Synthesized swine influenza NS1 antigen provides a protective immunity in a mice model

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ABSTRACT

Background: Swine influenza is an important infectious disease caused by an RNA virus of the Orthomyxoviridae family. It is a real threat to the porcine industry due to its devastating effect. Although the anti-influenza drugs are available, vaccination is the best choice for the disease control. The continuous occurrence of genetic mutations necessitates developing of stable and safe vaccines have the ability to induce both of cellular and humoral immunity.

Objectives: The aim of this study was to evaluate the protective effect of recombinant probiotic in a model mice.

Methods: Accordingly, lactobacillus pSIP409-pgsA-NS1 bacteria was constructed based on the conserved non-structural (NS1) protein. Enzyme-linked immunosorbent assay and flow cytometry techniques were used to determine the host immune response.

Results: The results revealed that, immunization of mice with the constructed pSIP409-pgsA-NS1 resulted in eliciting a systemic and mucosal humoral response in the form of increasing IgG, IgA, and B220+ B cells production adding to inducing up-regulation of the CD8+ T cells and the immunomodulatory cytokines (interleukin-4 and tumor necrosis factor alpha).

Conclusions: The matter that necessities further assessment in a porcine model to confirm the complete safety, and efficiency against the swine influenza virus infection.

Keywords: Lactobacillus plantarum; swine influenza virus; NS1 protein; immune response

INTRODUCTION

Presence of the influenza viruses is an actual threat to the worldwide health, the occurrence of genetic shift and drift is the main cause for evolving of mutant subtypes which results in high devastating outbreaks [1].

The high susceptibility of pigs to be infected with the different influenza viruses including the avian and the human types is due to presence of the α-2,3 and α-2,6 sialic acids receptors in the respiratory epithelium [2] which resulted in emergence of stable swine types with an
Recombinant probiotic provides protection against influenza virus

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Conflict of Interest
The authors declare no conflicts of interest.

Funding
This work was supported by the National Key Research and Development Program of China (2017YFD0500100, 2017YFD0501200), National Natural Science Foundation of China (31672528, 31972696, 31941018), Science and Technology Development Program of Jilin Province (20180520037JH, 20180200404NY, 20190301042NY), the Key Funds for Agriculture Ministry Key Laboratory of Healthy Freshwater Aquaculture (ZJK201808), "Thirteen Five-year Plan” for Sci & Tech Research Program of Jilin Education Department of P.R. China (JJKH20190943KJ).

important effect on both of the animal and human beings [3]. The phylogenetic studies of the different influenza viruses clarified intermingling of porcine isolates with avian or human isolates [4].

Most of the epidemiological studies conducted in Asia especially the South East Asian countries revealed the infection of pigs with different lineages including H3N2, H9N2, H1N2 while the H1N1 was the most predominant type [3,5-7]. Swine influenza H1N1 viruses which belong to the Orthomyxoviridae family are causing respiratory disorders, poor growth rate, low body weight gain, and immunosuppression of swine [8]. The occurrence of swine influenza infection depends mainly on different risk factors including; the severity of the viral strain, host age and sex, presence of secondary infections, biosecurity and management system of the farms [9].

The genome of swine influenza is a single-stranded RNA of negative-sense which encodes different proteins [10]. The non-structural protein 1 (NS1) is a polyfunctional virulence factor of the influenza viruses and having a significant role in its replication, virulence, and prevention of the host antiviral immune response [11] adding to the role as a post-transcriptional regulator in the influenza virus life cycle [12]. It has 2 main domains; the first is an RNA-binding domain found in the N-terminal region with a role in virus protection against the antiviral status produced by IFN α/β. While the second C-terminal one is an effector domain contains a number of other domains [13].

The role of the non structural proteins in the pathogenesis of the different viruses was elucidated previously [14,15]. Using of the NS1 as a base for immunization against its corresponding virus was optimistic. A single dose of immunization with non structural protein based Zika virus vaccine candidate not only provoked humoral and cellular responses but also provided protection against challenging with a lethal dose in mice model [14]. Also, using of the mutant NS1 swine influenza virus vaccines elicited cell-mediated cross-protection against antigenically divergent strains [16]. At the same side, Solorzano et al. (2005) cleared that all NS1 mutant swine influenza viruses were attenuated in pigs and such mutated viruses may have a great potential as live attenuated vaccine candidates against the swine flu in pigs [15].

Several reports discussed the function of dendritic cells (DCs) in inducing T cell polarization, B cells activation, scavenging and presenting of foreign antigens. Accordingly, it was considered to be the most potent functional antigen presenting cells (APCs) [17,19].

