ABSTRACT

BACKGROUND

Streptococcus pneumoniae causes pneumococcal disease, which is responsible for millions of deaths worldwide. Various pneumococcal vaccine candidates have been developed to prevent S. pneumoniae infection, one of which is an epitope-based vaccine. This study aimed to prove that intranasal immunization with each of the five S. pneumoniae RrgB epitopes can induce a mucosal immune response by increasing the β-defensin-2 concentration through upregulation of interleukin (IL)-22 expression.

METHODS

An experimental laboratory study was conducted using 28 male Wistar rats aged 3-4 months, that were randomly divided into 7 groups containing four rats each. Group 1 was given 40 mL of phosphate-buffered saline (PBS) only (control group). Group 2 was the adjuvant group that received 40 mL PBS containing 2 μg cholera toxin B (CTB), and groups 3-7 were immunized with 40 mL PBS containing a combination of adjuvant and one of the five different S. pneumoniae RrgB epitopes. The concentrations of IL-22 and β-defensin-2 from nasal rinse examination were measured by means of ELISA. The Kruskal-Wallis test, followed by the Mann-Whitney statistical test, were used for statistical analysis.

RESULTS

Rats immunized with the adjuvant-epitope combination had significantly higher β-defensin-2 and IL-22 levels than the control group (p=0.030; p=0.018, respectively), according to the Kruskal-Wallis test. And the Mann-Whitney statistical test, showed there was a significant increase in B-defensin-2 and IL-22 levels.

CONCLUSIONS

Intranasal immunization with epitope 1 of the S. pneumoniae RrgB can increase β-defensin-2 expression significantly and has a greater potential to be developed into a pneumococcal vaccine.

Keywords: Intranasal immunization, epitope-based vaccine, Streptococcus pneumoniae RrgB protein, β-defensin-2

INTRODUCTION

*Streptococcus pneumoniae* (pneumococcus) is a Gram-positive opportunistic pathogen that colonizes the human upper respiratory tract (URT), specifically the nasopharynx. *S. pneumoniae* can cause pneumococcal disease, including community-acquired pneumonia (CAP), sinusitis, acute otitis media, sepsis or bacteremia, and meningitis. In 2017, the World Health Organization (WHO) included *S. pneumoniae* in the 12 priority pathogens responsible for millions of deaths worldwide. Pneumococcal pneumonia is a lung infection of high morbidity and mortality, especially in children under five years old, the elderly above 65 years old, and immunocompromized individuals. The primary transmission mode of *S. pneumoniae* is direct contact with secretions from the respiratory system. It is more frequent during the colder months with dry air, when respiratory secretions are excessive, and there often is coinfection of influenza and respiratory syncytial virus (RSV) with *S. pneumoniae*.

Various pneumococcal vaccine candidates have been developed. Currently, two types of licensed pneumococcal vaccines are available to prevent pneumococcal infection, namely a 23-valent pneumococcal polysaccharide-based vaccine (PPV23) and a 13-valent pneumococcal conjugate vaccine (PCV13). PPV23 is a capsular polysaccharide vaccine containing 23 serotypes that cause 90% of invasive pneumococcal disease. However, PPV23 only induces a T cell-independent immune response that is less effective in children under two years. PCV13 is a capsular polysaccharide vaccine conjugated with the non-toxic diphtheria toxin cross-reactive material 197 (CRM197). PCV13 can induce good antibody response, T cell-dependent response, and immunological memory even in infancy. Nevertheless, this vaccine is more expensive and has limited serotype coverage. Both vaccines are invasive because they are administrated intramuscularly. Until now, there are 98 serotypes of *S. pneumoniae*. These vaccines have limited coverage of pneumococcal serotypes and cannot protect against unencapsulated or non-vaccine serotypes of *S. pneumoniae*. This condition can lead to a rapid increase in antibiotic-resistant non-vaccine serotypes. Advances in prevention or treatment of pneumonia have been made with the development of vaccines and antibiotics. However, it is certain that such strategy may not be able to cover all of the 98 known serotypes of *S. pneumoniae*. Another vaccine candidate is an intranasal epitope-based vaccine that has more advantages than conventional vaccines, including high specificity, good safety and stability, and ease of production and storage. The nasal mucosa is suitable for administering vaccines because it is an entry site for pathogens. Besides, it has nasal-associated lymphoid tissue (NALT), which can help induce the mucosal and systemic immune systems. The intranasal epitope-based vaccine is expected to be more effective in protecting against *S. pneumoniae* infection with a broader spectrum of serotypes, to induce mucosal and systemic immunity, and to be affordable and non-invasive. Previous in-silico studies have identified five epitopes of the *S. pneumoniae* RrgB (pilus backbone) protein with peptide lengths between 13-23 amino acids as a potential candidate for the pneumococcal vaccine.

