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Mucosal and systemic immunization with a novel attenuated pneumococcal vaccine candidate confer serotype independent protection against *Streptococcus pneumoniae* in mice

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ABSTRACT

Despite the availability of effective vaccines, *Streptococcus pneumoniae* is still one of the major infectious diseases causing substantial morbidity and mortality in children under 5 years old. In this study, we demonstrate the protective efficacy of *S. pneumoniae* SPY1, a novel live attenuated vaccine strain against pneumococcal infection in murine models. This strain was characterized by defects in three important pneumococcal virulence factors including capsule, teichoic acids and pneumolysin. The lactate dehydrogenase assays and *in vivo* animal experiments demonstrated a significantly attenuated virulence and a reduced nasopharyngeal colonization for the SPY1 strain. We also show that mucosal and systemic immunization with the live SPY1 strain induced protective immune responses against pneummococci. Mucosal immunization with SPY1 offered better protection against colonization challenge with strains TIGR4 and serotype 19F than systemic SPY1 immunization. In invasive infection models, mucosal vaccination with the SPY1 strain conferred complete protection against D39 and clinical serotype 6B and 3 strains. Notably, intranasal vaccination with the SPY1 strain conferred superior protection against pneumococcal invasive disease compared with the commercial available vaccines. SPY1 strain was shown to elicit high levels of serotype-independent antibodies and a mixed cellular immune response. Besides, the SPY1 serum was able to passively protect mice against invasive challenge with D39 strain, indicating the protective effect of the antibody-mediated responses. Together, the SPY1 strain may be a promising live vaccine strain to protect pneumococcal infection.

1. Introduction

Despite the wide use of efficacious conjugate and unconjugated polysaccharide vaccines, *Streptococcus pneumoniae* represents the largest cause of vaccine preventable death in children under 5 years old [1]. Globally, pneumococcal diseases are responsible for about 3–5 million deaths each year, and most of them are children under 5 years old and the elderly [2,3].

According to epidemiological data from the United States, 44% of clinical *S. pneumoniae* isolates are becoming resistance to penicillin in some states [4,5]. In Asian countries, the rate of resistance to penicillin has been increasing to more than 50%, close to 90% in some area [6–10]. This antibiotic-resistance situation of pneumococci has been worsening by multi-drug resistance pneumococcal isolates and vancomycin resistance strains [11–13]. Taken together, increasing antimicrobial drug resistance in pneumococci has prompted for a better strategy to control pneumococcal infection.

To the best of our knowledge, there are currently 93 recognized capsular serotypes of *S. pneumoniae* [14], significantly outnumbering the coverage of the PPV23 and PCV7 vaccines. Besides geographically varied protection [15] serotype replacement has been reported and received much concern [16–18]. Afterwards, PCV9, PCV10 and PCV13 were generated to cover some additional serotypes on the basis of PCV7 [19]. Nevertheless, to obtain cost effective, broad and long-lasting protection, the ideal vaccines would be protein or whole bacterium based formulations.

A number of proteins, either singly or in combination, have been shown to induce protection in animal models [20,21]. However,

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the development of protein based vaccines will increasingly rely on the identification and production of novel antigen candidates. As an alternative, attention has also been drawn to pneumococcal whole cell based vaccines [22]. In the year of 1999, Benedicte et al. first reported the protective effect elicited by mucosal delivery of the killed whole bacterial cells against pneumococcal stains [23]. To date, few live attenuated vaccines have been reported to be protective against pneumococci [20,24,25].

The present study examined the phenotypes of the novel live attenuated strain *S. pneumoniae* Y1 (SPY1). Besides, we tested the live SPY1 strain as a vaccine against pneumococcal colonization and invasive infection. Finally, mechanisms of systemic and mucosal immunization with SPY1 strain were investigated.

2. Materials and methods

2.1. Mice and bacteria

Female 6–8 weeks old BALB/c mice were obtained from the animal center of Chongqing Medical University. All experimental protocols were approved by the Ethics Committee of Chongqing Medical University (Reference number: 2011-032). All volunteers provided written informed consent for the collection of blood and subsequent use. Pneumococcal strains used in this study were listed in Table S1. Pneumococci were grown either in the C + Y medium or blood agar plates supplemented with 0.25 μg/ml of erythromycin.

2.2. Phenotypic characterization and transformation efficacy

The SPY1 strain was obtained when we performed transformation to construct the *SPD_1672* deletion mutant with previous described method [26]. The *SPD_1672* gene was deleted by allelic replacement by the *erm* cassette gene. The primers used for *SPD_1672* deletion were listed in Table S2. The colonies were selected on blood agar plates supplemented with 0.25 μg/ml of erythromycin.

