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 (1×10^9 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 word, 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* serotype7 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

Figure 1. *Serum antibody levels were measured by indirect ELISA two-weekly until 6 weeks. L1-L10: sub-libraries L1-L10 respectively; EV: empty vector pCDNA3.1(+).**, p<0.01 compared antibodies levels of sub-library-treated mice with empty vector-treated mice.*
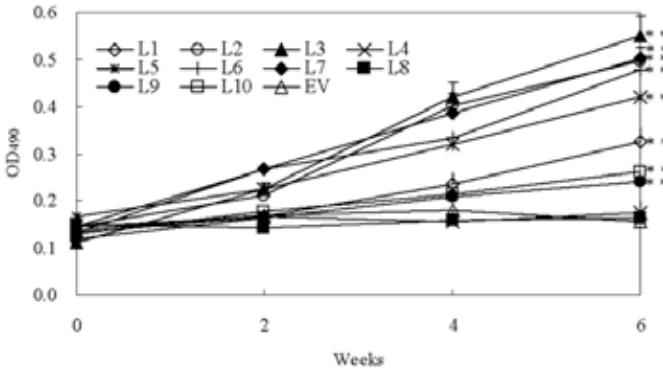
antigens (10 μg/ml) (Magnusson 1997). The levels of IFN-γ were determined using interferon-γ Test Kits (Dalian Pan-State Biotechnology Company, Dalian, China).

Two weeks after the third immunization, mice were challenged intraperitoneally with 1×109 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

Figure 2. *IFN-γ levels of the serum from peripheral blood detected by indirect ELISA. L1-L10: sub-libraries L1-L10; EV: empty vector pCDNA3.1(+). FI: first immunization; SI: second immunization; TI: third immunization. *, p<0.05, and ** p<0.01 compared IFN-γ levels of sub-libraries groups with empty vecto group.*
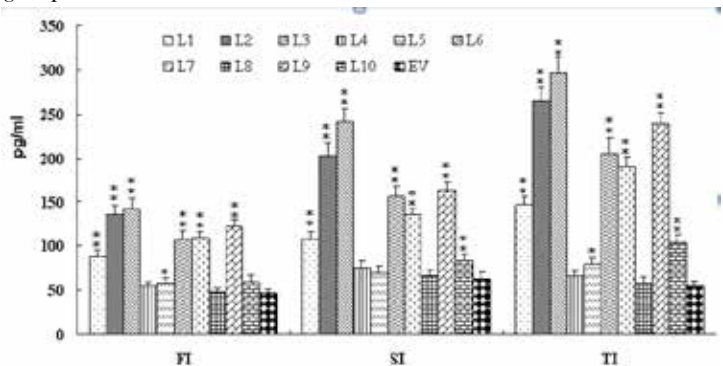
Table 1. *Viable bacteria recovered from mice after challenged*
Note: The mice in L8 and empty vector groups all died within 5 days post-challenge, so the data were not available in the table. A and B indicates p<0.05 and p<0.01 compared the viable bacteria with sub-library L5 treated, respectively; C indicates p<0.01 compaed percentages of survivors with empty vector group.

Immunization groups	Lung CFU/mg(mean ± SD)	No. of survivors (%)
L1	29433 ± 2515	20 C
L2	1073 ± 155 B	60 C
L3	203 ± 31B	70 C
L4	30763 ± 2692	30 C
L5	34206 ± 2692	30 C
L6	1306 ± 190 B	50 C
L7	4577 ± 280 B	40 C
L8	-	0
L9	24657 ± 2201 A	30 C
L10	26760 ± 2638 A	30 C
Empty vector	-	0

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 imminization (SI) and third imminization (TI). Furthermore, the IFN-γ levels between L4, L8 and the control group was not statisically 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).

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.