The intranasal immunization epitope of the *S. pneumoniae* RrgB protein will be received by antigen-presenting cells (APC) and presented to naïve T cells through the T-cell receptor (TCR). Furthermore, naïve T cells will differentiate into Th1, Th2, Th17, and T reg. Th17 will express CCR6 and produce various cytokines such as interleukin-22 (IL-22), interleukin 17A (IL-17A), interleukin-17F (IL-17F), interleukin-26 (IL-26), tumor necrosis factor-α (TNF-α), chemokine (CC motif) ligand 20 (CCL20), and granulocyte macrophage-colony stimulating factor (GM-CSF). Interleukin-22 is an important cytokine in mucosal immunity that can increase complement 3 (C3) deposition on *S. pneumoniae*, thereby increasing the phagocytosis of this pathogen by neutrophils. Besides, IL-22 can also increase
proliferation and repair of pulmonary epithelial cells after injury. Moreover, IL-22 and IL-17A are the most important cytokines that will synergistically induce the respiratory epithelial cells to produce antimicrobials like β-defensin-2, lipocalin-2 (Lcn2), mucin (MUC5B), and various chemokines such as CXCL5, CXCL9, CCL3, CCL-20, and G-CSF which can prevent *S. pneumoniae* infection. In a previous study by Widiatmaja et al., (13) there was no significant difference in IL-4 levels of Wistar rats induced with *S. pneumoniae* RrgB 225-270 protein. Another indicator of immune response that we want to measure is β-defensin-2 which is also an agent of innate, mucosal, and adaptive immunity that plays a role in preventing *S. pneumoniae* infection. In this connection, another study on Wistar rats showed that intranasal immunization with the 54 kDa hemagglutinin pilus proteins of *S. pneumoniae* enhanced the expression of β defensin 2. (14) We can use the measurement of β-defensin-2 expression in the respiratory mucosa to assess the mucosal immune response, which is important in protecting cells from *S. pneumoniae* infection. (15,16) There were inconsistent results in the previous studies, therefore, a study was performed to evaluate the effect of intranasal immunization on β defensin-2 through the upregulation of IL-22 expression. The purpose of this study was to evaluate the effect of intranasal immunization of the five *S. pneumoniae* RrgB epitopes as vaccine candidates for induction of the mucosal immune response by increasing the β defensin-2 concentration through the upregulation of IL-22 expression.

**METHODS**

**Research design**

This study was of an experimental in-vivo post-test only control group design and was carried out in the Microbiology, Pharmacology, and Biochemistry Laboratories, Faculty of Medicine, University of Jember, from January to December 2020.

**Epitopes of *S. pneumoniae* RrgB protein**

This study used the five epitopes of *S. pneumoniae* RrgB protein identified from previous in-silico studies as potential candidates for a pneumococcal vaccine. Epitope 1 contains 23 amino acids (VNHQVDVEYEVETKIPA LANY), epitope 2 comprises 16 amino acids (TVKVTVDVALEAGDY), epitope 3 has 19 amino acids (TFDLVNAQAGKVVQTVTLT), epitope 4 consists of 14 amino acids (PKVTYGKFKVKVN), and epitope 5 comprises 13 amino acids (DRAVAAYNAL TAQ). These epitopes were designed by and purchased from PT. Genetika Science Indonesia.

