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# Characterization of Pili Protein 67 kDa *Streptococcus pneumoniae*: New Candidate for Virulence Factor-Based Pneumococcal Antigen Vaccine

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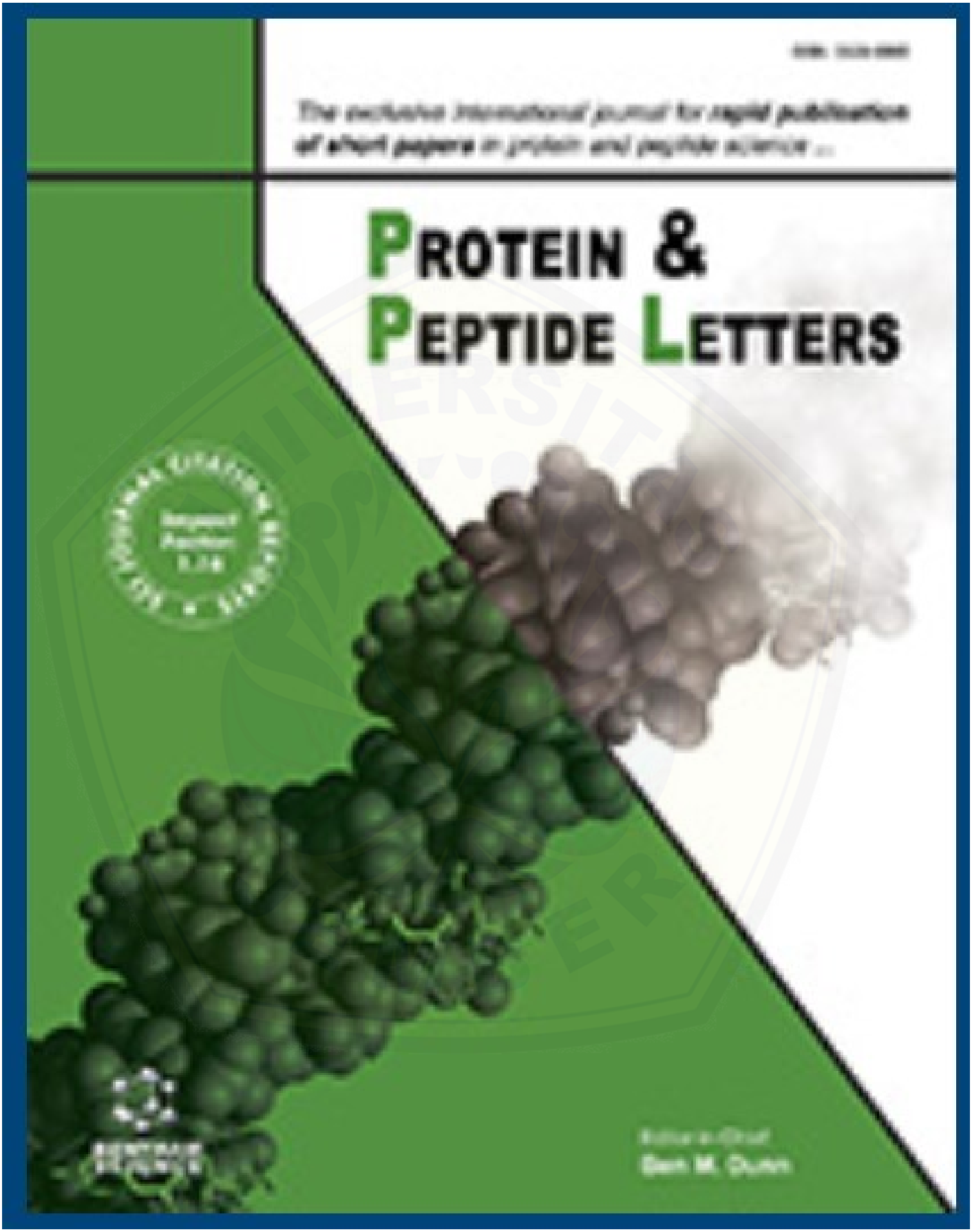

