Stimulation of Mucosal and Systemic Antibody Responses against Recombinant Transferrin-binding Protein B of *Actinobacillus pleuropneumoniae* with Chitosan after Tracheal Administration in Piglets

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ABSTRACT: This study evaluated the suitability of using a chitosan formulation as an adjuvant to enhance both the mucosal and systemic immune responses against recombinant transferrin-binding protein B (rTbp B) of *Actinobacillus pleuropneumoniae* via direct tracheal administration. The chitosan formulation was found to enhance mucosal immune response, as measured by the secretory IgA level in lung lavage fluid and lung homogenate extracts, and systemic immune response, as measured by the serum IgG level.

KEY WORDS: *Actinobacillus pleuropneumoniae*, chitosan, transferrin-binding protein.

There are large variations in the efficacy of parenterally (intramuscular, subcutaneous) administered vaccines for respiratory diseases. These types of vaccines are believed to act via these routes primarily by eliciting IgG antibodies, which exude into the lower respiratory tract. However, some studies have shown that both serum IgG and (local) secretory IgA antibodies are essential for effective immunity to the pathogens that cause respiratory diseases [14]. Nasal immunization not only presents antigens at the mucosal surface, where they may produce a local immune response, but the antigens may also pass into the circulatory system via the extensive submucosal blood supply in the nasal cavity and stimulate a systemic response [14]. Adjuvants are substances that enhance the ability of antigens to elicit an immune response [10]. Recombinant proteins are better-defined and safer components in both veterinary and human vaccines. However, they are often weakly immunogenic and require adjuvants to improve their efficacy. The importance of effective adjuvants has been demonstrated by the vast number of studies aimed at developing many adjuvant systems [29].

Chitosan, which is a cationic polysaccharide, is produced by the deacetylation of chitin. Compared with other natural polysaccharides, chitosan has high biodegradability, low toxicity, and bioadhesive properties and is neither an irritant nor an allergen in humans [25]. Toxicological studies have shown that chitosan is non-toxic both locally in terms of its interaction with the nasal membrane and systemically with the body as a whole [13]. Therefore, chitosan might have an ‘adjuvant’ effect on immune response either by increasing the contact time of the various antigens with the relevant tissue and/or by increasing the rate of transport across the membrane, which can provide better presentation of the antigens to the immune cells of the mucosal epithelial and nasal-associated lymphoid tissue (NALT) [14]. Chitosan suspensions or microparticles have been reported to have immune stimulating activity, such as increasing the accumulation and activation of macrophages and polymorphonuclear cells, suppressing tumor growth, promoting resistance to infections by microorganisms, inducing cytokines, augmenting antibody responses, and enhancing delayed-type hypersensitivity (DTH), cytotoxic T lymphocyte (CTL) responses, [2, 6, 11, 20, 21, 24, 26] and T cell responses [4,17]. Chitosan also has uses in transmucosal delivery of drugs and peptides [12].

Iron is one of the nutrients that must be obtained from the host for growth of pathogens. *Actinobacillus pleuropneumoniae* possesses a mechanism consisting of two different proteins that are expressed under iron-limiting conditions, transferrin-binding protein A and B (Tbp A and B), and that are specific for transferrin from the host [23]. Recent observations suggest that proteins of the Tbp family have important vaccine potential and are good candidates for vaccine components [7].

The nasal and tracheal routes for immunization require low doses of antigen and allow efficient and rapid transport of proteins across the epithelium to local lymphoid tissues [22]. This study describes the immune response elicited by administration of rTbp B in conjunction with an adjuvant formulation containing chitosan.

Animal and antigen: Newborn piglets were obtained from an animal farm of the Agro-Bioindustry Technical Support Center, Chonnam National University. The piglets were separated from their sow immediately after birth in order to avoid the transduction of antigen-specific maternal immunoglobulins through the colostrum and were maintained with artificial substitute milk. Preparation of rTbp B, which was found to be immunogenic, is described elsewhere [15].