**Intranasal immunization**

The sample of 28 male Wistar strain rats aged 3-4 months was randomly divided into 7 groups, each consisting of 4 animals. Group 1 was the control group and received 40 mL of phosphate buffered saline (PBS). Group 2 was the adjuvant group and received 40 mL PBS containing 2 μg cholera toxin B (CTB). Groups 3-7 were immunized with 40 mL PBS containing a combination of adjuvant and an antigen of the five different *S. pneumoniae* RrgB epitopes. A 20mL volume of the adjuvant-epitope combination was administered in each nostril. All rats were immunized on days 0, 7, and 14. All animal studies were conducted following guidelines and approval from the Institutional Biosafety Committee and Institutional Animal Care and Use Committee, Faculty of Medicine, University of Jember.

**Nasal rinse isolation**

Seven days after the third immunization, the rats were anesthetized with 10% ether by inhalation. After that, the rats were sacrificed by decapitation. Nasal rinse isolation was performed on a retrograde basis by inserting 1–2 mL of sterile saline through the trachea. We accumulated the
droplets of saline solution from the nostrils into a sterile Eppendorf tube.

**IL. 22 and β defensin 2 concentrations**

IL 22 and β defensin 2 concentrations in the nasal rinse were measured with an enzyme linked immunosorbent assay (ELISA) kit from Elabscience® according to the specified procedure. 100 μL samples were loaded into the wells, incubated for 90 minutes at 37°C, then the nasal rinse liquid and 100 μL of IL 22 or β defensin 2 antibodies were added. After having been left to stand for 1 hour at 37°C, the plate was rinsed with the washing solution. Then, 100 μL of horseradish peroxidase (HRP) conjugate was added and the plate incubated for 30 minutes at 37°C. Next, the plate was rinsed with the washing solution and 50 mL of substrate reagent was added and the plate incubated for 15 minutes at 37°C. The reaction was then quenched with 50 μL of the stop solution. Color expression was measured by optical density at a wavelength of 450 nm with an ELISA reader.

**Statistical analysis**

Descriptive data were presented as mean ± standard deviation. The between-group differences in IL 22 and β defensin 2 mean concentrations were assessed by the Kruskal-Wallis test, followed by the Mann-Whitney post-hoc test. The significance of the data is indicated by a p-value of <0.05.[17]

**Ethical clearance**

The Ethics Committee of the Faculty of Medicine, University of Jember, approved this study under No. 1313/H25.1.11/KE/2019.

**RESULTS**

The concentrations of IL 22 and β defensin 2 in the nasal rinse were measured by the ELISA method and the results are presented in Table 1. Rats immunized with the adjuvant-antigen combination had a higher concentration of β-defensin-2 than the control group, but there was no significant increase in IL-22 concentrations. The Kruskal-Wallis test showed that the mean concentrations of IL-22 (p=0.030) and β-defensin-2 (p=0.018) were significantly different between all groups. The test was followed by the Mann-Whitney post-hoc test to determine the groups with a significant difference.

The Mann-Whitney test results for the IL 22 concentrations are presented in Table 2, showing that the epitope 4 group had the lowest concentration that was significantly different from the other groups. The results of the Mann-Whitney test for the β defensin 2 concentrations are shown in Table 3. There was a statistically significant difference between the mean β-defensin-2 concentration in the control and the other treatment groups. These results prove that intranasal immunization, both with adjuvant alone and with a combination of one of the five epitopes and adjuvant, can significantly increase the concentration of β-defensin-2 in the respiratory tract mucosa of Wistar rats. We can also see that the mean concentration of β-defensin-2 in the epitope 1 group was significantly different from that in the control group and the epitope 3, epitope 4, and epitope 5 groups. These results indicate that epitope 1 of the S. pneumoniae RrgB 214-236 pilus consisting of the 23 amino acids VNHQVGDVVEYEIVTKIPALANY as the antigen in combination with the adjuvant can increase β-defensin-2 expression significantly compared to the other epitopes.