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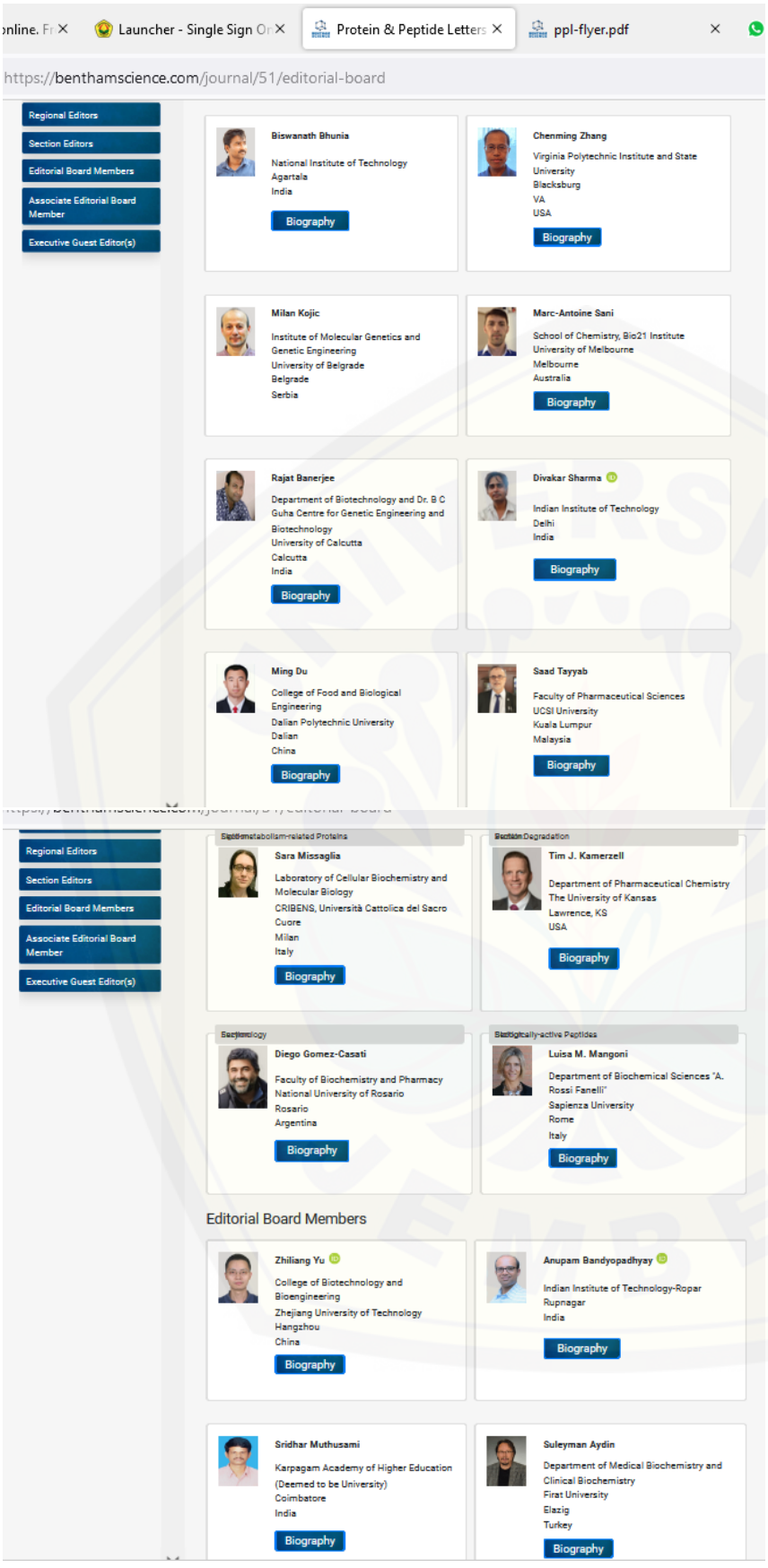

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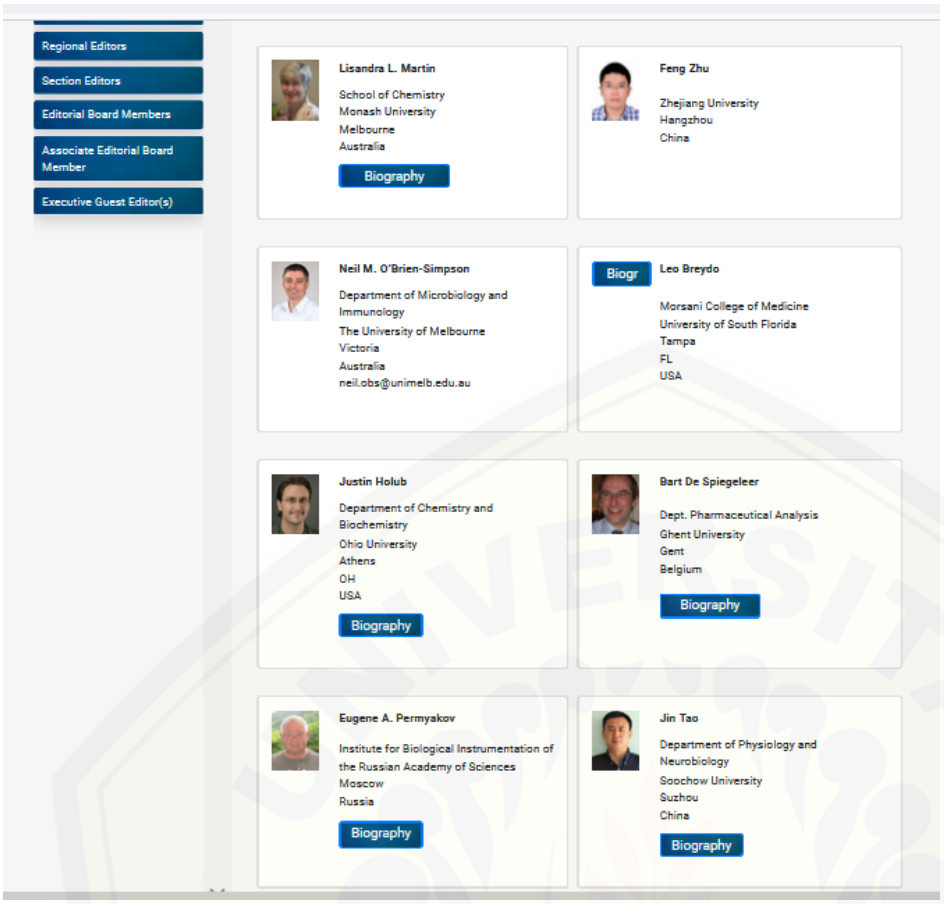