Successful using of Lactobacillus plantarum including the pgsA gene and the DCpep system for producing of an exogenous protein and subsequent using as an effective vaccine against some of the important infectious diseases was proved in previous studies [17,20]. It was reported that, using of lab animal models in studying the pathogenesis of some infectious microbes, host immune response, assessment of the newly produced vaccines and medications is necessary [21,22].

Therefore, a pgsA was used to anchor the NS1 protein of swine influenza virus (SIV) on lactobacillus, pSIP409-pgsA-NS1/NC8 displayed the NS1 protein has been constructed previously [23] as a candidate for developing a safe and an effective swine flu oral formulations (vaccine), the immune response was determined in a mice model using the flow cytometry and enzyme-linked immunosorbent assay (ELISA).
MATERIALS AND METHODS

Ethics statements for experimental mice

Six-week-old female C57BL/6 mice were purchased from Beijing Huafukang biotechnology Co., Ltd. Conduction of mice experimentation was done and evaluated according to the Animal Care and Ethics Committees of Jilin Agriculture University number (JLAU20140627). All applicable international and national guidelines for the care and use of mice were followed.

Construction of recombinant bacteria

The pMD18-T-NS1 and pMD18-T-pgsA-NS1 were kept in our laboratory. The NS1 gene was from the A/swine/Tianjin/3/2011(H1N1) strain. The *Escherichia coli*-*Lactobacillus* shuttle plasmid pSIP-409 and *L. plantarum* NC8 were kindly provided by A.Kolandaswamy (Madurai Kamaraj University, India). The pMD18-T-NS1, pMD18-T-pgsA-NS1 and pSIP-409 plasmids were digested with *Xba*I and *Hind*III. Purification of the pgsA-NS1 (or NS1) and linearized plasmid pSIP-409 were ligated using a T4 DNA ligated kit (TransGen, Beijing, China) and transformed into *E. coli* Trans5α competent cells (TransGen, Beijing, China). The positive pSIP-409-NS1 and pSIP-409-pgsA-NS1 plasmids were transformed into *L. plantarum* NC8 by electroporation.

Vaccination and challenge

Ninety female C57BL/6 mice of 6-weeks-old were randomly divided into 5 groups (n=18) as follow: PBS group, pSIP409-pgsA group, pSIP409-NS1 group, pSIP409-pgsA-NS1 group and inactivated vaccine group, respectively. Briefly, the lactobacillus was induced overnight in each group, and the bacteria precipitate was collected at 6,000 rpm for 5 minutes at the next day followed by washing with a sterile PBS three times. The mice in each group were treated with intensive feeding (1.0 × 10⁹ CFU/200 μL). The first immunization time was at the first, the third, and the fifth day. While, the strengthening time was at the 20th, 22th, and the 24th day. In the PBS group, 200 μL PBS was administered as a negative control. In the first day, the inactivated vaccine group was given intramuscular inactivated vaccines of H1N1 subtype influenza (15 μg each of the respective hemagglutinin antigens: A/California/7/2009 (H1N1) pdm09 virus) as a positive control (Fig. 1). Serum, tracheal flushing fluid, ileal contents and fresh excreta were taked from all groups two weeks after booster vaccination.

After 2 weeks of immunization, the mice in each group were anesthetized by intraperitoneal injection with pentobarbital. Each mouse was infected with a 1 × 10⁵ EID₅₀ H1N1 subtype of influenza virus (A/Puerto Rico/8/1934) by nasal drip. Weight changes in the mice were monitored and the death of the mice was recorded daily. To detect the lung viral titer, samples were taked from animal at 5 days after challenge and was titrated in SPF embryonated chicken eggs.

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**Percent identity**

| Divergence | A  | B  | C  |
|-----------|----|----|----|
| A         | 82.2 |    | 46.9 |
| B         | 28.8 |    | 40.6 |
| C         | 32.7 |    | 47.9 |

*Fig. 1. Comparisons of the NS1 amino acid similarity of among influenza viruses.*

A is the NS1 from A/California/7/2009(H1N1) strain, B is the NS1 from A/PuertoRico/8/1934(H1N1) and C is the NS1 from A/swine/Tianjin/3/2011(H1N1). The NS1 amino acid similarity of different viruses were compared using DNAStar software.