For the preliminary study, blood samples from the newborn piglets were collected and tested for the absence of *A. pleuropneumoniae* Tbp B-specific maternal IgG and IgA.
Color development was quenched by thorough washing in 0.05% Tween-20, pH 7.4) containing 1% skim milk for 1 hr. The membrane was washed three times with PBST before being incubated with the serum for 1 hr at 37°C. After a further three washes with PBST, the membrane was incubated for 1 hr with HRP-conjugated goat anti-pig IgG (Serotec, Oxford, U.K.) or HRP-conjugated goat anti-pig IgA (Koma Biotechnology, Seoul, Korea). The membrane was again washed three times in PBST, washed once with PBS, and then developed in a diamobenzidine (DAB) substrate buffer containing a DAB concentration (Serotec) until brownish bands were observed. Color development was quenched by thorough washing in PBS.

**Adjuvant preparation:** One ml of vaccine was prepared by mixing 500 µl of PBS containing 200 µg of purified Tbp B with or without 500 µl of a 4% chitosan (Sigma, St. Louis, MO, U.S.A.) in 4% acetic acid solution (final conc. 2% chitosan in 2% acetic acid). Eighteen 7-day-old piglets were divided into 6 groups, as shown in Table 1. The piglets were immunized with/without 100 µg of purified Tbp B in 500 µl via the tracheal or intramuscular (IM) route with/without chitosan on days 1 and 15. The controls were immunized with chitosan only via the 2 routes.

**Serum, lung lavage fluid, and homogenate extracts:** After immunization, the animals were bled by cardiac section under mild anesthesia using Azaperone (Stresnil, Dong-bang, Seoul, Korea), and serum samples were then prepared. The animals were sacrificed by electric shock after collection in 2% acetic acid. Eighteen 7-day-old piglets were immunized with/without 100 µg of purified Tbp B in 500 µl via the tracheal or intramuscular (IM) route with/without chitosan on days 1 and 15. The controls were immunized with chitosan only via the 2 routes.

**Western blot assay and ELISA for IgG and IgA detection:** Tbp B-specific IgG and IgA induction was confirmed by a Western blot assay, as described above. For Tbp B-specific IgA detection, HRP-conjugated goat anti-pig IgG (Koma Biotechnology) was used as the secondary antibody. To check the cross-reactivity of the two antibodies (reactivity of anti-pig IgA with pig IgG and vice versa), HRP-conjugated goat anti-pig IgA was used for detection of Tbp B-specific IgG and HRP-conjugated goat anti-pig IgG was used for detection of Tbp B-specific IgA. The antibody (IgA and IgG) titers were determined by ELISA, as described previously [8]. Briefly, 96-well plates (Maxisorp™, Nunc, Roskilde, Denmark) were coated with rTbp B (1.25 µg/well/90 µl). The plates were blocked with PBS containing 1% skim milk and washed with PBST. The lung lavage fluid and homogenate extracts for IgA titration were prepared with 1:10, 1:100, and 1:1,000 dilution, while the sera for IgG analysis were prepared with 1:100, 1:200, 1:400, and 1:800 dilution. The IgA titration from IM administration was determined using a 1:10 dilution. The antibody samples were incubated in 90 µl PBST for 1 hr at 37°C, the PBST was emptied, and then the samples were washed 3 times with PBST. The bound IgA was detected using HRP-conjugated goat anti-pig IgA (Koma Biotechnology) diluted 1:500, while the bound IgG was detected using HRP-conjugated goat anti-pig IgG (Serotec) diluted 1:500. The secondary antibody was incubated in 90 ml PBST for 1 hr at 37°C, the PBST was emptied, and then the samples were washed 3 times with PBST. Tbp-specific IgG was detected in the lung lavage fluid and homogenate extracts, and Tbp B-specific IgA was detected in the sera.

The presence of any bound secondary antibody was detected using a substrate solution [0.1 M citric acid buffer, pH 4.0, 10 µl; ABTS stock solution (ABTS 100 mg in 4.5 ml DW), 250 µl; H2O2, 50 µl], and the plates were developed in the dark at room temperature for 15 min. The absorbance at 405 nm was read using an ELISA reader (Multiskan EX, Thermo LabSystems, Beverly, MA, U.S.A.). The results are expressed as the mean ± standard deviation (SD) of the end-point OD values at 405 nm. Samples without secondary antibodies were used as a negative control. The differences in the mean antibody values between groups were examined using the Student’s t-test. P values < 0.01 or 0.05 were considered significant. Values lower than the negative control were evaluated as “not induced”.

