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Protective Efficacy of Calcium Phosphate Nanoparticle Adsorbed Bivalent Subunit Vaccine of *Pasteurella multocida* against Homologous Challenge in Mice

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ABSTRACT

Background: Swine pasteurellosis, caused by *Pasteurella multocida* capsular types A and D, causes heavy economic loss to the pig farmers. The vaccine presently used is a bacterin of *Pasteurella multocida* capsular type B that is proven to be effective against bovine pasteurellosis. However, its efficacy against swine pasteurellosis is questionable.

Methods: The present study was carried out to evaluate the efficacy of calcium phosphate nanoparticle adjuvanted bivalent subunit vaccine prepared from *Pasteurella multocida* capsular types A and D along with a monovalent subunit vaccine prepared from *Pasteurella multocida* capsular type B in mice. The Alum precipitated bacterin vaccine was used as the control.

Result: The bivalent subunit vaccine showed significantly higher serum IgG response than either of the other two vaccines. The calcium phosphate nanoparticle adjuvanted vaccines could elicit 100% protection in mice against homologous challenges but the aluminum hydroxide adjuvanted bacterin vaccine could not elicit significant protection. Based on this preliminary work, it was concluded that the bivalent subunit vaccine would be a better option for immunization of swine against swine pasteurellosis.

Key words: Calcium phosphate nanoparticle, Capsular type A and D, Outer membrane protein, *Pasteurella multocida*.

INTRODUCTION

Swine pasteurellosis is caused by *Pasteurella multocida* capsular types A and D, while the capsular type B:2 of *P. multocida* is the causative agent of haemorrhagic septicaemia (HS) that affects mainly cattle, buffalo, sheep and goat. Swine pasteurellosis is responsible for significant economic loss to the pig industry in the north-eastern region of India. The disease occurs in acute septicaemic as well as subacute pneumonic forms. Pneumonic forms are caused by *P. multocida* of capsular types A and D Djordjevic *et al.* (1998) cited in Hazarika *et al.*, (2010).

Presently in India, an alum-or oil-adjuvanted killed vaccine prepared from *P. multocida* serotype B: 2 (P_{52} strain) is used for control of swine pasteurellosis in endemic areas. This vaccine is unable to elicit sufficient protective immune response against swine Pasteurellosis as it is prepared from P_{52} strain of serotype B: 2 of *P. multocida* that is efficient in controlling bovine pasteurellosis. These vaccines have certain limitations, *viz*. alum precipitated vaccine induces local reactions, elicits IgE antibody responses and generally fail to induce cell mediated immunity. Therefore, the duration of immunity induced is only for 4 to 6 months. Alum type adjuvants are not effective for all antigens. Whereas, oil adjuvanted vaccines being too viscous are difficult to inject in animals particularly during herd vaccination and it has site-specific and pyrogenic responses. The commercial vaccines prepared from *P. multocida* serotype B: 2 (P_{52} strain) has been successful in controlling HS but this serotype is generally not involved in swine pasteurellosis. This necessitates the exploration of other vaccine candidates that may ensure adequate protection against *P. multocida* capsular types A and D. The inactivated form of vaccine is widely used throughout the world. The protein is an integral part of the outer membrane of gram-negative bacteria and is found to be associated with immunity (Morton *et al.*, 1995; Pati *et al.*, 1996; Srivastava, 1998). Many different studies on outer membrane proteins (OMPs) have shown that they are major immunogens against homologous challenges in mammal (Gong *et al.*, 2013). The OMPs being non-live