**DISCUSSION**

β-defensin-2 is a very important antimicrobial in innate and adaptive immunity to prevent S. pneumoniae infection. The mechanism of β defensin 2 as an antimicrobial is through several ways: (a) bacterial membrane depolarization; (b) the formation of holes in the membranes of microorganisms resulting in cell leakage; (c) activating the enzymes and pathways for the degradation of bacterial walls; (d) damaging the cell wall; and (e) preventing attachment of bacteria to the host cell.
Table 1. Distribution of IL-22 and β-defensin-2 levels in the nasal rinse of rats after intra-nasal immunization, by treatment groups

<table>
<thead>
<tr>
<th>Variable</th>
<th>Treatment groups</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C (n=4)</td>
<td>A (n=4)</td>
</tr>
<tr>
<td>IL-22 (pg/mL)</td>
<td>20.80 ± 2.02</td>
<td>20.00 ± 1.15</td>
</tr>
<tr>
<td>β-defensin-2 (pg/mL)</td>
<td>396.19 ± 143.59</td>
<td>650.23 ± 27.58</td>
</tr>
</tbody>
</table>

Note: C: control. A: adjuvant; E1: epitope 1; E2: epitope 2; E3: epitope 3; E4: epitope 4; and E5: epitope 5; IL: interleukin

Epitope 1  VNHQVGDVVEYEIVTKIPALA
Epitope 2  TVKVTVDDVALEAGD
Epitope 3  TFDLVNAQAGKVQTV
Epitope 4  PKVVTYGGKKFVKVN
Epitope 5  DRAVAAYNALTAQ
epithelium.\(^{(15)}\) β-defensin-2 also has a role in adaptive immunity: (a) chemotactic activity to recruit immature dendritic cells and memory T cells from the circulation through the CCR1 and CCR5 and CCR6 receptors; (b) formation of defensin-antigen complexes that aid the presentation to dendritic cells; (c) induction of the maturation of dendritic cells by β-defensin-2, resulting in the production of IL-2, TNF-α, and IL-1 by macrophages and monocytes.\(^{(18)}\)

The regulation of β-defensin-2 expression in the respiratory epithelial cells involves multiple signaling pathways, including phosphatidylinositol-3-kinase (PI3K), mitogen-activated protein kinases (MAPKs), and protein kinase C (PKC). The bacterial antigens will induce the expression of β-defensin-2 in the respiratory epithelial cells through nuclear factor interleukin 6 (NF-IL6), the transcription factor NF-κB, myeloid ELF-1-like factor (MEF), or activated protein 1–3 (AP1–3). Other factors that induce β-defensin-2 expression are the Toll-like receptors (TLRs) 1–6 in intracellular vesicles and on the epithelial surface, and TLR 3,7,8,9 in endosomes or the endoplasmic reticulum. A previous study found that pulmonary epithelial cells infected with *Legionella pneumophila* produced defensin-2. Its expression was mediated by TLR2 and TLR5 receptors, then activated via the MAPK (p38, JNK), transcription factor NF-κB, and AP-1 pathways.\(^{(18)}\)

β-defensin-2 is expressed mainly in all epithelial layers of the human body, including the conjunctiva, respiratory tract, digestive tract, urogenital tract, mucous membranes, skin, and peripheral blood. In the respiratory tract, β-defensin-2 is induced by bacterial, viral, or fungal infection and proinflammatory cytokines such as interferon-gamma (IFN-γ), tumor necrosis factor-alpha (TNF-α), interleukin 1α (IL-1α),

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**Table 2. Results of the Mann-Whitney test of IL 22 concentrations**