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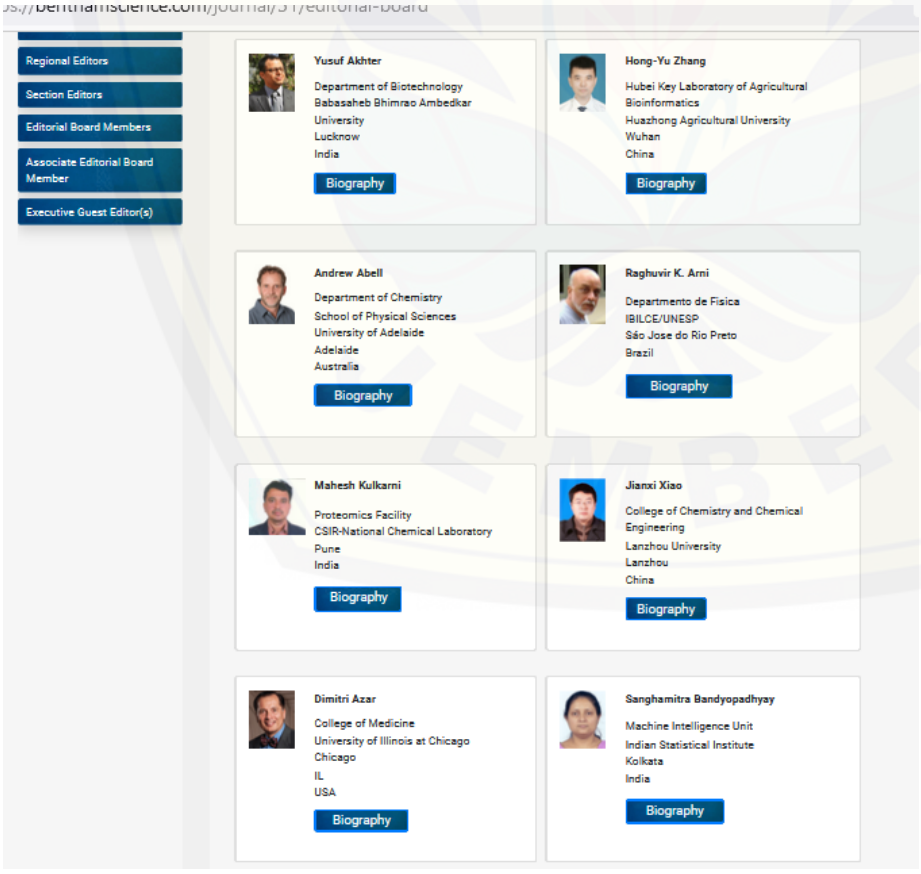

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RESEARCH ARTICLE

# Characterization of Pili Protein 67 kDa *Streptococcus pneumoniae*: New Candidate for Virulence Factor-Based Pneumococcal Antigen Vaccine

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**Abstract:** ***Introduction*:** *Streptococcus pneumoniae* is a Gram-positive diplococci bacteria that causes infectious diseases such as otitis, meningitis, and pneumonia. *Streptococcus pneumoniae* has various virulence factors, one of which is pilus. In addition to being immunogenic, pilus *S. pneumoniae* also plays a role in bacterial adhesion to host cells and biofilm formation. The *S. pneumoniae* pilus found in this study consisted of several proteins with various molecular weights, one of which was a 67 kDa protein.

***Objective*:** This study aimed to determine the characteristics of the 67 kDa pilus protein, including its capacity as hemagglutinin and adhesin and its amino acid sequence (AA).

***Methods*:** The LCMS/MS method is used to determine the AA sequence of the 67 kDa pilus protein. The AA structure was analyzed through BLASTP by matching it with the sequence of the protein data bank of *S. pneumoniae* (taxid: 1313). The ProtParam tool from ExPASY was used to calculate various physical and chemical parameters of the protein, while for evaluating its immunogenicity, the VaxiJen V2.0 online server was used.

***Results*:** The results of this study indicate that the 67 kD a pilus protein, is an anti-hemagglutinin protein and has a role as an adhesin protein. Adhesion tests show the action between protein concentration and the number of bacteria attached to enterocyte cells. LCMS/MS test results obtained by BLASTP showed that the 67 kDa pilus protein had three AA sequences (ITYMSPDFAAPTLAGLDDATK, AEFVEVTK, and LVVSTQTALA), which had similarities with the A backbone chain of *S. pneumoniae* pilus. The physicochemical test showed that the protein is hydrophilic and nonpolar, while the antigenicity test showed that the protein is antigenic.

***Conclusion*:** Based on these characteristics, it can be concluded that the 67 kDa *S. pneumoniae* pilus protein can be used as a vaccine candidate for pneumococcus.

**Keywords:** Pilus 67 kDa, *S. pneumoniae*, vaccine, virulence factors, gram-positive, antigen.

## 1. INTRODUCTION

*Streptococcus pneumoniae* is a common primary cause of bacterial pneumonia, otitis media, meningitis, and sepsis in children worldwide. Pneumococcal pneumonia causes around 826,000 deaths in children under five years of age [1]. There were 156 million cases of pneumococcal infection which generated more than 2 million deaths in 2004 [2]. *Streptococcus pneumoniae* caused around 30-50% cases of infant mortality due to pneumonia infections in 2010. Pneumonia was the primary cause of infant mortality caused by *S. pneumoniae*. Antibiotics are the main treatment for pneumonia, however because of the increasing rates of antimicrobial resistance (AMR), prevention is often the best form of treatment. For example, it has been proven that the *Haemophilus influenzae* type B (Hib) and *S. pneumoniae* (pneumococcus) conjugate vaccine can not only prevent life-threatening diseases caused by these bacteria but also reduce the use of irrational antibiotics and AMR [3, 4].