**Western blot assay:** The absence of maternal immunoglobulins was confirmed by a Western blot assay using serum samples from newborn piglets (data not shown). After immunization via the tracheal route, the IgA antibody responses elicited by the rTbp B/chitosan formulation were

<table>
<thead>
<tr>
<th>Group</th>
<th>Formulation</th>
<th>Administration route</th>
<th>Number</th>
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<tbody>
<tr>
<td>A</td>
<td>Tbp B with chitosan</td>
<td>Tracheal</td>
<td>3</td>
</tr>
<tr>
<td>B</td>
<td>Tbp B with chitosan</td>
<td>Intramuscular</td>
<td>3</td>
</tr>
<tr>
<td>C</td>
<td>Tbp B without chitosan</td>
<td>Tracheal</td>
<td>3</td>
</tr>
<tr>
<td>D</td>
<td>Tbp B without chitosan</td>
<td>Intramuscular</td>
<td>3</td>
</tr>
<tr>
<td>E</td>
<td>Chitosan only</td>
<td>Tracheal</td>
<td>3</td>
</tr>
<tr>
<td>F</td>
<td>Chitosan only</td>
<td>Intramuscular</td>
<td>3</td>
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STIMULATION OF ANTIBODY RESPONSES AGAINST RECOMBINANT TBP B OF A. PLEUROPNEUMONIAE WITH CHITOSAN

stronger than those without chitosan (Fig. 1A). A signal was only observed in the piglet sample immunized with chitosan (Fig. 1A, lane 1). Tracheal route administration with the chitosan formulation induced not only antigen-specific IgA production but also IgG in the serum sample (Fig. 1B), indicating that tracheal administration can also induce a systemic humoral response. Controls showed no antibody responses. No detectable bands were generated when HRP-conjugated goat anti-pig IgA was used for detection of Tbp B-specific IgG and vice versa (data not shown).

ELISA: The recombinant protein vaccine with the chitosan conjugation elicited statistically higher IgA titers when administrated directly via the tracheal route than those without chitosan. Analysis of the anti-Tbp B IgA levels in the lung lavage fluid and homogenate extracts demonstrated that the piglets immunized with the rTbp B/chitosan formulation generated significantly higher antibody titers than the same antigen without chitosan; there was a 4-fold increase in the lung lavage fluid (1:10 and 1:100 dilution, Fig. 2, \( P < 0.01 \)) and a 3-fold increase in the lung homogenate extracts (1:10 dilution, Fig. 3, \( P < 0.01 \)). The serum IgG titers with the chitosan formulation were approximately twice those without chitosan (1:100 dilution, Fig. 4, \( P < 0.05 \)). This confirms the Western blot assay results. Control groups were immunized with chitosan only. Furthermore, the serum IgG titer with the chitosan conjugation was statistically higher than without chitosan conjugation in the intramuscular administration experiment (1:100 dilution, Fig. 5, \( P < 0.01 \)). No Tbp-specific IgG was detected in the lung lavage fluid and homogenate extracts and no Tbp B-specific IgA was detected in the sera (Table 2).

It has been demonstrated that certain soluble antigens,
such as filamentous hemagglutinin from *B. pertussis* [5], can induce immune responses via the nasal route. Most antigens delivered mucosally are poorly immunogenic and can induce immunological tolerance [16]. This study found that nasal administration of rTbp B without chitosan induced very weak immune responses. In contrast, the Tbp B/chitosan formulation had considerably enhanced immunogenicity for tracheal immunization in piglets. In addition, we demonstrated that tracheal immunization of piglets with the rTbp B/chitosan formulation induces both serum and mucosal immune responses. These findings are similar to those of previous reports [1, 9, 17, 27, 28]. The chitosan microparticles showed excellent loading and release characteristics for the protein vaccine [27], which indicates that these microparticles can be used for multiple vaccines. At least three immunizations were previously required to induce a relatively modest antibody response [18]. However, significantly high levels of antibody titers were induced in our study with only one booster dose. The different chitosan concentration (2% in our study vs 0.5% in McNeela *et al.* [18]) might be an explanation for this.