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Control</th>
<th>Adjuvant</th>
<th>Epitope 1</th>
<th>Epitope 2</th>
<th>Epitope 3</th>
<th>Epitope 4</th>
<th>Epitope 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.56</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adjuvant</td>
<td>0.77</td>
<td>0.24</td>
<td>0.39</td>
<td>0.04</td>
<td>0.02</td>
<td>0.02</td>
<td>0.02</td>
</tr>
<tr>
<td>Epitope 1</td>
<td>0.25</td>
<td>0.08</td>
<td>0.08</td>
<td>0.04</td>
<td>0.02</td>
<td>0.02</td>
<td>0.02</td>
</tr>
<tr>
<td>Epitope 2</td>
<td>0.15</td>
<td>0.15</td>
<td>0.08</td>
<td>0.77</td>
<td>0.39</td>
<td>0.02</td>
<td>0.02</td>
</tr>
<tr>
<td>Epitope 3</td>
<td>0.02*</td>
<td>0.02*</td>
<td>0.02*</td>
<td>0.02*</td>
<td>0.04*</td>
<td>0.04*</td>
<td>0.04*</td>
</tr>
<tr>
<td>Epitope 4</td>
<td>0.47</td>
<td>0.24</td>
<td>0.56</td>
<td>0.39</td>
<td>0.25</td>
<td>0.15</td>
<td>0.15</td>
</tr>
</tbody>
</table>

\(^{*}\) means that there is a significant difference (p <0.05)

Epitope 1: VNHQVGDVVEYEIVTKIPALA; Epitope 2: TVKVTVDDVALEAGD; Epitope 3: TFDLVNAQAGKVQTV; Epitope 4: PKVVTYGKVFVKVN; Epitope 5: DRAAAYNALTAQ

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**Table 3. Results of the Mann-Whitney test of β defensin 2 concentrations**

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Control</th>
<th>Adjuvant</th>
<th>Epitope 1</th>
<th>Epitope 2</th>
<th>Epitope 3</th>
<th>Epitope 4</th>
<th>Epitope 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.02*</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adjuvant</td>
<td>0.04*</td>
<td>0.08</td>
<td>0.08</td>
<td>0.77</td>
<td>0.39</td>
<td>0.02</td>
<td>0.02</td>
</tr>
<tr>
<td>Epitope 1</td>
<td>0.02*</td>
<td>0.39</td>
<td>0.04*</td>
<td>0.77</td>
<td>0.39</td>
<td>0.02</td>
<td>0.02</td>
</tr>
<tr>
<td>Epitope 2</td>
<td>0.04*</td>
<td>0.39</td>
<td>0.02*</td>
<td>0.77</td>
<td>0.39</td>
<td>0.02</td>
<td>0.02</td>
</tr>
<tr>
<td>Epitope 3</td>
<td>0.02*</td>
<td>0.77</td>
<td>0.04*</td>
<td>1.00</td>
<td>0.77</td>
<td>0.39</td>
<td>0.02</td>
</tr>
<tr>
<td>Epitope 4</td>
<td>0.02*</td>
<td>0.77</td>
<td>0.04*</td>
<td>1.00</td>
<td>0.77</td>
<td>0.39</td>
<td>0.02</td>
</tr>
<tr>
<td>Epitope 5</td>
<td>0.02*</td>
<td>0.77</td>
<td>0.04*</td>
<td>1.00</td>
<td>0.77</td>
<td>0.39</td>
<td>0.02</td>
</tr>
</tbody>
</table>

\(^{*}\) means that there is a significant difference (p <0.05)

Epitope 1: VNHQVGDVVEYEIVTKIPALA; Epitope 2: TVKVTVDDVALEAGD; Epitope 3: TFDLVNAQAGKVQTV; Epitope 4: PKVVTYGKVFVKVN; Epitope 5: DRAAAYNALTAQ
interleukin 1β (IL-1β), and interleukin 6 (IL-6). β-defensin-2 is expressed in the mucous membrane from the oral cavity to the epithelium of the lungs. A previous study showed that cytokines IL-17 and IL-22 produced by Th17 cells are more powerful in controlling the expression of β-defensin-2 on mucosal surfaces than are other cytokines.

Interleukin-22 is an IL-10 family cytokine produced by the lymphoid lineage cells CD4+ and CD8+ T cells, NK cells, γδ T cells, and innate lymphoid cells (ILCs). In mucosal immunity, Th17 cells are a significant source of IL-22. IL-22 has two heterodimeric transmembrane receptors, namely IL-10R2 and IL-22R1, which subsequently activate the ERK, JAK/STAT3, and JNK pathways that induce the expression of β-defensin-2. Various factors regulate IL-22 secretion. The main inducers of IL-22 are IL-23 and IL-1β, which are produced by dendritic cells and macrophages. On the contrary, the expression of IL-22 is inhibited by TGF-β, inducible costimulator (ICOS), and IL-27.