Pneumococcal conjugate vaccine (PCV) is an effective vaccine to prevent pneumonia, but the protective efficacy of PCV is limited. This limitation is caused by its composition based on the geographical prevalence and virulence of specific pneumococcal serotypes, so new vaccine candidates are needed that have the potential to protect against all serotypes of *S. pneumoniae*. Instead of the whole cell's vaccine, a protein-based pneumococcal virulence factor can be developed as a potential vaccine candidate. To create an ideal vaccine, several criteria are needed: immunogenic properties; must be possessed by all *S. pneumoniae* serotypes; involve humoral and cellular immune responses, and provides long-term protection [5]. Vaccine candidates are taken from protein that plays a role in the bacterial

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colonization process so that it can be used to block its colonization to the host through the adhesion process [6]. This process is played by proteins on the surface of bacteria such as pili. Pilus is a multimeric filamentous surface structure consisting of protein subunits with LPxTG motifs. The subunits are targeted by sortases so that the two different sortases (housekeeping or A-type and pilus-specific or C-type) are involved in pilus assembly. Pilus-specific sortase catalyzes the polymerization of pili by covalently connecting pilins, while housekeeping sortase covalently connects the assembled pilus to the cell wall [7, 8].

*Streptococcus pneumoniae* has two types of pili, type 1 and type 2. Type 1 pilus consists of three proteins: RrgA; RrgB; and RrgC. Type 2 pilus consists of two pilus proteins, pitA, and pitB proteins [8, 9]. Type 1 pilus has helped in the pathogenesis of the infectious process, namely adhesion, colonization, and facilitating the formation of microcolonies and biofilms. Studies conducted by Mufida *et al*, 2018, have shown that the adhesin protein strengthens bacterial colonization of host cells. The research indicated that *S. pneumoniae* has some pili proteins with 67, 54, 25, and 11 kDa molecular weight. Of all these proteins, 54 kDa is a hemagglutinin protein that has similarities with the backbone pilin (RrgB) from *S. pneumoniae* [10]. Meanwhile, 67 kDa's protein, which is one of the constituent pilus proteins that have diverse characteristics, plays the same role in the pathogenesis of *S. pneumoniae* transmission. For this reason, we need to do further research on this 67 kDa protein.

## 2. MATERIALS AND METHODS

### 2.1. Subject

In this research, we used *S. pneumoniae* isolates of pneumoniae patient obtained from the Balai Besar Laboratorium Kesehatan Surabaya, East Java, Indonesia.

### 2.2. Breeding Bacteria

The method used is a modification of that used in the study by Sumarno *et al*. [11]. After identification, *S. pneumoniae* was propagated on BAP medium by incubation at 35 °C for 18-24 h. The culture was transferred to a two-component medium consisting of a BHI liquid medium to which 5% sheep blood was added to obtain TCG and incubated in $CO_2$ incubators or an anaerobic jar with a candle at 35 °C for 18-24 h.

### 2.3. Pilus Isolation

The cultured bacteria were collected in a 100 ml tube to which TCA was added so that the concentration was 3%, was shaken for 30 min, then left at room temperature for 1 h, and then centrifuged at 4 °C 5635 x g for 30 min. Three grams of bacteria were suspended in 6 ml of PBS at pH 7.4 and then placed in a pilus cutter tube. The pilus was cut using a bacterial pilus cutter at 3913 x g rpm at 4 °C for 30 seconds. The bacteria suspension was then centrifuged at 2,2539 x $10^{-4}$g for 15 min. The supernatant was conserved, and the bacterial pellet was suspended with PBS pH 7.4; the process was repeated four times more. All supernatants containing pilus protein were mixed [11].

### 2.4. SDS-PAGE Pilus *S. pneumoniae* 67 kDa Protein

To determine the molecular weight of pilus protein, SDS-PAGE was performed using a 12% separating gel and 4% stacking gel. Before the sample was put into the well, it was given a buffer and then heated for 5 min. A total of 20 µl protein samples were inserted into the gel pit. Electrophoresis was carried out for 60 min, 125 volts, at room temperature in which the electrode was placed in a Tris buffer with pH 8.3. Brilliant coomassie blue is used for gel staining (for 30 min) followed by destaining [11, 12].

### 2.5. Purification of *S. pneumoniae* Pilus Protein 67 kDa

Purification of pilus protein was carried out by electroelution using gel pieces. Pilus proteins that had been characterized for molecular weight were cut off in the dominant band. The cut protein band is inserted into a cellulose membrane containing an electrophoretic running buffer. Electroelution uses a horizontal electrophoresis tool with a voltage of 125 mV for 120 min. Fractions from electroelution were then dialyzed in d 2 L PBS pH 7.4 for 2 × 24 hours at 4 °C [13, 14].