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ELISA
Specific antibodies were tested according to the published method [17]. One hundred μL of the NS1 antigen (4 μg/mL) was wrapped up in a 96-well plate followed by overnight incubation at 4°C. The 96-well plate was then blocked with 1% bovine serum albumin (BSA) in PBST buffer (PBS with 0.1% Tween-20) at room temperature for one hour. After washing, all samples (100 times dilution) were added and incubation at 37°C for one hour was conducted. Horseradish peroxidase (HRP) goat anti-mouse IgG or IgA (Sigma) was added for one hour at 37°C. After washing the plate, 100 μL 3,3-,5,5-tetramethylbenzidine (TMB, Sigma) was incubated for 15 minutes at 37°C. The last 50 μL 2 N-H2SO4 was used to terminate the enzymatic reaction. All samples were tested at OD492 nm.

Flow cytometry
PP cells were separated from groups of mice and single cell suspension was prepared according to published methods [20]. 1 × 106 cells were incubated with the monoclonal antibodies PE-B220 (clone RA3-6B2), FITC-CD3 (clone 145-2C11), APC-CD4 (clone OX-35), and PE-CD8 (clone 53-6.7) (BD Pharmingen). The suspension was mixed and incubated at 4°C with light avoidance for 30 min. After the ending of the incubation, washing with FACS twice was carried out before measuring with the flow cytometry instrument detection (BD LSR Fortessa, USA).

Cytokine assay
The level of the tumor necrosis factor alpha (TNF-α) and interleukin (IL)-4 was determined using the ELISA (R&D). The ELISA method was following the previously published protocol [24]. The linear regression equation of the standard curve was calculated. Finally, the corresponding sample concentration was obtained.

Statistical analysis
All data were analyzed using GraphPad Prism 5.0 software. Significance was evaluated using the analysis of variance (ANOVA). The value of p < 0.05 means a significant difference.

RESULTS
Recombinant bacteria trigger the production of antigen-specific IgG in the immunized mice
The specific IgG response against the non-structural protein was detected in the serum of mice using ELISA (Fig. 2). The obtained results showed a presence of a marked increase in the titer of the pSIP409-pgsA-NS1 group, in comparing with the groups immunized with PBS (p < 0.001), pSIP409-pgsA (p < 0.001), pSIP409-NS1 (p < 0.05) respectively. But, there was no significant difference with the inactivated vaccine group. The obtained data elucidated that, oral gavage immunization of mice with pSIP409-pgsA-NS1 significantly induced the production of specific IgG antibodies against the NS1.

Recombinant bacteria enhance secretory IgA production in the immunized mice
ELISA was used to determine the level of the produced secretory IgA. Regarding the upper respiratory tract cleaning fluid of mice in the different immunized groups, it was cleared that the titer of the SIgA was found to be significantly high in the pSIP409-pgsA-NS1 group, in comparing with the groups immunized with PBS (p < 0.001), pSIP409-pgsA (p < 0.001),
pSIP409-NS1 ($p < 0.05$) respectively. Meanwhile, there was no significant difference with inactivated vaccine group (Fig. 3A). Concerning the level of the SIgA in the intestinal tract and mouse stool cleaning fluid Interestingly, the same pattern like the upper respiratory tract was
found except that inactivated vaccine group had significantly increased titer in compared to the pSIP409-pgs A-NS1 group (Fig. 3B). On the other hand, in the mouse stool cleaning fluid, there was no clear significant difference between these 2 groups (Fig. 3C). So, it could be concluded that vaccination with pSIP409-pgsA-NS1 triggered secretory antibody production either in the mice upper respiratory, intestinal tracts, and stool cleaning fluids.

**Recombinant bacteria induce CD8⁺ T cell immune response in the immunized mice**

To evaluate the T cell immune response induced by the recombinant lactobacillus, Peyer’s patches (PP) from each group of immunized the mice was isolated and detected by flow cytometry. The data showed that immunization with the pSIP409-pgsA-NS1 elicited a higher frequency of CD3⁺CD8⁺ T cell in PP than pSIP409-NS1 (p < 0.05), and inactivated vaccine group (p < 0.01) (Fig. 4). However, we did not find that the frequency of CD3⁺CD4⁺ T cell in PP has changed dramatically in all groups of animal (data not shown).

**Recombinant bacteria induce B cell immune response in immunized mice**

As an indicator of the humoral immune response, the B cells response was determined in the cells of the PP (Fig. 5) of the tested mice. It was cleared that immunization with the pSIP409-pgsA-NS1 elicited B220⁺ B cells level significantly higher than the pSIP409-NS1 (p < 0.05), the pSIP409-pgsA (p < 0.05), and the PBS (p < 0.01). Meanwhile there was no statistically significant difference with the inactivated vaccine group. The obtained data confirmed the importance of pSIP409-pgsA-NS1 in inducing B cells humoral response at the local levels.

