inoculated *P. multocida* strains could be re-isolated from the internal organs including heart blood of all the mice died within 24 to 48 hrs of inoculation. Re-isolation of *P. multocida* from the dead mice confirmed the death due to the inoculated *P. multocida* organisms.
Mice have been used for a long time as an experimental animal to determine the pathogenicity of *P. multocida* strains (Jaisunder and Kumar, 1999) [22]. Variation in pathogenicity of different strains of *Pasteurella multocida* was in agreement with the findings of a number of researchers (Dev Sharma, 2001; Sharma and Rahman, 2003) [2, 25].
Such variation might be due to the difference in virulence potential of the organisms, general condition of the animals, difference in antigenic structure of the organisms and number of times the organisms sub-cultured on laboratory media before inoculation in the mice taken for pathogenicity trial.
Majority of the inoculated mice (28.33%) were killed during 24 hrs of inoculation. Re-isolation of the inoculated *P. multocida* capsular type A from different internal organs, *viz.*, heart blood, liver, lung and spleen, could be possible in all the mice died within 24 to 48 hrs of inoculation. On the other hand, the mice died within 24 hrs did not reveal presence of the isolates in their heart blood and internal organs. Similar observations were also made by various workers (Dev Sharma, 2001; Sharma and Rahman, 2003) [2, 25]. Death as well as recovery of the organisms from lungs and heart blood of experimentally inoculated mice died within 24-48 hours indicated that mice died due to acute phase of bacteremia caused by *Pasteurella multocida* organisms.

3.2 Immunological study of various vaccine preparations
Humoral immune response to OMV extract vaccine and bacterin vaccine of P. *multocida* type A was studied by immunizing piglets with the vaccines.
The comparative immunogenicity of the two different vaccine preparations of *P. multocida* was determined by monitoring the corresponding serum antibody titres at different time intervals up to 90 days in different vaccinated groups. The mean serum antibody titres were monitored by indirect ELISA. Details of the immunological studies of the vaccine preparations are shown in Table 4 and Fig 2. The mean serum antibody titres in the vaccinated groups were expressed in log $_{10}$ scale.

Table 4: Mean Elisa Antibody Titer (Log_{10}) Of Serum of Pig for Bacterin and Omv of Post-Immunization on Different Days

Types of Vaccine	Mean ± SE of log_{10} antibody titer of different days of post-immunization.						
	0^{th}	7^{th}	14^{th}	21^{st}	28^{th}	60^{th}	90^{th}
Bacterin (n=6)	1.62±0.36	3.20±0.34	4.20±0.21	5.60±0.39	6.10±0.14	5.90±0.25	5.70±0.23
OMV (n=6)	1.72±0.39	3.60±0.21	3.90±0.49	5.30±0.34	5.90±0.26	6.40±0.19	6.10±0.12
Control (n=6)	1.60±0.42	1.60±0.43	1.64±0.41	1.70±0.35	1.72±0.92	1.72±0.40	1.62±0.16

3.2.1 Response to OMV Extract
All the serum samples collected from the groups of animals immunized with *P. multocida* type A OMV extract and bacterin vaccine were subjected to indirect ELISA. Details of the mean serum antibody titres in the vaccinated groups at different time intervals are presented in Table. 4.
The groups of pigs immunized with single dose of OMV extract vaccine showed a sharp increase in the ELISA antibody titre from the very first week (3.60±0.21) of immunization. The serum antibody titre reached peak on the 60th day post vaccination (6.40±0.19) and started to decline on 90^{th} day post vaccination.

3.2.2 Response to Bacterin Vaccine
Similar trend was observed in immunized pig following vaccination with bacterin of the same strain of *P. multocida* used for OMVs preparation till 7^{th} day (3.20±0.34) post-immunization. The peak titre reached on the 28^{th} day (6.10±0.14) post-vaccination. From the 60^{th} day, there was a gradual decrease in the mean serum antibody titre (5.9±0.25) till 90^{th} day (5.70±0.23) post immunization. In both the vaccine preparations, the antibody titre showed gradual decreases up to 90^{th} days post vaccination. This finding indicated the need of further standardization of the dose, the time of booster vaccination and collection of serum samples beyond 90 days post vaccination to obtain a prominent anamnestic response following booster.

3.3 Statistical Analysis
The statistical analysis of the mean serum antibody titre in the animals vaccinated with OMVs extract (Group A)

revealed no significant difference from the day pre-immunization (0 day) to 90th day post immunization Similarly no significant difference in the antibody titre between the days of serum collection vaccinated with bacterin vaccine was observed. The mean antibody titre in the animals of control Group C was not statistically different at any of the time intervals during the study.

The statistical analysis of the mean antibody titre following vaccination with the two preparations revealed no significant difference at different days of immunization within the group. No significant difference could be observed between the two vaccine preparations throughout the entire period of immunization trial. (Table 5).

Since, IgM is the first Ig isotype expressed by mature B cells, an increase in the IgM titre is first sign of induction of humoral immune response (Boes, 2000) [26]. A pronounced induction of IgM titre of at least 10-fold, after immunization with OMVs derived from nontypeable *H. influenzae* or *V. cholerae* was reported by Roier *et al.* (2012) [27] and Schild *et al.* (2009) [28]. 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 could record the median IgM antibody titers to *P. multocida* OMVs and showed only a mild increase that peaked on day 28 followed by slight decline. In contrast to IgM, median IgA and IgG1 antibody titre to *P. multocida* of the immunization groups increased and showed the highest level at the endpoint of the experiment on day 39.