### 2.6. The Hemagglutination Assay

The hemagglutination titer was determined by the interaction between the electroeluted protein and the erythrocytes of mice. 50 ul of PBS was added to each well on the microplate except for the first one to add 50 ul of protein solution, then serially diluted. As a negative control, 50 ul of erythrocyte suspension was added to the last well, shaken for 15 minutes, and then placed at room temperature. The results of the hemagglutinin test of the sample were read if the control well shows a red dot. An anti-hemagglutination test was also performed in this study. The difference with the hemagglutination test was only in the components of each well. 50 L of PBS and 54 kDa of *S. pneumoniae* pilus protein, which is a hemagglutinin protein, were introduced into the well. After 50 L of 67 KDa protein was added it was continued with serial dilutions. Finally, 50 ul of erythrocyte suspension was added to each well, shaken for 15 minutes, and placed at room temperature [15].

### 2.7. Isolation of Enterocyte Cells in Mice

Isolation of enterocyte cells was carried out by the Weisser method that had been modified. Mice were anesthetized using chloroform, and then the small intestine was taken, and washed with PBS pH 7.4, which contained 1 mM dithiothreitol, at 4 °C until it looked clean. After that, it was put into a liquid containing 1.5 mM KCl, 9.6 mM NaCl, 27 mM Na Citrate, 8 mM $KH_2SO_4$, and 5.6 mM $Na_2HPO_4$ with pH 7.4. Then, put in a shaker incubator for 15 min at 37 °C. The next step is to remove the supernatant, while the tissue is added to a liquid containing 1.5 mM EDTA and 0.5 mM dithiothreitol. The mixture is shaken vigorously for 15 min at 37 °C, then the supernatant is removed. The tissue was washed with PBS and centrifuged for 5 min at 157 x g. The process was repeated three times [16].

### 2.8. Adhesion Test

Modified adhesion test of Nagayama, in the adhesion test of *S. pneumoniae*, bacteria were bred in BAP at 37 °C, 5% $CO_2$ for 24 h. Next, the harvested bacteria were suspended with PBS, and the bacterial content was made OD 1 using a 600 nm wavelength spectrophotometer. Preparation of pilus protein dose was made as much as 0 µg, 25 µg, 50 µg, 100 µg, and 200 µg, respectively. 300 µl of epithelial cell

suspension (cell count) was mixed with 0, 25, 50, 100, and 200 µg of electroeluted protein and then gently shaken at 37 °C for 30 minutes. That mixture was added to the bacterial suspension as much as 300 µl and then incubated with a shaking incubator for 30 minutes at 37 °C. Centrifugation was carried out at 352 x g at 4 °C for 3 minutes. After washing with PBS twice, the precipitate was added with 50 µl of PBS. The suspension was then applied to a slide for Gram staining. Bacterial adhesion to epithelial cells was calculated by observing the object under a microscope at 1000x. magnification [17].

### 2.9. Production of *S. pneumoniae* Protein Antibodies 67 kDa

The pilus protein used as an antigen was the *S. pneumoniae* pilus hemagglutinin protein. The protein was injected intraperitoneally into mice at a dose of 50 µg/50 µL plus Complete Freund's Adjuvant with the same volume at the first immunization. For boosters (at the 2nd and 3rd immunization), the Incomplete Freund's adjuvant with the same dose as antigen was given. One week after the last immunization, the mice were euthanized and antibodies were isolated from the serum [18].

### 2.10. Western Blotting

The western blotting analysis was performed to detect the presence of proteins identified by antibodies. The SDS-PAGE gel was immersed in a transfer buffer before transferring to the membrane. Furthermore, a pile resembling a sandwich composed of two layers of Whatman paper, SDS-PAGE acrylamide gel, PVDF membrane, and foam. The pile above must be tight, and there was no bubble. Semidry blotting (Bio-Rad) is used in this procedure for 1 h at 100 mA. The NC membrane is immersed in 5% non-fat dry milk that has been mixed in a blocking buffer for 1h at room temperature. Before incubating for 24 h at 4 °C with mice serum in a blocking buffer (1:100). The membrane was washed with 0.05% Tween in PBS three times. After that, the membrane was incubated with alkaline-phosphatase rabbit anti-mouse IgG secondary antibodies at a dilution of 1:200 for 2 h at room temperature. Finally, the membrane was washed using TBST Nitro-blue tetrazolium-bromo-4-chloro-3 indolyl phosphate three times [19].

### 2.11. In-gel Protein Digestion and Liquid Chromatography-mass Spectrometry (LC-MS/MS) Analysis

Trypsin was used for in-gel digestion from the protein sample to extract the peptide using standard procedure [20]. Electrospray ionization mass spectrometry was used to analyze peptides. Insert the tryptic peptides into column C18 300 SB, 5 m, and linear water gradient/acetonitrile / formic acid spectrometer 0.1% used to separate it. The identity of 67 kDa protein was obtained by LCMS/MS method and its physicochemical properties by in silico analysis.

### 2.12. MS data Analysis

To search for protein databases, we used BLASTP (https://blast.ncbi.nlm.nih.gov/Blast.cgi). This program compares the protein sequence to a sequence database by entering the name, tax ID, or protein query. BLASTP was also used to compare all novel proteins and human proteins [20].

### 2.13. Antigenicity and Epitope Mapping Analysis

For searching and predicting epitopes from protein sequences, we used Kolaskar and Tongaonkar antigenicity (http://www.iedb.org), and to predict linear B-cell epitope, we used the DiscoTope method [21].