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vaccine candidates require an efficient adjuvant system for eliciting efficient immune response (Cox and Coulter, 1997). An ample amount of work has been carried out on oil and alum adjuvants, which have respective drawbacks of poor syringability and site-specific reactions (Cox and Coulter, 1997). Nanoparticles have recently been shown to possess significant potential as a drug and vaccine delivery system. Studies have shown significant adjuvanticity of calcium phosphate nanoparticles when complexed with whole capsid proteins of virus and recombinant protein of bacteria (He *et al*., 2000; He *et al*., 2002; Tamuly *et al*., 2014). Calcium phosphate is a natural component the body and known to act by depot effect and due to its smaller size, facilitates efficient uptake by macrophages. The potential advantages of calcium phosphate nanoparticles as an adjuvant are-1). Reduce systemic side effect. 2). Efficiently deliver antigens to antigen presenting cells (APCs), especially dendritic cells 3). Provide sustained release of the encapsulated antigens *i.e*. depot effect (Akagi, Baba and Akashi, 2011).

In the present study, the whole outer membrane proteins obtained from capsular types A and D (isolated from upper respiratory tract of pig) or from P_{52} vaccine strain of *P. multocida* were conjugated with calcium phosphate nanoparticles and their protective efficacy was evaluated in mice.

MATERIALS AND METHODS

Place of the work

The work was carried out in Department of Animal Biotechnology, C.V.Sc, AAU, Khanapara, Guwahati in the year 2015.

Bacterial strains and media

The bacterial strains used in the present study were P_{52} strain (capsular type B:2) of *P. multocida*, swine isolates of capsular types A and D of *P. multocida* obtained from the repository of Department of Veterinary Microbiology, College of Veterinary Science, Assam Agricultural University.

Isolation of whole outer membrane protein

The whole outer membrane proteins were purified from revived isolates of capsular types A, B and D of *P. multocida*. The whole OMPs were extracted from all the three strains as per the method described by Choi-Kim *et al.* (1991). The protein was quantified by the method described by Lowry *et al.* (1951). The presence of proteins were documented by one dimensional SDS-PAGE, using 5% stacking gel and 12% separating gel (Sambrook and Russel, 2001).

Preparation of calcium phosphate nanoparticle-adsorbed outer membrane protein conjugate (CAP-Omp)

One mg of OMP (0. 5 mg of OMP each from *P. multocida* of capsular types A and D) was lyophilized in sterile conical flasks. The conjugation of OMPs with calcium phosphate nanoparticle was carried out using the method described by Tamuly *et al.* (2014).

Immunization protocol in mice

The study was approved by the Institutional Animal Ethics Committee of Assam Agricultural University (India). The 24 numbers of Swiss Albino mice were divided randomly into four groups containing six mice in each group as depicted in Table 1. The booster dose was administered on 14th day after primary immunization (dppi). The serum samples were collected weekly up to 28 dppi. The anti-IgG response was determined by indirect ELISA (Tamuly *et al*., 2014).

Challenge studies in mice

All groups were inoculated intra-peritoneally with virulent *P. multocida* capsular types A or D at the dose rate of 100 × LD_{50} on 28th day post primary vaccination. The animals were observed for mortality or any kind of clinical symptoms for 72 hours. The re-isolation of organism was attempted from the heart blood of dead mice.

Statistical analysis

The difference in humoral immunity between the groups were analyzed by two way ANOVA followed by pair-wise t test with Bonferroni correction. The analysis of results of the animal challenge study was performed by Chi-square test with Yates's correction. The analysis was performed with the statistical software R (R Core Team, 2019).

RESULTS AND DISCUSSION

Protein profile of outer membrane protein of *Pasteurella multocida* capsular types A, D and B

Protein profiles of OMPs were studied by employing 12.5% SDS-PAGE. Coomassie brilliant blue stained gel revealed six prominent OMP bands of capsular type A having the relative molecular weights (Mr) ranging from 28.88 to 63.7 kDa; seven prominent OMP bands of capsular type D having Mr ranging from 23.88 to 94.76 kDa and three prominent OMP bands of serotype type B:2 (P_{52} strain) having Mr ranging from 20.38 to 47. 95 kDa. One OMP of Mr 47.95 kDa was found to be commonly expressed in all the three capsular types. On the other hand, the OMP of molecular

Table 1: Groups of mice injected with different vaccine formulations and their route of inoculation.