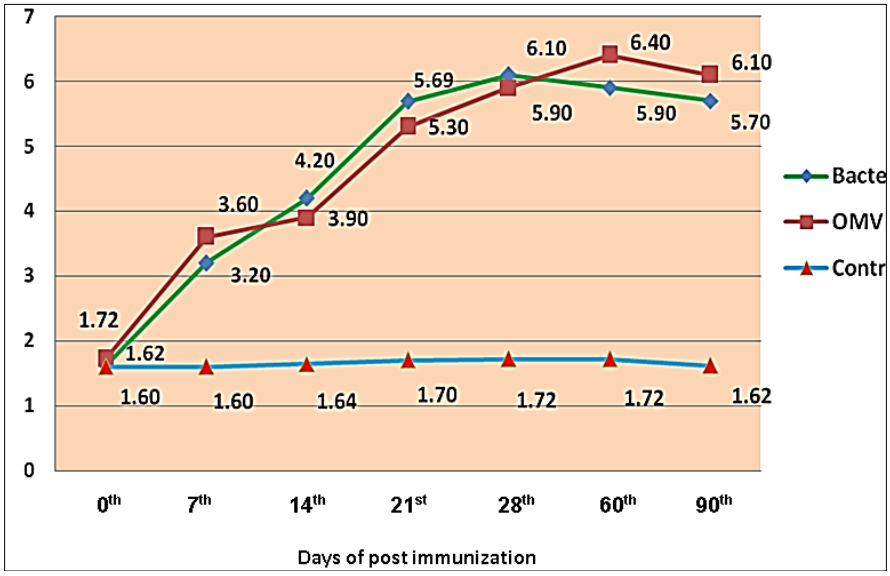

Fig 2: Graphical Representation of Post Vaccination Elisa Titre In Pig

Table 5: Analysis of Variance For *(Pasteurella Multocida)* Specific Antibody of Omv And Bacterin In Between The Groups and Days of Post Vaccination

S.S. due to	d. f.	S.S.	M.S.S.	F
Group (G)	3	1.07	0.36	4.50^{NS}
Days (D)	6	214.37	42.87	535.87^{NS}
G x D	18	0.73	0.05	0.625^{NS}
Error	216	16.49	0.08	

NS Non-significant.

3.4 Passive Mouse Protection Test

3.4.1 Mouse protection test for serum against OMV vaccine

The antiserum against OMV vaccine collected on the 21st and 28th day of post vaccination were found to be highly protective producing 100 per cent protection in mice. The 14th, 60th and 90th day serum samples were found to produce (83.33%) protection in mice. However, the 0th and 7th day serum samples were found to be non-protective in mice. (Table. 6).

Table 6: Passive Mouse Protection Test in Mice

Observation (hr)	Type of antisera	No. of mice	Post challenged mortality in mice following inoculation with pig serum of different days of immunization						
			0th	7th	14th	21st	28th	60th	90th
24	OMV	6	4	3	-	-	-	1	1
48			2	3	1	-	-	-	-
72			-	-	-	-	-	-	-
Total no. of animal protected			0 (0)	0 (0)	5 (83.33)	6 (100)	6 (100)	5 (83.33)	5 (83.33)
24	Bacterin	6	3	2	1	-	-	-	-
48			3	4	1	1	-	2	1
72			-	-	-	-	-	-	1
Total no. of animal protected			0 (0)	0 (0)	4 (66.66)	5 (83.33)	6 (100)	4 (66.66)	4 (66.66)
24	Control	6	4	3	2	4	2	3	4
48			2	3	4	2	4	3	2
72			-	-	-	-	-	-	-
Total no of animal protected			0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)

* Figures in parenthesis indicate percentages.

3.4.2 Mouse protection test for serum against bacterin vaccine

100 percent protection was also recorded in mice group inoculated with 28th day serum sample against bacterin vaccine. The 14th, 60th and 90th day post vaccination serum samples could produce 66.66 per cent protection in mice. 83.33 % protection was recorded in 21st day post vaccination serum and similar finding were also reported by Sundar and Kumar (1999) [22]. The 0th and 7th day serum sample was found to be non-protective in mice resulting in death in mice at different time interval (Table 6).

The animals in the control group died post challenged with *P. multocida*. Inoculated *P. multocida* strains could be re-isolated from the internal organs including heart blood of all the mice died post inoculation. Reisolation of *P. multocida* from the dead mice confirmed the death due to the inoculated *P. multocida* organisms.

These findings confirmed the protective efficacy of the both OMV and bacterin vaccine against *P. multocida* in mice. The protective immunity induced by the vaccine preparation might be attributed to the higher immunogenicity of OMV extracts of *P. multocida*.

4. Conclusion

From the results of the present study, it could be concluded that Outer membrane vesicles (OMVs) of the most pathogenic *P. multocida* was found superior to bacterin based on post vaccinal antibody titre. No significant difference could be observed between OMV and bacterin preparations of *P. multocida* capsular type A in respect of antibody titre. However, the protection conferred by OMV preparation was found quicker and superior to conventional bacterin preparation which might be attributed to the higher immunogenicity of OMV extracts of *P. multocida*. The finding also indicates the need for further standardization of the dose, the time of booster vaccination and collection of serum samples beyond 90 days post vaccination to obtain a prominent anamnestic response following booster.

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