### 2.14. Physiochemical Analysis

The physiochemical analysis by *in silico* analysis can describe molecular weight, theoretical pI, amino acid composition, instability index, aliphatic index, and the grand average of hydropathicity (GRAVY) of the protein target sequence, was performed using the ProtParam tool (http://web.expasy.org/protparam/) [22].

## 3. RESULT

### 3.1. Identification and Detection of Protein

*Streptococcus pneumoniae* pilus protein 67 kDa was visualized by SDS-PAGE (Figure **1**). A protein band known as 67 kDa protein is cut for electroelution and dialysis. The results of dialysis in the form of a protein solution were tested for their ability to agglutinate erythrocytes (hemagglutination test) and also the ability of adhesion to epithelial cells (adhesion test), and a western blotting test showed protein pilus detected by antibody-protein 67 kDa (Figure **2**).

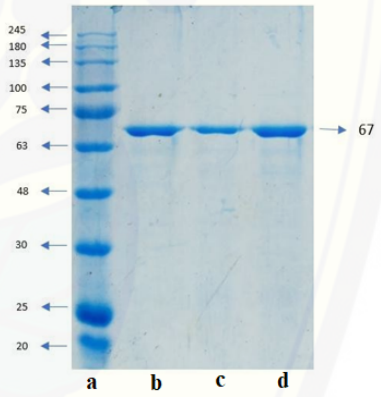


**Figure 1.** *Streptococcus pneumoniae* pilus protein profile from SDS-PAGE gel. (**a**) marker protein, (**b**) 1st cut pilus (**c**) 2nd cut pilus, (**d**) 3rd cut pilus protein fraction. *(A higher resolution/colour version of this figure is available in the electronic copy of the article).*

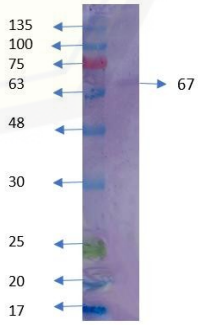


**Figure 2.** Western blotting result of pilus protein *S. pneumoniae* 67 kDa detected with antibody anti-67 kDa of *S. pneumoniae* pilus protein. *(A higher resolution/colour version of this figure is available in the electronic copy of the article).*

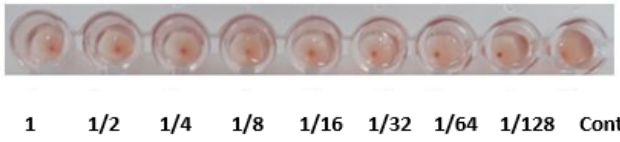


**Figure 3.** The results of the hemagglutination test on *S. pneumoniae* pilus protein 67 kDa. The negative results (red dots) indicate that the protein is not a hemagglutinin protein. *(A higher resolution/colour version of this figure is available in the electronic copy of the article).*


**Figure 4.** The result of the anti-hemagglutination test of *S. pneumoniae* pilus protein 67 kDa using *S. pneumoniae* 54 kDa as hemagglutinin protein. The positive result (no red dot) showed that the protein pilus 67 kDa is not a hemagglutinin protein. *(A higher resolution/colour version of this figure is available in the electronic copy of the article).*

### 3.2. Hemagglutination Test

The 67 kDa pilus protein is a non-hemagglutinin protein because, from the hemagglutination test, the protein does not agglutinate erythrocytes (Figure **3**). The characteristics of non-hemagglutination of these proteins are also shown in anti-hemagglutination tests using *S. pneumoniae* 54 kDa pilus protein (Figure **4**). The 54 kDa protein as a hemagglutinin protein (the results of previous studies) can bind to erythrocytes so that no sediments will form at the bottom of the plate (red dot). The resulting test shows that in the first dilution of 67 kDa pilus protein, agglutination of erythrocyte was also exposed to 54 kDa hemagglutination protein. Hemagglutination still occurred until three times dilution, and it disappeared after four times dilution, so it can be concluded that the 67 kDa pilus protein was able to inhibit the effect of 54 kDa protein in the hemagglutination process (Figure **4**).

### 3.3. Adhesion Test

The adhesion test was carried out to determine the function of 67 kDa pilus protein in the process of adhesion of bacteria to host cells. This is a test of competition by 67 kDa protein that analyzes the number of bacterial cellsadhesion to epithelial cells. The results of the adhesion test showed that the 67 kDa pilus protein influenced the adhesion process. The higher the dose of 67 kDa pilus protein, the fewer bacteria were attached (Figures **5a-f**). Linear regression test showed $R^2$ 0.618 with $\alpha = 0.00$ (Figure **6**).

### 3.4. MS Analysis for Pilus Protein *S. pneumoniae* 67 kDa

MS data suggested that three peptides from the 67 kDa band were matched with chain A backbone pilus *S. pneumoniae* (Table **1**).

### 3.5. Antigenicity and Epitope Mapping Analysis

The antigenicity analysis result showed that chain A backbone pilin of *S. pneumoniae* proteins using Kolaskar and Tongaonkar antigenicity (http://www.iedb.org) have a polyantigenic region (Table **2**). Epitope mapping analysis showed that the protein has polyepitope regions (Table **3**). The proteins of pili protein *S. pneumoniae* were not similar to human surface cell proteins.