Bacon *et al.* [3] reported that chitosan in PBS substantially enhances the local and systemic immune response against influenza viruses after nasal co-administration with chitosan in solution, which is almost the same as the conditions of our experiment. On the other hand, Jabbal-Gill *et al.*

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**Table 2.** Comparison of the immunoglobulin titers induced by two different administration routes (tracheal and intramuscular) with/without Tbp B or chitosan

<table>
<thead>
<tr>
<th>Administration Route</th>
<th>Source of antibody</th>
<th>IgA&lt;sup&gt;a&lt;/sup&gt;</th>
<th>IgG&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>With chitosan</td>
<td>Without chitosan</td>
</tr>
<tr>
<td>Tracheal</td>
<td>Lung lavage</td>
<td>1.14 (0.07&lt;sup&gt;a&lt;/sup&gt;)</td>
<td>0.28 (0.01&lt;sup&gt;a&lt;/sup&gt;)</td>
</tr>
<tr>
<td></td>
<td>Homogenate extract</td>
<td>0.83 (0.05&lt;sup&gt;a&lt;/sup&gt;)</td>
<td>0.28 (0.02&lt;sup&gt;a&lt;/sup&gt;)</td>
</tr>
<tr>
<td></td>
<td>Serum</td>
<td>N/I&lt;sup&gt;b&lt;/sup&gt;</td>
<td>N/I&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Lung lavage</td>
<td>N/I&lt;sup&gt;b&lt;/sup&gt;</td>
<td>N/I&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Serum</td>
<td>1.00 (0.11&lt;sup&gt;a&lt;/sup&gt;)</td>
<td>0.38 (0.02&lt;sup&gt;a&lt;/sup&gt;)</td>
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a) standard deviation; b) not induced (value lower than the negative control, 0.03); c) IgA=1:10 dilution, IgG=1:100 dilution.

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**Fig. 4.** Results of the ELISA study for comparison of the IgG titers immunized via the tracheal route. Diluted (1:100) sera were used as the primary antibodies for piglets immunized via the tracheal route with (black, left) or without (grey, right) chitosan.

**Fig. 5.** Results of the ELISA study for comparison of the IgG titers. Diluted (1:100) sera were used as the primary antibodies for the piglets immunized via the intramuscular route with (left) or without (right) chitosan.
al. [14] and McNeela et al. [18] reported comparable results against cross-reacting material for diphtheria toxin in mice and guinea pigs, respectively. It is unclear whether the chitosan microparticles in the present are more efficient than those of the chitosan formulations used by these authors because of the differences in the antigens, formulations, and doses used. Nevertheless, it is unlikely that this system is more potent than previously used chitosan formulations because no IgA formed after nasal administration of the present microparticles.

Jabbal-Gill et al. [14] and McNeela et al. [18] reported that chitosan delivery systems might be potent as oral vaccine delivery systems, but not as nasal vaccine carriers. However, considering these [14, 18] and other previous results [3, 17], a chitosan formulation in solution form could be used as a carrier and/or adjuvant for nasal vaccination. When chitosan is administered nasally in combination with a well-known adjuvant, muramyl dipeptide (MDP), the adjuvant formulation results in upregulation of immune response. However, when administered via the intramuscular route, co-administration of chitosan and MDP appears to limit responsiveness to the antigen [19], suggesting that the mechanism of adjuvantisation and adjuvant synergy differ according to the route of immunization and the components used in co-administration.

The precise mechanism by which chitosan enhances the immunogenicity of rTbp B following tracheal immunization is unclear. It has been reported that chitosan can enhance the nasal absorption of polypeptides, such as insulin in rats, and this is believed to be due to transient opening of the tight junctions in the nasal membranes [13, 17]. In a similar manner, chitosan might also increase the absorption of antigens for nasal immunizations, thereby allowing the antigen to gain rapid access to the NALT.

Therefore, chitosan could represent a next generation delivery system for mucosal vaccine administration. When administered directly to piglets via the tracheal route, chitosan appears to enhance both systemic (IgG) and local (IgA) immune response to recombinant protein antigens. However, further research is needed to determine the mechanism of chitosan activity as an adjuvant.

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REFERENCES