**Recombinant bacteria provide protection for immunized mice against the H1N1 virus Challenge**

For identifying the protective effect of the pSIP409-pgsA-NS1 against the swine influenza infection. The weight losses and survival rate of the examined mice groups were tested. Concerning the body weight loss, it was found that both of the inactivated vaccine and the pSIP409-pgsA-NS1 treated groups were the least groups with the peak at the 9th day post challenge with no significant difference between them adding to their fast recovery rate. Meanwhile, the other PBS and pSIP409-pgsA groups lost more than 30% of their body weight in the same period until the time of death (Fig. 6A). Regarding the survival rate, all animals at the PBS and pSIP409-pgsA groups died by the 8–10 days post challenge. The survival rates of the inactivated vaccine and the pSIP409-pgsA-NS1 immunized groups were 50% and 40%
respectively with no statistical difference between them (Fig. 6B). While the survival rate of the pSIP409-NS1 group was only 20%. The obtained results elucidate the protective effect of the produced lactobacillus pSIP409-pgsA-NS1 against the swine influenza H1N1 virus infection through decreasing weight loss and increasing the survival rate of the tested mice.

Concerning the viral load, it was determined in the lung of the different tested groups. The lowest viral titers were detected in the inactivated vaccine administered group followed by pSIP409-pgsA-NS1 and the pSIP409-NS1 with no significant difference between them. Meanwhile, a clear significant variance was found between the pSIP409-pgsA-NS1 and the pSIP409-pgsA (\( p < 0.001 \)) and the PBS (\( p < 0.001 \)) groups respectively (Fig. 6C). The obtained results showed the potency of the pSIP409-pgsA-NS1 in decreasing viral shedding.

**Recombinant bacteria improve cytokines production in immunized mice**

The serum level of IL-4 and TNF-\( \alpha \) cytokines before and 2 weeks post challenge was detected by ELISA (Fig. 7A and B) respectively. Generally, the level of the 2 cytokines was raised post challenge in compared with the level before the challenge. Regarding the difference between the groups, the cytokines level of the pSIP409-pgsA-NS1 and the pSIP409-NS1 group was significantly high in comparing with the PBS and the pSIP409-pgsA (\( p < 0.001 \)), pSIP409-NS1 group (\( p < 0.05 \)) respectively. While the level was not significantly increased in the killed vaccine treated group in comparing with pSIP409-pgsA-NS1 group. The same attitude was found when the level of TNF-\( \alpha \) had been tested.

**DISCUSSION**

The obtained results clarified that immunization of mice with pSIP409-pgsA-NS1 significantly induced humoral response due to the production of specific serum IgG against the NS1. Our results agree with that published by [25] who cleared that mice immunization with maize seeds expressed H3N2 nucleoprotein (NP) resulted in eliciting humoral immunity in the form of IgG2a antibody titer which was highly produced rather than the IgG1 level. Vaccination with the recombinant influenza A PR8 viruses carrying a synonymous codon deoptimized NS segment resulted in immune responses that able to combat homologous and heterologous influenza infection [26]. Interestingly, vaccination with a live-attenuated H9N2
influenza vaccine having NS1 truncation showed an increase in the IgG production started from 7 days post immunization in comparing with the chickens that were immunized with the inactivated vaccine [27]. Also, the live attenuated genetically modified NS1 of the H5N2 (H5N2 NS1-LAIVs) vaccine administration was able to induce significant serum neutralizing IgG in the assessed mice [28].

Inducing of strong mucosal immunity is essential for providing protection against influenza virus infection [29], especially that the respiratory tract is the main route of infection, its lining epithelium is the most predilection site for influenza virus multiplication and the resulted nasal discharge is the main source of infection [30,31]. Immunization with the pSIP409-pgsA-NS1 elicited a strong secretory IgA response in the upper respiratory, intestinal, and stool cleaning fluids of the examined mice groups. Previous reports declared that using of intranasal administration of split virus vaccine combined with R4Pam2Cys

Fig. 6. Recombinant bacteria provided protection against the H1N1 virus. Two weeks after the booster immunization, mice from each group were challenged with $1 \times 10^5 \text{EID}_{50}$ of the H1N1 virus, and weight loss (A) and mortality (B) were monitored two weeks. Lung viral titer (C) was determined at 5 days after challenge with the H1N1 virus.

ns, not significant; PBS, phosphate-buffered saline.

