Cross Protection Experiment in Mice Immunization with *Actinobacillus pleuropneumoniae* serotype 7 Genomic Expression Library

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**ABSTRACT**

*Actinobacillus* (A.) *pleuropneumoniae* is a causative agent of porcine pleuropneumonia, which has been reported in most countries. A genomic expression library of *A. pleuropneumonia* serotype 7 was constructed to identify potential vaccine candidates. Plasmid DNA from the sub-libraries (L1 to L10) was used to immunize BALB/c mice. Then, 2 weeks after the final immunization, the groups of mice were challenged with *A. pleuropneumonia* serotype 1 (1×10⁹ cfu). Mice immunized with one of the sub-libraries (L3) had a higher level of antibodies and IFN-γ production than others. This sub-library (L3) resulted in a significant reduction in the amount of *A. pleuropneumoniae* recovered from mouse lungs and the survival rate of the L3 group was the most highest. Our study demonstrates that a genome expression library of *A. pleuropneumoniae* serotype 7 offers a novel approach for screening possible cross-serotype protection vaccine candidates against *A. pleuropneumoniae* serotype 1 infection.

**INTRODUCTION**

*Actinobacillus pleuropneumoniae* (A. *pleuropneumoniae*) is a bacterial pathogen that causes both acute and chronic forms of porcine contagious pleuropneumonia (PCP) in swine (Bosse 2002, Ramjeet 2008). Pigs surviving the disease suffer from reduced growth rates and frequently become asymptomatic carriers of the pathogen; they are the main cause of bacterial dissemination. Fifteen different serotypes of *A. pleuropneumoniae* have been identified, and the prevalence of the different serotypes varies...
widely throughout the world, although different serotypes can be isolated in one area or even in one herd (Schaller 2001).

Currently, vaccination is considered to be a potential tool for the prevention of PCP (Ramjeet 2008). Currently, the commercial vaccines for PCP are still primarily killed whole cell bacterins and Apx toxins-based subunit vaccines, which generally reduce mortality from A. pleuropneumoniae infection, but frequently fail to induce cross-serotype immunity (Higgins 1985, Jolie 1995). Live attenuated vaccines (Bei 2007, Piedrafita 1999) and DNA vaccines appear to hold much promise for the future vaccination, especially DNA vaccines, which can induce systemic immune responses, are currently the subject of intense investigation in the field of vaccine research (Chiang 2009, Lu 2011).

But the survival for animals immunized with the DNA vaccine was not significant compared to the groups immunized with commercial inactivated vaccine (Chiang 2009). So more than able to provide cross-protection between serotypes, building a DNA vaccine can provide protection of the antigen should be selected. Many new approaches have been used in the last decade to identify potential bacterial components to be included in subunit vaccines or potential genes to be inactivated in live vaccine strains (Baltes 2004, Fuller 1999).

A high-throughput genomics methodology for vaccine design, genomic expression library immunization (ELI) was first reported by Barry et al in 1995 (Barry 1995). Since the first study, other research has shown identical results in a wide range of pathogens and challenge animal models (Fachado 2003). An expression library and a phage expression library of the A. pleuropneumoniae genome were constructed and screened to identify potential vaccine components (Gerlach 1992).

Here, we study the feasibility of ELI to induce a cross-serotype protective immune response against A. pleuropneumoniae. The purpose of the present work was to generate a genomic library of A. pleuropneumoniae serotype 7 and to analyze the efficacy of vaccination with this library in eliciting cross-serotype protective immunity.

**MATERIALS AND METHODS**

Genomic DNA was extracted from A. pleuropneumoniae serotype 7 L25-4 (Chinese field isolate). A genomic expression library was constructed by partially digesting genomic DNA of A. pleuropneumoniae serotype 7 L25-4 with Sau3A I restriction endonuclease (NEB, Ipswich, MA) to obtain 1- to 2-kb fragments. The vector pcDNA3.1(+) was digested with restriction endonuclease BamHI. The genomic fragments and digested vector were were purified using a gel extraction mini kit (Shanghai Watson Biological Engineering Company, Shanghai, China) and transformed cells were plated on Luria-Bertani (LB)-ampicillin agar so that an average of about 1,000 colonies grew on each 50-mm plate.

A total of 10 plates, termed “sub-libraries,” were selected for this study Colonies from each sub-library were harvested from each plate. Plasmids were purified from each sub-library using an EndoFree plasmid maxi kit and were quantified by spectrophotometer at 260 nm.

Eleven groups of 6-8 weeks old female BALB/c mice (10 mice each) were randomly assigned and 10 groups were immunized in both tibialis anterior muscles with 100 µg of the DNA for each sub-libraries three times at 2-week intervals. The control group were given empty vector pcDNA3.1(+)(EV). Animal experiments were performed in accordance with animal ethics guidelines and approved protocols. The Animal Ethics Committee approval number was Heilongjiang-SYXK 2006-032.