10

DISCUSSION

Currently, 15 serotypes of *A. pleuropneumoniae* have been described, and these serotypes show significant differences in pathogenicity and immunogenicity (Blackall 2002, Cruijsen 1995, Haesebrouk 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 lunge.
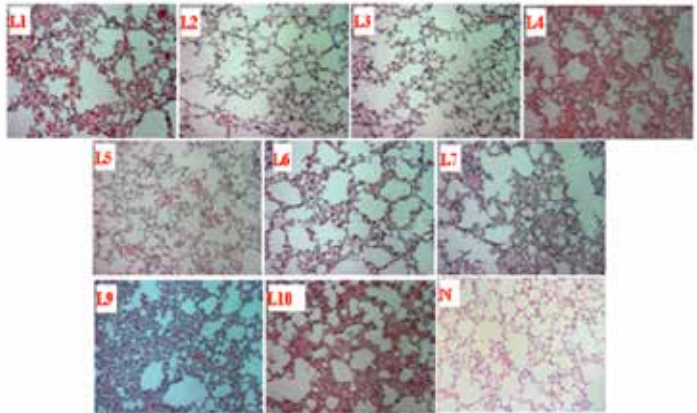
The capacity of DNA vaccination to generate a strong humoral and cellular immune response has been described (Piedrafita 1999, Donnelly 1997, Rainczuk 2003).

In our study vaccination of mice with *A. pleuropneumoniae* serotype 7 sub-libraries induced a humoral immune response to A. leuropneumoniae 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.

ACKNOWLEGEMENT

REFERENCE

1. Baltes N, Gerlach GF (2004) Identification of genes transcribed by *Actinobacillus pleuropneumoniae* in necrotic porcine lung tissue by using selective capture of transcribed sequences. *Infect Immun* 72 (11): 6711-6716.

2. Barry MA, Lai WC, Johnston SA (1995) Protection against mycoplasma infection using expression-library immunization. *Nature* 377 (6550): 632-635.

3. Bei W, He Q, Zhou R, Yan L, Huang H, Chen H (2007) Evaluation of immunogenicity and protective efficacy of *Actinobacillus pleuropneumoniae* HB04C(-) mutant lacking a drug resistance marker in the pigs. *Vet Microbiol* 125 (1-2): 120-127.

4. Blackall PJ, Klaasen HL, van den Bosch H, Kuhnert P, Frey J (2002) Proposal of a new serovar of Actinobacillus pleuropneumoniae: serovar 15. *Vet Microbiol* 84 (1-2): 47-52.

5. Bosse JT, Janson H, Sheehan BJ, Beddek AJ, Rycroft AN, Kroll JS, Langford PR (2002) Actinobacillus pleuropneumoniae: pathobiology and pathogenesis of infection. *Microbes Infect* 4 (2): 225-235.

6. Byrd W, Harmon BG, Kadis S (1992) Protective efficacy of conjugate vaccines against experimental challenge with porcine Actinobacillus pleuropneumoniae. *Vet Immunol Immunopathol* 34 (3-4): 307-324.

7. Chiang CH, Huang WF, Huang LP, Lin SF, Yang WJ (2009) Immunogenicity and protective efficacy of ApxIA and ApxIIA DNA vaccine against *Actinobacillus pleuropneumoniae* lethal challenge in murine model. *Vaccine* 27 (34): 4565-4570.

8. Cruijsen T, van Leengoed LA, Ham-Hoffies M, Verheijden JH (1995) Convalescent pigs are protected completely against infection with a homologous *Actinobacillus pleuropneumoniae* strain but incompletely against a heterologous-serotype strain. *Infect Immun* 63 (6): 2341-2343.

9. Donnelly JJ, Ulmer JB, Shiver JW, Liu MA (1997) DNA vaccines. *Annu Rev Immunol* 15: 617-648.

10. Fachado A, Rodriguez A, Molina J, Silverio JC, Marino APMP, Pinto LMO, Angel SO, Infante JF, Traub-Cseko Y, Amendoeira RR, Lannes-Vieira J (2003) Long-term protective immune response elicited by vaccination with an expression genomic library of Toxoplasma gondii. *Infect Immun* 71 (9): 5407-5411.