Groups	Material injected	Route	Dose (per mice)	
			Primary	Booster
Group A	CAP-OMP (A+D)	s/c	100 µg	50 µg
Group B	CAP-OMP (P_{52})	s/c	100 µg	50 µg
Group C	CAP-Void	s/c	-	-
Group D	Alum precipitated bacterin vaccine	i/m	4×10^8 cfu	4×10^8 cfu

weight 28. 88 kDa was found to be commonly expressed in capsular type A and P_{52} strain of *P. multocida* (Fig 1).

Production of *P. multocida* outer membrane protein loaded calcium phosphate nanoparticle

The conjugate of protein and calcium phosphate nanoparticle was characterized by transmission electron microscopy. Majority of nanoparticles were of spherical morphology with diameter ranging from 39.9 nm to 80 nm (Fig 2A). A total of 10 µg protein could be loaded in 1 mg of calcium phosphate nanoparticles. The zeta potential of the CAP-OMP was found to be -22. 7 (Fig 2B).

Induction of anti-IgG response in mice

The mean IgG titre against OMP of capsular type A in the group of mice vaccinated with CAP-OMP- (A+D) increased up to 14 days after the primary vaccination. Following booster vaccination on the 14th day, it increased slightly up to the 21st day which started declining on 28th day post primary vaccination; but there was no significant difference between the IgG titres on 21st day and 28th day ($p>0. 05$).

The mean IgG titre against OMP of capsular type D in the same group increased up to 14 days and was maintained till 28 days as there were no significant difference between the titres recorded on 14th day and 21st day as well as between 21st day and 28th day ($p>0. 05$).

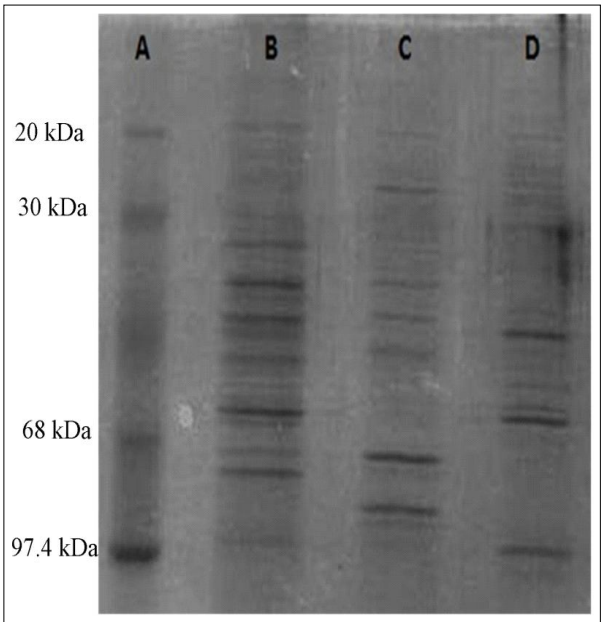
The mean IgG titre against OMP of capsular type A was found to be significantly higher in the group injected with CAP-OMP- (A+D) than that of the group injected with CAP-OMP-(P_{52}) ($p<0.01$). The mean IgG titre of the group of mice injected with bacterin vaccine was found to be significantly lesser compared to that of the other two vaccinated groups (Fig 3A).

The mean IgG response in all the groups against OMP of capsular type D increased up to 7 days post-primary vaccination and then it was maintained up to the 28th day. The mean IgG titre of the alum-adjuvanted bacterin vaccinated group rose up to 14 days but it started declining from 21 days even after booster vaccination on the 14th day post-primary vaccination (Fig 3B).