After immunization, blood samples were drawn from the vaccinated mice and serum was obtained. The serum antibody titers were determined using indirect enzyme linked immunosorbent assay (ELISA) (Furesz 1997). ELISA microtitre plates were coated with A. pleuropneumoniae serotype 1 strain 72-1 (Chinese field isolate) total
antigens (10 μg/ml) (Magnusson 1997). The levels of IFN-γ were determined using interferon-γ Test Kits (Dalian Pan-State Biotechnology Company, Dalian, China). Two weeks after the third immunization, mice were challenged intraperitoneally with 1×10^9 colony forming units (CFU) of 72-1 (serotype 1). The number of the surviving mice was observed and recorded 5 days post-challenge. Two of each group animals were sacrificed on day 6 post-challenge. The left lungs were extracted from the mice and separated into two parts. One part was homogenized in PBS. Each homogenate was serially dilated in PBS and were plated on PPLO agar plates incubated at 37°C for 48 h, then the number of CFU was counted. The other part of the lung was fixed by formalin followed by hematoxylin and HE staining for the observation of histological changes using light microscopy (Olympus, Tokyo, Japan).

The data from the experiments were expressed as the mean ± standard deviation (S.D.). All data analysis was performed with SAS software (Version 9.0; SAS Inst., Cary, NC) to compare the differences between different groups. P-values <0.05 were considered statistically significant; P-values <0.01 were considered highly statistical significant.

RESULTS

An *A. pleuropneumoniae* serotype 7 L25-4 was constructed and a total of 10 sub-libraries were selected for this study, with an average of 1,000 clones.

As the results showed in Fig 1, after immunization eight sub-libraries induced antibodies, and the titers were significantly higher in the L3, L7, L2, and L6 groups than the control groups (P <0.01). Sera from mice vaccinated with sub-libraries L4 and L8 showed no detectable antibody titers compared to the control group. The antibody level of the sera from mice immunized with sub-library L3 was significantly higher than...
the other groups.

The concentration of IFN-γ in each group was summarized in Figure 2. On first immunization (FI), the IFN-γ levels in the mice immunized with sub-library L1-L3, L6, L7, and L9 were significantly different from the control group (P < 0.01), and the differences were increased on second immunization (SI) and third immunization (TI). Furthermore, the IFN-γ levels between L4, L8, and the control group was not statistically significant (P > 0.05).

Two of immunized animals in each group were sacrificed on day 6 post-challenge. The mice in the L8 and the empty vector groups all died within 5 days post-challenge, so the data were not available. In addition, the bacterial loads in sub-libraries L1-L4, L6-L7, and L9-L10 were compared with L5. The viable bacteria in L1 and L4 were not lower than L5 (P > 0.05). Furthermore, in L2, L3, L6, and L7 the numbers of the bacteria recovered from the lungs of mice substantially reduced compared with L5 (P < 0.01). And the sub-library L3 showed the lowest colonization compared with other groups (Table 1).

<table>
<thead>
<tr>
<th>Immunization groups</th>
<th>Lung CFU/mg (mean ± SD)</th>
<th>No. of survivors (%)</th>
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<tbody>
<tr>
<td>L1</td>
<td>29433 ± 2515</td>
<td>20 C</td>
</tr>
<tr>
<td>L2</td>
<td>1073 ± 155 B</td>
<td>60 C</td>
</tr>
<tr>
<td>L3</td>
<td>203 ± 31 B</td>
<td>70 C</td>
</tr>
<tr>
<td>L4</td>
<td>30763 ± 2692</td>
<td>30 C</td>
</tr>
<tr>
<td>L5</td>
<td>34206 ± 2692</td>
<td>30 C</td>
</tr>
<tr>
<td>L6</td>
<td>1306 ± 190 B</td>
<td>50 C</td>
</tr>
<tr>
<td>L7</td>
<td>4577 ± 280 B</td>
<td>40 C</td>
</tr>
<tr>
<td>L8</td>
<td>-</td>
<td>0</td>
</tr>
<tr>
<td>L9</td>
<td>24657 ± 2201 A</td>
<td>30 C</td>
</tr>
<tr>
<td>L10</td>
<td>26760 ± 2638 A</td>
<td>30 C</td>
</tr>
<tr>
<td>Empty vector</td>
<td>-</td>
<td>0</td>
</tr>
</tbody>
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Table 1. Viable bacteria recovered from mice after challenged
Note: The mice in L8 and empty vector groups all died within 5 days post-challenge, so the data were not available in the table. A and B indicates p < 0.05 and p < 0.01 compared the viable bacteria with sub-library L5 treated, respectively; C indicates p < 0.01 compared percentages of survivors with empty vector group.