The capsule was examined by transmission electron microscopy (TEM). For teichoic acids analysis, bacteria were digested with 10 mg/ml lysozyme (Sigma), followed by boiled for 10 min in the protein loading buffer, and subjected to western blot analysis with TEPC-15 (IgA, κ chain, Sigma) as the primary antibody. Total proteins were used as loading control. Total RNA isolation and PCR procedures were conducted as described previously [27]. mRNA level of *ply* was determined by quantitative real time-PCR. SPY1 and WT D39 strains were washed twice with phosphate-buffered saline (PBS) and subjected to analyze the hemolytic activity as described elsewhere [26].

Transformation experiments were carried out as previously described with some modifications [28]. Bacteria were grown to an OD_{620} = 0.1, and induced in the presence of competence-stimulating factor peptide (CSP; 100 ng/ml) for 10 min at 37 °C. Chromosomal DNA from a tetracycline-resistant strain (1 μg/ml) was added into the mixture for 30 min at 30 °C. DNase at a final concentration of 10 μg/ml was used to digest excessive DNA. The mixture was incubated for an additional 90 min at 37 °C to allow expression of the tetracycline resistance gene. Serial dilutions were plated on blood plates containing 2.5 μg/ml tetracycline to determine the transformation efficiency which was calculated as previously described [29]. Natural transformation was performed in the same manner as described above without the use of CSP.

2.3. Virulence evaluation

The lactate dehydrogenase (LDH) assay was used to analyze cytotoxicity property of the SPY1 strain as previously described [24]. Bone marrow-derived macrophage cells were prepared according to a previous protocol [30]. Supernatants were collected and subjected to LDH assay with LDH cytotoxicity assay kit (Beyotime institute of Biotechnology). Mice were intraperitoneally (IP) injected with 5×10^7 colony-forming units (CFU) of wild type D39 strain or SPY1 vaccine strain. Survival was monitored over time.

2.4. SPY1 colonization on the respiratory tracts and histological analysis

BALB/c mice were intranasally (IN) inoculated with 10^7 CFU of SPY1 or wild type D39 strain. Nasal washes, lungs and blood samples were collected at the indicated time points post infection. Nasal washes were collected from nostrils of mice [31]. Nasal washes and homogenized lung tissues were plated on blood agar plates to obtain viable counts. For histological examination, lungs were obtained at 24 h and 36 h post-infection, fixed in 4% (v/v) formalin saline solution, and embedded in paraffin. Sections were examined microscopically with a 40× objective following hematoxylin–eosin staining.

2.5. Immunization and sample collection

BALB/c mice were randomly divided into groups as follows: a negative control group was IN received cholera toxin (CT; 1 μg) in 30 μl PBS; the positive control (PC) was subcutaneously (SC) immunized with PPV23 (Chengdu Institute of Biological Products Co., Ltd, China) or PCV7 (Prevnar); and the experimental groups were either SC vaccinated with 100 μl SPY1 ($l0^8$ CFU) mixed with equal volume of 100 μl aluminum adjuvant or IN immunized with SPY1 ($l0^8$ CFU) plus CT (1 μg) in 30 μl PBS respectively. Unless otherwise indicated, IN represents intranasal immunization with SPY1 plus CT. For SC immunization, mice were vaccinated three times at an interval of two weeks; whereas for IN immunization, mice were immunized once per week for four consecutive weeks. PCV7 was used as the positive control against serotype 6B infection; whereas PPV23 was used for other strains.

Serum and saliva samples were collected one week or three months after the final immunization. Blood samples were collected from the tail of mice. Saliva samples were induced by carbachol (30 μl of 0.1 mg/ml). All samples were stored at −80 °C for future assays.

2.6. Antibody titers and IgG isotypes determined by enzyme-linked immunosorbent assay (ELISA)

96-well ELISA plates were coated with either SPY1, D39, TIGR4, R6, serotype 19F, 14, 6B or 3 as previously described [20]. For saliva samples, protein concentration was determined using BCA protein assay kit (Beyotime institute of Biotechnology) and adjusted to the same level. For measurement of protein antigen specific IgG titers, antibody levels were determined as previously described by using plates coated with the indicated proteins [26].

2.7. Animal studies

For colonization protection studies, two weeks after the last immunization, vaccinated mice were IN challenged with *S. pneumoniae* strains 19F (10^8 CFU) or TIGR4 (10^8 CFU). Three days later, nasal washes were collected, and lungs were homogenized in 1 ml of sterile PBS buffer. All samples were plated on blood agar plates to form viable counts after overnight incubation at 37 °C.

To evaluate whether the vaccines were effective in preventing invasive infections, BALB/c mice were IN challenged with 10^7 CFU, 5×10^8 CFU and 7×10^6 CFU of D39, serotype 6B and 3 two weeks after the last immunization. Also, vaccinated mice were administrated with $3 \times LD_{50}$ dose of D39. For long-term protection, BALB/c

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