Challenge study

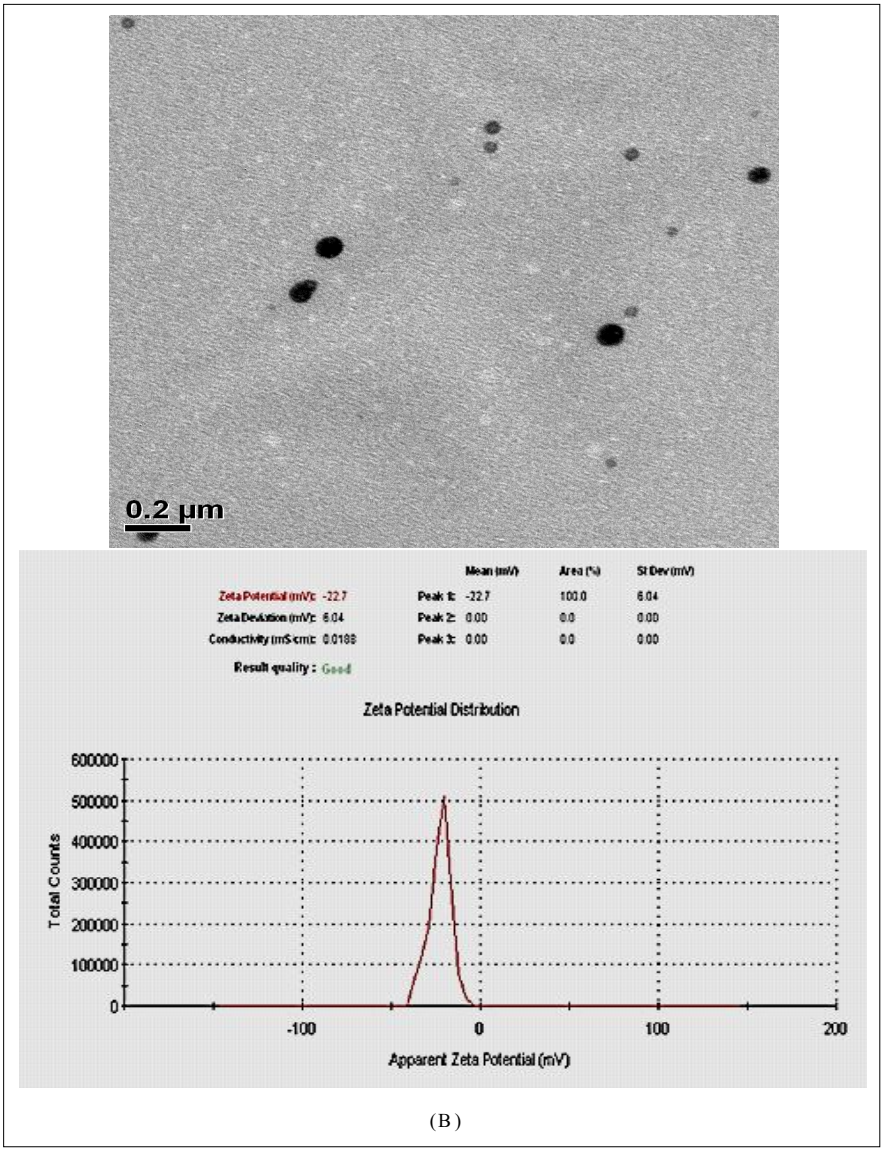
Both the calcium phosphate nanoparticle conjugated vaccines could elicit 100% protection against the homologous challenge but the bacterin vaccine could elicit only 50% and 66.67% protection against *Pasteurella multocida* capsular type A and D respectively. The organism was isolated from the heart blood of dead mice and plated on the blood agar plate that was confirmed by PCR (Fig 4).