The number of surviving mice in each group was recorded 6 days post-challenge. Ten mice all died after 5 days post-challenge in the L8 and the empty vector groups. The survival of the other sub-libraries L1 to L7, L9, and L10 (>20%) was higher than the empty vector group (0%). And seven percent mice (70%) survived in the L3 and six percent mice (60%) in L2 group. The results indicate that the sub-library L3 can significantly improve the survival rate than the other sub-libraries (Table 1).

The mice in L8 and the empty vector groups all died after challenged, there were not the histopathologies. In L3 the HE-stained lung tissue sections revealed inconspicuous pathologic alterations (Fig. 3). The L1, L4, and L10 groups exhibited severe pathologic alterations. This included fibrinous, lung haemorrhage and fibrin exudation. The L2 group exhibited relatively mild pathologic alterations, i.e., mild congestion of small blood vessels. Pathological changes were more severe in the L5-L7 and L9 groups. The L5 and L6 groups presented congestion and mild fibrin exudates, and the L7 and L9 sub-library-vaccinated groups showed alveolar fibrin exudates.
DISCUSSION
Currently, 15 serotypes of *A. pleuropneumoniae* have been described, and these serotypes show significant differences in pathogenicity and immunogenicity (Blackall 2002, Cruijisen 1995, Hae-sebrouk 1996). Therefore, vaccines against a specific serotype do not confer protection from infection by other serotypes (Ramjeet 2008). At the same time as inactivated whole-cell bacterial vaccines appeared, gene deletion live vaccines, protein subunit vaccines, DNA vaccines and other kinds of new vaccines also began to appear (Byrd 1992, Jansen 1995, Tonpitak 2002). Consequently, recent research on *A. pleuropneumoniae* vaccination has mainly focused on finding antigens that are highly conserved among all serotypes and could be purified and used as potential subunit vaccines to overcome the problem of failed of cross-protection. ELI has the advantage of almost all of the genome of a pathogen antigen being presented simultaneously rather than one or several antigenic components (Donnelly 1997).

The data presented here demonstrate that the application of ELI is a viable approach against *A. pleuropneumoniae* because immunization with *A. pleuropneumoniae* serotype 7 genomic libraries induced antibodies that afford cross-serotype protection in mice. There have been reports of using *A. pleuropneumoniae* genomic expression library immunization and screening to obtain a new virulence protein (Gerlach 1992). However, this is the first study, to our knowledge, that uses ELI of *A. pleuropneumoniae* serotype 7 for generating a functional immune response against *A. pleuropneumoniae* serotype 1.

Figure 3. Histopathology of lung from immunized mice after challenge (HE staining; 200× magnification). In L8 and empty vector groups the mice all died within 5 days post-challenge, so there were not the histopathologies. L1-L7 and L9-L10: L1-L7 and L9-L10 group respectively. N: one figure from a normal mice lung.

The capacity of DNA vaccination to generate a strong humoral and cellular immune response has been described (Piedrafita 1999, Donnelly 1997, Rainczuk 2003).

In our study vaccination of mice with *A. pleuropneumoniae* serotype 7 sub-libraries induced a humoral immune response to *A. pleuropneumoniae* serotype 1 antigens; this was characterized by the presence of specific IgG in sera. The IFN-γ secretion levels from the sera of sub-library-immunized mice demonstrated that the sera from these mice secreted a certain amount of IFN-γ. In our experiments, the reduction of CFU in lungs after challenge could be, to some extent, due to the in vivo expression of several *A. pleuropneumoniae* serotype 1 antigens. Inoculated DNA itself can also induce antigen-specific T lymphocytes that secrete IFN-γ and show cytotoxic potential (Piedrafita 1999, Furesz 1997).

The effective immune response elicited after immunization with our genomic library is specific. The animals that were vaccinated with the 10 sub-libraries were infected with *A. pleuropneumoniae* serotype 1, and the survival of three of the sub-library-treated mice (L3, L2, L6) was significantly higher than in the other groups. In particular, the 10
mice that were treated with the sub-library L3 plasmid had only three deaths. This means that sub-library L3 shows resistance to *A. pleuropneumoniae* serotype 1 infection in terms of antigen components present, and these antigen components have been expressed.

In summary, we have obtained a sub-library (L3) of *A. pleuropneumonia* serotype 7 that induced a strong cross-serotype antibody response and conferred significant cross-serotype protection against *A. pleuropneumoniae* serotype 1. Although the cross-serotype protection is partial, it is substantially greater than what has been achieved previously. Our results open the door for the application of ELI to identify and characterize new cross-serotype protective antigens against *A. pleuropneumonia* infection; and support the original idea that ELI might be a generally applied vaccine discovery system.

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**REFERENCE**