* $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.  

https://doi.org/10.4142/jvs.19411
induced a high humoral response characterized by the presence of high significant levels of IgA [32]. On the other side, oral administration of MNP vaccine failed in raising the IgA secretion, confirming poor mucosal immune response in the tested mice [25]. The secretory Abs have a role in counteracting virus assembly through binding the newly synthesized viral proteins. Also, due to the polymeric nature of the secretory IgA, it provides sufficient protection against its homologue and any resulted drifted viruses. On the other side, the IgG antibodies that diffused from the serum to the mucus afford protection against homologous virus infection only [29]. Different studies showed that, administration of inactivated intramuscular vaccines did not produce high levels of antigen-specific mucosal IgA antibody response in the respiratory tract [33], adding to its limitation of using in the presence of maternal-derived antibody [34].

Fig. 7. Changes of cytokine production in the serum of mice before and post-challenge with influenza virus. IL-4 (B) and TNF-α (A) was test by enzyme-linked immunosorbent assay.
IL, interleukin; ns, not significant; PBS, phosphate-buffered saline; TNF-α, tumor necrosis factor alpha.
*p < 0.05, **p < 0.01 and ***p < 0.001.
The CD4+ T lymphocytes and CD8+ T cytotoxic lymphocytes not only provoke the adaptive immunity against the influenza virus infection but also help in viral clearance and cross-protective immune response [35]. Also, its activity correlates with the absences of virus shedding, decreasing the risk of fever, and reducing the influenza clinical signs [36]. Detection of CD3+CD8+ in PP of the tested mice showed the high significance of the pSIP409-pgsA-NS1 in inducing CD8+ T cells response in comparing with the other groups. A similar response was obtained when a modified vaccine encoding internal nucleoprotein and matrix proteins were used to immunize pigs [37]. The observations of [38,39] cleared that the inactivated vaccines are not completely effective against the heterologous influenza infections due to lacking the ability to stimulate the host cellular immunity and inadequate levels of mucosal IgA secretion. Taken together, variable studies indicated that CD8+ T cells can play a role in the protection against IAV infections, these T cells are long-lived and are able to cross-react with multiple IAV strains. Thus, induction of these T cells may be the basis of broadly reactive universal influenza vaccines. On the other side, multiple reports clarified that, using of whole inactivated vaccine in high dose resulted in triggering remarkable amount of influenza A virus-specific CTLs in mice especially when administered with intramuscular route rather than the intranasal one [40,41].

To declare the capacity of the constructed pSIP409-pgsA-NS1 in inducing cytokines production. The obtained data revealed the presence of a remarkable induced level of IL-4 and TNF-α in the pSIP409-pgsA-NS1 treated group in comparing with the other groups but to an extent lower than the inactivated vaccine group. Vaccination with a recombinant swinepox virus co-expressing hem-agglutinating genes of swine influenza viruses rSPV/H3-2A-H1 resulted in the production of a remarkable level of IL-4 in the guinea pigs lab animal model [42]. Also in a different study, the expression level of different kinds of cytokines including IL-4 in peripheral blood mononuclear cells of an epitope-based antigen vaccinated pigs was significantly raised in comparing with the other tested groups [43]. Furthermore, vaccination with maize-expressed H3N2 nucleoprotein resulted in increasing production of both IL-4 and interferongamma cytokines [25]. The obtained results elucidate the role of pSIP409-pgsA-NS1vaccination in inducing the T-helper 2 immunity. It is important to record that up-regulation of cytokines will stimulate T cells to improve cytotoxic T cells responses [44].

Mice body weight and survival rates were measured as indicators for vaccine efficiency, it was found that the pSIP409-pgsA-NS1 and the inactivated mice groups had recovered the transit body weight loss rapidly after lethal challenging with the H1N1 virus with 40%–50% survival rate in comparing to the other groups that significantly lost their body weight till the time of death. The obtained results were previously recorded when a formulation of inactivated vaccine in combination with the (R4Pam2Cys) as an adjuvant was used to protect against challenge with influenza A virus in mouse model experiment [32]. Also, when the intranasal H1-H3-H5-H7 virus-like particle cocktail vaccine was used to protect against challenging with eight different IAV strains [45]. Using of (H5N2 NS1-LAIVs) vaccine had the potency to provide mice protection against the infection with the homologous H5N2, and the heterologous H5N1 experimental infection [28].

In conclusion, lastly, it could be concluded that the newly developed recombinant pSIP409-pgsA-NS1 bacteria induced the humoral immune response in the mice model. Adding to the protection of the vaccinated mice against the lethal challenge with H1N1 subtype of influenza virus infection. Further evaluation in a porcine model is needed before being used as safe, and protective mucosally delivered vaccine to provide protection against the swine influenza virus.
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