The SDS-PAGE banding patterns of OMP of *Pasteurella multocida* of capsular types A, D and B in the present study revealed significant differences which could be the possible reason behind the less efficiency of conventional vaccine prepared from capsular type B (P_{52} strain) in protecting pigs against swine pasteurellosis. As both the capsular types of A and D are involved in swine pasteurellosis, it would be prudent to use a bivalent vaccine containing the immune components of both the capsular types. The OMPs of *P. multocida* have been studied by various workers for their importance in development of subunit vaccine as the OMPs are known to play important role in interaction of bacteria with the host's epithelial cells and in virulence (Pati *et al.*, 1996). The alum based adjuvants have been used for many years but they face the drawback of causing site-specific inflammatory reactions (Prasannavadhana *et al.*, 2014). Nanoparticle based adjuvants efficiently stimulate the antigen uptake by APCs (He *et al.*, 2002; Sahdev *et al.*, 2014). Joyappa *et al.* (2009) reported loading of 50 µg plasmid DNA per mg of calcium phosphate nanoparticle-DNA conjugate while in our study only 10 µg of OMP could. The variation of loading of DNA and protein (as in present study) could be due to the difference in biophysical properties of DNA and protein. The method of preparation of calcium phosphate nanoparticles is easier and can be efficiently used for production of vaccine in industrial scale. It takes nearly 18 hours to prepare the calcium phosphate nanoparticle-OMP conjugate. On the other hand, the double micro-emulsion method described by Bisht *et al.* (2005) for conjugation of calcium phosphate nanoparticle with DNA appears to be more difficult to perform and expensive. In addition, the zeta potential of calcium phosphate nanoparticle were found to be -22.7 indicating its stability in aqueous suspension. The magnitude of zeta-potential is an indicator of electrostatic



(Lane A = molecular marker; Lane B = *P. multocida* capsular type A; Lane C= *P. multocida* capsular type D; Lane D= *P. multocida* capsular type B:2).

Fig 1: Coomassie Brilliant Blue stained SDS-PAGE (12. 5%) profile of outer membrane proteins of *P. multocida* of different capsular types along with standard MW markers.



(B)

Fig 2: (A) Electron micrograph of CAP-OMP complex at one hour stirring (150, 000 × magnification), (B) Zeta potential of CAP-OMP.

repulsion among the particles in dispersion. The smaller zeta-potential indicates instability of particles and can aggregate in dispersion due to higher attractive forces among the particles (Chirayil *et al.*, 2017). The zeta potential ranging from 20-40 mV is considered to have a good stability and are less prone to aggregation (Samimi *et al.*, 2019). In addition, the zeta potential also influences the interaction of nanoparticle with antigen presenting cells (Jia *et al.*, 2018).

The present study has indicated that the vaccine containing bivalent OMPs of types A and D of *P. multocida* elicited better IgG response compared to that of the vaccine formulation containing OMPs of P_{52} strain. This could be due to significant antigenic variations observed in the OMPs of capsular types A, D or B. However, both the vaccine formulations were able to elicit 100% protection against both homologous or heterologous challenges. This could probably be due to the presence of common OMP band of relative molecular weight 47. 95 kilodaltons in all the three capsular types. This protein might be the 47 kDa hypothetical protein reported by Wheeler (Wheeler, 2009), which is known to be expressed by *P. multocida* in wide range of hosts. It was reported to be one of the immunogenic porin proteins that is involved in long chain fatty acid transport across the cell membrane (Prasannavadhana *et al.*, 2014). On the other hand, the bacterin vaccine containing alum adjuvant could not elicit significant level of protection against the challenges either with capsular types A or D. This could be due to masking of the immunogenic OMPs by the lipopolysaccharide of the outer membrane of