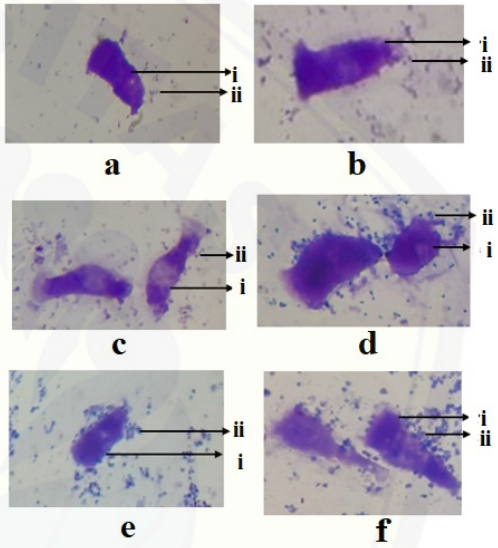


**Figure 5.** The results of adhesion test with various concentrations of 67 kDa pilus protein from *S. pneumoniae*: (**a**) 200 µg; (**b**) 100 µg; (**c**) 50 µg; (**d**) 25 µg; (**e**) 12.5 µg; (**f**) 0 µg. (i) enterocyte cells (columnar epithelial cell). (ii) *S. pneumoniae* (coccus gram-positive bacteria). *(A higher resolution/colour version of this figure is available in the electronic copy of the article).*

### 3.6. Physiochemical Analysis

The results of the physicochemical analysis showed that the protein with a molecular weight of 71364.09 Da (71.3 kDa) was the main structure of chain A of the backbone of the *S. pneumoniae* protein. Theoretical PI is 5.01 and the aliphatic index is 82.06. GRAVY is -0.347, showing hydrophilic character. The instability index is 15.18 (<40), indicating that the protein is stable.

## 4. DISCUSSION

*Streptococcus pneumoniae* is the leading cause of deaths from respiratory infections in children. One of the virulence factors of *S. pneumoniae*, which plays a role in the adhesion process, is pilus. Both gram-positive and gram-negative bacteria have pilus, which is divided into two based on its ability to hemagglutinate erythrocytes namely hemagglutinin and non-hemagglutinin proteins. The characteristics of the

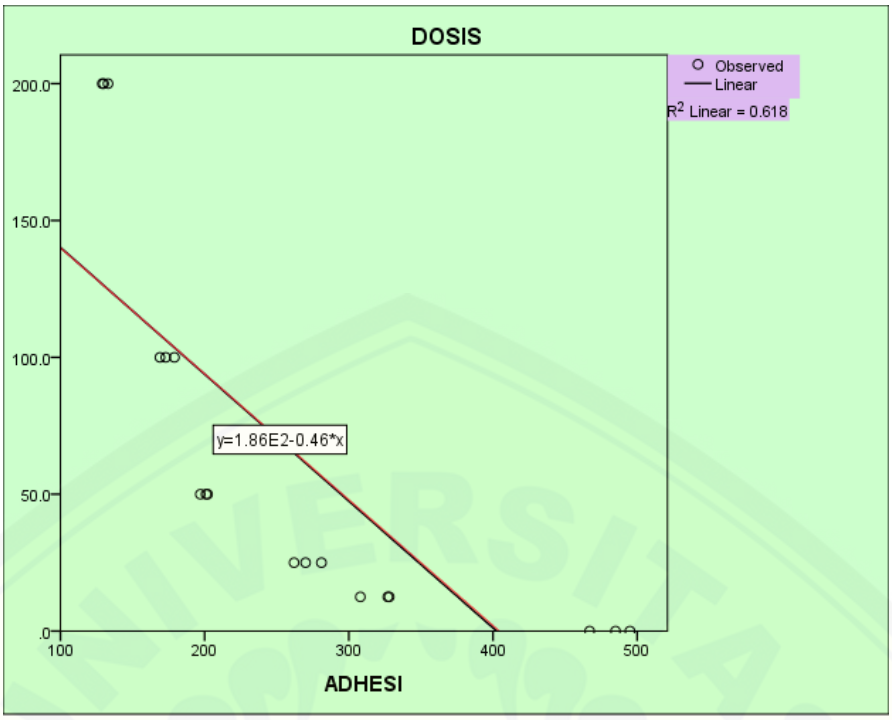


**Figure 6.** Linear regression test between the dose of 67 kDa pilus and the number of bacterial cells that adhesion to epithelial cell ($R^2$=0.618). *(A higher resolution/colour version of this figure is available in the electronic copy of the article).*

**Table 1. Three peptides from pilus of *S. pneumoniae* 67 kDa are identical with backbone pilin (RrgB).**

| AA Residues | Query Coverage (%) | Identity (%) | E-value | Accession |
|---|---|---|---|---|
| ITYMSPDFAAPTLAGLDDATK | 56 | 100 | 4.5 | 3RPK A |
| AEFVEVTK | 50 | 100 | 4.7 | 3RPK A |
| LVVSTQTALA | 90 | 66.7 | 2.5 | 3RPK A |

pilus are also confirmed by a study of Shigella sp. that is directly proportional to the ability to colonize host cells. Adhesion to host epithelial cells is an initiation process before bacterial colonization occurs [23, 24]. Research on *Bordetella pertussis* shows that this bacterium has a hemagglutinin protein which also acts as an adhesin making it a potential candidate for a *Bordetella pertussis* vaccine [25, 26].

