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Live recombinant *Salmonella* oral vaccine against avian influenza viruses

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KEY MESSAGE

A live recombinant *Salmonella* oral vaccine platform against avian influenza viruses was constructed.

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Introduction

In 1997, the avian influenza virus (AIV) subtype H5N1 was first reported to infect humans.¹ Up to 3 March 2015, the cumulative number of confirmed human cases of H5N1 was 784, with 429 deaths. There is no evidence that H5N1 viruses can transmit efficiently from person to person.² Therefore, the key to prevention of a human H5N1 pandemic is to first control the H5N1 virus in birds.

Traditionally, inactivated influenza vaccines have been used prophylactically against pandemic influenza. However, their cost is high and yield is low, and they are difficult to administer on a large scale. The use of live, attenuated *Salmonella* vaccines has been extensively studied. *Salmonella* is a Gram-negative, intracellular bacterium.³ It has advantages as a live antigen delivery vector.⁴ First, it can be modified to express a wide range of antigens. Second, live *Salmonella* vaccine is easy to administer; it can be mixed with food and given to birds orally. Third, after oral administration, *Salmonella* can penetrate the Peyer's patches via M cells in the gastrointestinal tract and colonise the mesenteric lymph nodes that contain various antigen-presenting cells.³ This can generate a range of immune responses, including mucosal and systemic immunity. Fourth, live *Salmonella* vaccine can be easily and cheaply produced.⁵

This study constructed analogous sets of live recombinant *Salmonella* Typhimurium vaccine strains expressing a model antigen—enhanced green fluorescent protein (EGFP). The advantages and deleterious or synergistic effects of these vaccine strains were identified by comparing their immunogenicity in mice.

Methods

To develop an effective, live recombinant *Salmonella* oral vaccine against avian influenza viruses,

EGFP was used as a model antigen to study the relationship between immunogenicity and different antigen expression systems in *Salmonella*. Six 'single-recombinant' strains were constructed: three cytoplasmic expression strains based on a high or low copy plasmid, or the chromosome expression cassette (HP, LP and CP); two outer-membrane expression strains developed from a high copy plasmid or chromosome (HO, CO); and one eukaryotic expression plasmid, strain E. Several different pairs of EGFP-expression systems were combined to produce five additional 'double-recombinant' strains. The antigen expression levels, and immunogenic properties of these single and double-recombinant strains were systematically investigated in mice.

Based on the study of *Salmonella*-EGFP strains, three *Salmonella*-AIV strains were constructed: one chromosome-based *Salmonella* AIV strain (YBS010), in which multiple antigens of AIV (HA, NA, M2, NP) were inserted into the chromosome of *Salmonella* typhi Ty21a using an optimised recombinant method; one high-copy-plasmid-cytoplasmic-expression strain (C-HAOP); and one high-copy-plasmid-outer-membrane expression strain (O-HAOP). Their immune responses in mice and chicken were investigated via ELISA, ELISpot, haemagglutinin inhibition (HAI), and H5N1 virus challenge.

Results

Six 'single-recombinant' and five 'double-recombinant' *Salmonella*-EGFP strains were constructed to compare the immunogenic properties of various recombinant *Salmonella* strains. Maintaining high antigen expression levels was important for eliciting a strong B cell response. If the antigen (such as EGFP) is soluble and easily expressed in *Salmonella*, a low-copy plasmid-based strategy should be used, as it can provoke both

strong B cell and T cell responses. Furthermore, the stability of antigen expression is important for eliciting a strong T cell response against the recombinant vaccine strains. If a T cell response is preferred, a eukaryotic plasmid, or chromosome-based expression strategy may achieve better results. Nonetheless, a combination of two expression strategies did not enhance the immune response.

Based on the study of *Salmonella*-EGFP strains, three live recombinant *Salmonella* oral vaccine candidates against AIV were constructed. A chromosome-based *Salmonella* AIV strain YBS010 was first constructed. In the YBS010 strain, four antigen genes of AIV (HA, NA, M2, NP) were cloned and recombined into the chromosome of *Salmonella* typhi Ty21a strain using the novel recombinant method,⁶ and their expression was confirmed. Immunogenicity assays in mice (ELISA and ELISpot) indicated that they were not strong enough to generate protective B cell and T cell responses against the AIV (data not shown). According to the study of *Salmonella*-EGFP strains, a weak B cell response may be due to the low expression level of four antigens expressed by the single copy chromosome-based expression cassettes. A low T cell response was probably due to codon bias or inability to express soluble, correctly folded epitopes in YBS010.

Then, two *Salmonella*-HA strains were constructed including one cytoplasmic expression strain (C-HAOP) and one outer-membrane expression strain (O-HAOP). Both strains are based on high-copy plasmids. Previous studies have reported that many antigens from eukaryotic viruses (such as the HA protein from H5N1) cannot be readily expressed in a soluble form within the cytoplasm of *Salmonella*. Thus, the codons of the HA epitope were first optimised according to the *Salmonella* codon bias. Nonetheless, codon optimisation did not solve the insolubility problem of the HA epitope in C-HAOP strain. This may account for failure of the following immune test in mice models.