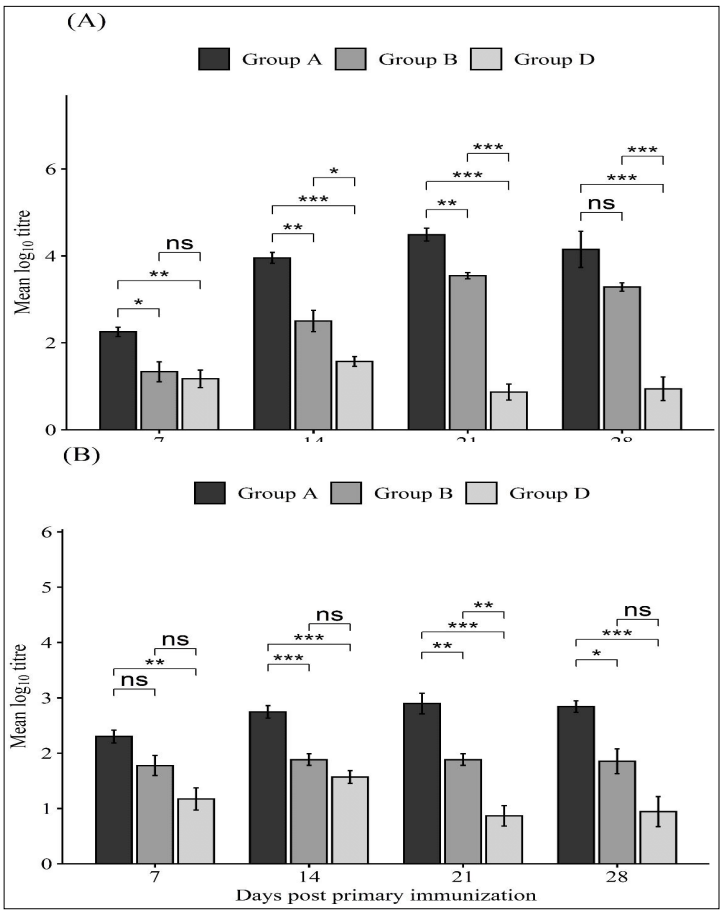
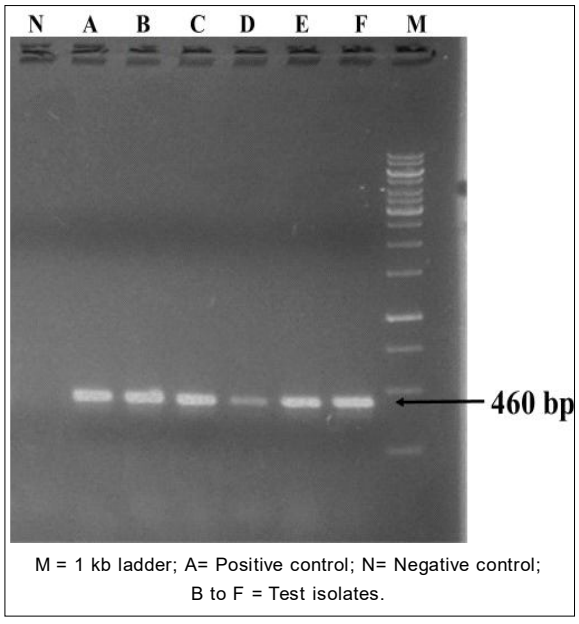


Fig 3: The graphical representation of mean log_{10} IgG titre of different groups of mice against outer membrane protein of *Pasteurella multocida* capsular type A (A) or D (B).



M = 1 kb ladder; A= Positive control; N= Negative control; B to F = Test isolates.

Fig 4: *Pasteurella multocida* specific PCR showing an amplified product of 460 bp in Agarose gel electrophoresis.

the intact inactivated bacteria in addition, many of immunogenic OMPs get denatured during the process of inactivation (Arshadi *et al.*, 2020).

Although both the vaccines containing either the OMP of P_{52} strain or bivalent OMP of capsular types A and D of *P. multocida* showed 100% protection, the later vaccine formulation showed significantly higher IgG response against capsular type A in mice.

CONCLUSION

As the first report of use of bivalent OMP based vaccine using calcium phosphate nanoparticles, the present study has shown that the vaccine formulation could elicit better immune response compared to that of alum adjuvanted bacterin in mice. Further study is required to assess its protective efficacy in pigs against swine pasteurellosis.

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Conflict of interest

All authors declare that they have no conflict of interest.

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