Interleukin-22 is essential for human immunity against extracellular pathogens, such as *S. pneumoniae*. The function of IL-22 can be categorized into three major areas. First, IL-22 initiates epithelial proliferation that maintains and restores the integrity of the epithelial barrier function during the invasion of pathogens. Second, IL-22 induces the production of inflammatory mediators such as IL-1β, IL-6, serum amyloid A, granulocyte-colony stimulating factor (G-CSF), and lipopolysaccharide-binding protein. Third, IL-22 synergistically with other cytokines, such as IL-17 or TNF-α, induces the expression of antimicrobial peptides (AMPs), including β-defensin-2, β-defensin-3, S100A7, S100A8, and S100A9, that are critical for mucosal immunity in the airways, the skin and the intestine.

In our study, there was a statistically significant difference between the mean β-defensin-2 concentrations in the control group compared to the other treatment groups. This signifies that intranasal immunization of adjuvant alone or a combination of each of the five different epitopes of *S. pneumoniae* RrgB pilus with an adjuvant can significantly increase the concentration of β-defensin-2 in the respiratory tract mucosa of Wistar rats. The mean β-defensin-2 concentration in the epitope 1 group was the highest and significantly different from that in the control group and the epitope 3, epitope 4, and epitope 5 groups. These results indicate that a combination of adjuvant and epitope 1 of the *S. pneumoniae* RrgB 214-236 pilus containing the 23 amino acids VNHQVDVVEYIVTKIPALANY as the antigen can increase β-defensin-2 expression significantly and has a greater potential to be developed into a pneumococcal vaccine than have the other epitopes. Previous studies have shown that synthetic epitope-based vaccines usually must consist of 20-30 amino acids to induce a good immune response. In addition, the longer cytotoxic T lymphocyte epitope can expand the target range to increase the peptide’s immunogenicity. In this study, these characteristics are found in epitope 1, which has 23 amino acids and is the longest epitope compared to the other four epitopes.

There has been no research about the effect of intranasal immunization with RrgB epitopes on β-defensin-2 concentrations. A previous study that can be used for comparison is the research by Mufida et al., in which there was an increase in β-defensin-2 concentration through the upregulation of IL-22 and IL-17A expression in the nasal rinses of Wistar rats immunized intranasally with the 54 kDa *S. pneumoniae* hemagglutinin pilus which is identical to the RrgB protein. In that study, the concentration of β-defensin-2 in the adjuvant group was not significantly different from the control group, therefore it was concluded that the cholera toxin subunit B (CTB) adjuvant does not increase β-defensin-2 expression and only acts as an immunomodulator. These results differ from those of the present study, where the β-defensin-2 concentration in the adjuvant group was relatively high and significantly different from that of the control group.
Cholera toxin subunit B is a non-toxic homopentamer protein antigen, that is often used as an adjuvant for mucosal vaccines, both intranasal and oral. Cholera toxin subunit B-based vaccines can elicit a stronger mucosal immunity because CTB has high affinity for monosialotetrahexosylganglioside (GM1) receptors on leukocytes and mucosal epithelium, especially M cells.\(^{22}\) Besides, CTB can increase antigen uptake in the mucosa and stimulate the expression of CD40, CD80, and CD86 and the secretion of IL-1 and IL-6 by APCs such as B cells, dendritic cells, and macrophages. This feature is intended to reduce the minimum antigen dose for causing an sIgA and IgG adaptive immune response. Cholera toxin subunit B has a low direct effect on Th17 cells, but other studies have shown that CTB stimulates the secretion of IL-17A by CD4 + T cells and the differentiation of Th17 cells. Mature Th17 cells do not express IL-22 but express IL-17F and IL-17A. CTB via the cAMP-PKA pathway can induce the secretion of IL-6, TGF-β, IL-17A, and GM-CSF.\(^{11}\) IL-17A is an essential cytokine that induces the secretion of β-defensin-2 in the epithelial cells of the respiratory tract mucosa. The ability of IL-17A to stimulate β-defensin-2 expression has been investigated in-vitro in human tracheobronchial tissue. It is proven that IL-17A induces a stronger β-defensin-2 expression than other cytokines, especially TNFα, IL-1α, IL-1β, and IL-6.\(^{16}\) In our study, CTB administration as an adjuvant may have increased IL-17A, IL6, and TGF-β secretion that in turn may have raised β-defensin-2 concentration. The increase in TGF-β can inhibit IL-22 expression. A previous study found that increased TGF-β leads to the loss of Th22 cells that produce IL-22 in the intestinal mucosa.\(^{23}\) Similarly, in our study there was an increase in β-defensin-2 concentration in the adjuvant group, but no increased IL-22 concentration in all groups.