According to our in vivo immune response assays, no detectable B cell or T cell responses were observed when mice were immunised with the C-HAOP strain (Table 1). For O-HAOP strain, the use of the Lpp-ompA system resulted in the successful export of the HA epitope to the outer membrane fraction. Results from ELISA, HAI, and ELISpot assays in mice indicated that this strain was capable of inducing better HA-specific B cell and T cell responses than the corresponding C-HAOP strain (Tables 1 to 3) that expresses the HA antigen in the cytoplasm. The use of the Lpp-OmpA system probably helped the HA protein fold into a soluble conformation, or resulted in the export of increased amounts of HA protein, causing notable enhancements in immunogenicity. Although the

TABLE 1. HA-specific IgG responses in infected mouse sera

Group	No. responding / total No. (mean IgG titre)		
	Day 7	Day 28	Day 42
O-HAOP	1/10 (100)	3/10 (160)	3/10 (250)
C-HAOP	0/6 (0)	0/6 (0)	0/6 (0)

TABLE 2. Haemagglutinin inhibition (HAI) in infected mouse sera

Group	No. responding / total No. (mean titre)		
	Non-infection	HO	O-HAOP
O-HAOP	1/10 (100)	3/10 (160)	3/10 (250)

TABLE 3. IFN-γ responses to recombinant SL7207-HA strains

Group	Mean ratio (total mice No.)
HO	0.69 (n=5)
O-HAOP	2.56 (n=5)
C-HAOP	0.91 (n=5)

TABLE 4. HA-specific IgY responses in infected chicken sera

Group	No. responding / total no. (mean titre)		
	HO (1011)	O-HAOP (1010)	O-HAOP (1011)
Titre	0/4 (0)	0/4 (0)	0/4 (0)

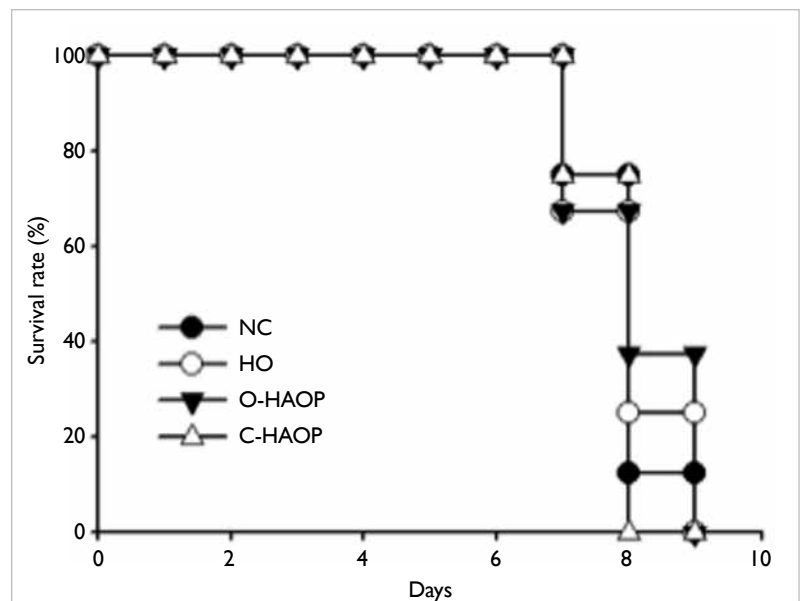


FIG. Challenge result of mice infected with C-HAOP and O-HAOP strains using the dose of 50 LD₅₀ H5N1 virus. Mice immunised with the HO strain and non-infected mice were negative controls. Mice were observed for sick and survival rates for 21 days. The survival rate per day after challenge was the challenge result.

O-HAOP strain increased the immune response in mice, this strain was still not strong enough to protect mice from the H5N1 virus challenge (Fig). In the chicken humoral immune response study, O-HAOP strain could not induce the significant HA-specific IgY response (Table 4). This may be due to the drawback of outer-membrane expression system in *Salmonella*-EGFP strains.

The total amounts of antigen synthesised and exported to the outer membrane were low, and the high-copy plasmid was not as stable as low-copy plasmid or chromosome-based expression cassette. Therefore, optimisation of this vaccine candidate is needed. Recombinant flagella can remain at the site of administration for many hours and are efficiently presented to the immune system to provoke the production of specific antibodies against the inserted foreign antigen and protection against challenge infection. The optimised recombinant technology can be used to integrate multiple copies of HA epitope into the *Salmonella* flagellin gene (*fliC*) to create a chromosome-based SL7207-*fliC*-HA strain. Chromosome-based expression cassette was more stable than plasmid-based expression cassette, which could help the flagellin-HA gene express constitutively. Flagellin-fused HA repeats would solve the insolubility problem of HA in the C-HAOP strain (cytoplasmatically expressed HA strain) and result in an increased expression level of HA compared with O-HAOP (outer membrane fused HA). The live, chromosome-based SL7207-*fliC*-HA strain could be orally administered to mice and chicken to test its immunogenic efficiency as described before.

Discussion

A method to integrate and express multiple H5N1 antigen genes in *Salmonella* was developed. It can serve as a general approach for future construction and optimisation of *Salmonella* recombinant strains.

The construction strategies of recombinant *Salmonella* vaccine strains for various needs and different forms of antigens were proposed. Such information can help guide the development of live recombinant *Salmonella* oral vaccines against AIV as well as other pathogens. If an epidemic arises,

relevant vaccines can be synthesised in a very short time.

Using the optimised recombinant method, several live *Salmonella* vaccine candidates against AIV were tested in animal models. These candidate vaccines stimulated a specific immune response against H5N1, although the efficacy was too low to protect the animals. Further optimisation of the vaccine candidates is necessary for rapid production during an epidemic. If AIV drifts or shifts to other subtypes, relevant vaccines can also be produced quickly.

As the vaccine can be administered orally, it is more convenient for mass administration in the poultry industry and avoids the use of hypodermic needles. The *Salmonella*-based vaccines also have the advantage of being easy to manufacture, as bacterial cultures need simple media and do not require special equipment.

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