11. Fuller TE, Shea RJ, Thacker BJ, Mulks MH (1999) Identification of in vivo induced genes in Actinobacillus pleuropneumoniae. *Microb Pathog* 27 (5): 311-327.

12. Furesz SE, Mallard BA, Bosse JT, Rosendal S, Wilkie BN, MacInnes JI (1997) Antibody- and cell-mediated immune responses of Actinobacillus pleuropneumoniae-infected and bacterin-vaccinated pigs. *Infect Immun* 65 (2): 358-365.

13. Gerlach GF, Anderson C, Potter AA, Klashinsky S, Willson PJ (1992) Cloning and expression of a transferrin-binding protein from Actinobacillus pleuropneumoniae. *Infect Immun* 60 (3): 892-898.

14. Haesebrouk F, Van de Kerkhof A, Dom P, Chiers K, Ducatelle R (1996) Cross-protection between *Actinobacillus pleuropneumoniae* biotypes-serotypes in pigs. *Vet Microbiol* 52 (3-4): 277-284.

15. Higgins R, Lariviere S, Mittal KR, Martineau GP, Rousseau P, Cameron J (1985) Evaluation of a Killed Vaccine Against Porcine Pleuropneumonia Due to Haemophilus pleuropneumoniae. *Can Vet J* 26 (2): 86-89.

16. Jansen R, Briaire J, Smith HE, Dom P, Haesebrouck F, Kamp EM, Gielkens ALJ, Smits MA (1995) Knockout mutants of *Actinobacillus pleuropneumoniae* serotype 1 that are devoid of RTX toxins do not activate or kill porcine neutrophils. *Infect Immun* 63 (1): 27-37.

17. Jolie RA, Mulks MH, Thacker BJ: Cross-protection experiments in pigs vaccinated with *Actinobacillus pleuropneumoniae* subtypes 1A and 1B. *Vet Microbiol* 1995, 45 (4): 383-391.

18. Lu YC, Li MC, Chen YM, Chu CY, Lin SF, Yang WJ (2011) DNA vaccine encoding type IV pilin of *Actinobacillus pleuropneumoniae* induces strong immune response but confers limited protective efficacy against serotype 2 challenge. *Vaccine* 29 (44): 7740-7746.

19. Magnusson U, Bosse J, Mallard BA, Rosendal S, Wilkie BN (1997) Antibody response to *Actinobacillus pleuropneumoniae* antigens after vaccination of pigs bred for high and low immune response. *Vaccine* 15 (9): 997-1000.

20. Piedrafita D, Xu D, Hunter D, Harrison RA, Liew FY (1999) Protective immune responses induced by vaccination with an expression genomic library of Leishmania major. *J Immunol* 163 (3): 1467-1472.

21. Rainczuk A, Scorza T, Smooker PM, Spithill TW (2003) Induction of specific T-cell responses, opsonizing antibodies, and protection against Plasmodium chabaudi adami infection in mice vaccinated with genomic expression libraries expressed in targeted and secretory DNA vectors. *Infect Immun* 71 (8): 4506-4515.

22. Ramjeet M, Deslandes V, Goure J, Jacques M (2008) *Actinobacillus pleuropneumoniae* vaccines: from bacterins to new insights into vaccination strategies. *Anim Health Res Rev* 9 (1): 25-45.

23. Schaller A, Djordjevic SP, Eamens GJ, Forbes WA, Kuhn R, Kuhnert P, Gottschalk M, Nicolet JL, Frey J (2001) Identification and detection of *Actinobacillus pleuropneumoniae* by PCR based on the gene apxIVA. *Vet Microbiol* 79 (1): 47-62.

24. Tonpitak W, Baltes N, Hennig-Pauka I, Gerlach GF (2002) Construction of an *Actinobacillus pleuropneumoniae* serotype 2 prototype live negative-marker vaccine. *Infect Immun* 70 (12): 7120-7125.