The limitations of this study are that the mechanism and pathway underlying the improvement of β-defensin-2 expression after immunization with \textit{S. pneumoniae} RrgB epitopes are still unclear. Bacterial antigens such as the epitopes of the \textit{S. pneumoniae} RrgB pilus can induce the expression of β-defensin-2 in the respiratory epithelial cells to involve multiple signaling pathways through the NF-IL6, NF-κB, MEF, AP1–3, PI3K, MAPKs, or PKC and stimulate proinflammatory cytokines such as IL-22, IL-17A, IL-1α, IL-1β, IL-6, or TNFα, and IFN-γ.\(^{18}\) To know the mechanism of the increase in β-defensin-2 expression by the RrgB epitopes, further research is needed to measure the concentration of other proinflammatory cytokines that induce β-defensin-2 expression. Another limitation is that there is no group immunized with antigen alone; in further studies it is necessary to include such a group to ensure that the increase in β-defensin-2 is caused only by antigen. External factors such as inflammation and infection can affect the expression of β-defensin-2, such that routine health examinations on the experimental animals need to be carried out. The selection of adjuvant also needs to be considered, because CTB as an adjuvant may stimulate IL-17A, IL6, and TGF-β secretion that increases β-defensin-2 concentration.\(^{11}\) All these factors can affect the results of the study.

The study by Gentile et al.\(^{24}\) also proved that intraperitoneal immunization with the \textit{S. pneumoniae} RrgB protein significantly increased serum IgG and protected experimental animals from bacteremia. These results indicate that intranasal immunization with the \textit{S. pneumoniae} RrgB pilus epitope in our study also may have the potential to increase mucosal and systemic immunity.

The high concentration of β-defensin-2 in nasal rinses of Wistar rats, especially in the epitope-1 group indicates that intranasal immunization with the \textit{S. pneumoniae} RrgB protein epitope can increase mucosal immunity and potentially protect against \textit{S. pneumoniae} infection. In future studies to prove the RrgB protein epitope as a candidate for a new pneumococcal vaccine, the sIgA concentration in the respiratory tract mucosa and the serum
IgM or IgG concentrations should be measured to determine the adaptive mucosal immune response and the systemic immune response, respectively. Our present study is expected to be the beginning of a new breakthrough to solve the problem of pneumococcal vaccines and *S. pneumoniae* infection.

**CONCLUSIONS**

This study demonstrated that intranasal immunization with the *S. pneumoniae* RrgB epitope could induce a mucosal immune response by increasing β-defensin-2 expression in the nasal rinse of Wistar rats. Combining adjuvant and the *S. pneumoniae* RrgB 214-236 pilus epitope-1 as antigen comprising the 23 amino acids VNHQVGDVVEYEIVTKIPALANY can increase β-defensin-2 expression significantly and has a greater potential to be developed into a pneumococcal vaccine compared to the other epitopes.

**CONFLICT OF INTEREST**

There is no conflict of interest in the writing and publishing of this manuscript.

**ACKNOWLEDGEMENT**

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**CONTRIBUTORS**

DCM, BH, DA, MAS, and YA contributed to the design of this study. DCM, BH, and ADS contributed to performing experiments and data collection and analysis. DCM, BH, ADS, DA, MAS, and YA contributed to the article’s writing and critical revision, and approved the final manuscript.

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