Pilus in *S. pneumoniae*, type 1 pneumococcus, plays an important role *in vivo* research of rats. The results of the study show that the state of active pilus is thermosensitive. This pilus gene is suppressed by members of the Snf2 proteins family [23]. In this study, the results showed that the 67 kDa *S. pneumoniae* protein is an anti-hemagglutinin protein because it inhibits the activity of the hemagglutinin protein (Figures **3** and **4**). Although it is an anti-hemagglutinin protein, the 67 kDa protein is an adhesin protein. The adhesion test showed that the higher the concentration of pilus protein (67 kDa), the fewer bacteria attached to the epithelial cells (Figures **5a-f**), and the statistical analysis of protein concentration and adhesion index gave $R^2$ 0,618. The results of the adhesion test show that the ability to do adhesion is not only determined by the ability to do hemagglutination. Many adhesin (or pilus) proteins are known, and many of them could be anti-hemagglutinin [27].

Pilus pneumococcus consists of three proteins: RrgA; RrgB; and RrgC, each of which is stabilized and covalently polymerized by intramolecular isopeptide bonds to be extended fibers. The RrgB protein is a type 1 *S. pneumoniae* backbone (BP) pilus, which has an important role in the formation of pilus, and if RrgB is absent, then pilus will not be formed. *In vivo* RrgB has been proven to protect mouse models with sepsis and pneumonia so that they can be potential candidates for protein-based vaccines. The RrgB forms a polymeric pilus rod consisting of several hundred BP subunits arranged in a string. This protein consists of four domains, such as Ig, namely D1-D4, with the N-terminal located at D1 and the C-terminal located at D4 [28-30].

Our present study showed *S. pneumoniae's* 67 kDa protein identity with chain A backbone pilus *S. pneumoniae*.

**Table 2. Antigenic regions of chain A backbone pilus of *S. pneumoniae* proteins.**

| S. No. | Start Position | End Position | Peptide Sequence | Peptide Length |
|---|---|---|---|---|
| 1 | 9 | 30 | TMLAALLLTASSLFSAATVFAA | 22 |
| 2 | 36 | 44 | SVTVHKLLA | 9 |
| 3 | 65 | 71 | KVGVLPA | 7 |
| 4 | 134 | 150 | LPAAKYKIYEIHSLSTY | 17 |
| 5 | 161 | 182 | SKAVPIEIELPLNDVVDAHVYP | 22 |
| 6 | 212 | 234 | VNHQVGDVVEYEIVTKIPALANY | 23 |
| 7 | 253 | 268 | TVKVTVDDVALEAGDY | 16 |
| 8 | 278 | 284 | DLKLTDA | 7 |
| 9 | 357 | 362 | APIPAG | 6 |
| 10 | 367 | 372 | FDLVNA | 6 |
| 11 | 375 | 384 | GKVVQTVTLT | 10 |
| 12 | 442 | 455 | PKVVTYGKKFVKVN | 14 |
| 13 | 464 | 469 | AEFVIA | 6 |
| 14 | 490 | 497 | KQLVVTTK | 8 |
| 15 | 501 | 510 | DRAVAAYNAL | 10 |
| 16 | 529 | 537 | AYNAAVIAA | 9 |
| 17 | 552 | 558 | VVKLVSD | 7 |
| 18 | 566 | 573 | TGLLAGTY | 8 |
| 19 | 582 | 589 | AGYALLTS | 8 |
| 20 | 594 | 600 | EVTATSY | 7 |
| 21 | 619 | 625 | TKVVNKK | 7 |
| 22 | 636 | 644 | TIIFAVAGA | 9 |
| 23 | 647 | 655 | MGIAVYAYV | 9 |

Similarities are taught by three peptides, namely ITYMSPDFAAPTLAGLDDATK, AEFVEVTK, and LVVSTQTALA (Table **1**). Chain A backbone pilus *S. pneumoniae* is a full-length RrgB protein. This result is supported by a study, which showed that the RrgB monomer has a molecular weight of about 65 kDa. In addition to citing the D1 domain, citing a full-length and D2-D4 fragment structure of RrgB may also be relevant [31, 32].

The results of this study are reinforced by the results of studies from Gentile *et al*. [31]. In his research, active and passive immunization procedures were performed to test the RrgB (D1-D4) domain. The results state that the domain of protection level that commensurates with the full-length RrgB protein is the D1 domain, meaning that this domain is most effective compared to other domains. Based on spectrum analysis, the D1 domain also has many regions that do not contain intramolecular isopeptide bonds and are shared with other fold domains such as Ig. This shows that the flexibility of D1 conformation is very important for the process of protein-antibody recognition.

Antibody-protein interactions in the humoral immune response have an important role. Antibodies released by B cells bind to antigens. The specific part of the antigen recognized by the antibody is called a B-cell epitope. Identification of B cell epitopes is a prerequisite for the creation of epitope-based vaccinations. *In silico* bioinformatics is one of the methods for extracting B cell epitopes from immunogenic proteins. This method offers a promising and cost-effective approach to identifying potential B-cell epitopes in target vaccine candidates. In this study, the results of the analysis of B-cell epitopes showed that the chain A backbone pilus of chain A *S. pneumoniae* has polyantigenic and

**Table 3. Epitope regions of chain A backbone pilus of *S. pneumoniae* proteins.**

| S. No. | Start Position | End Position | Peptide Sequence | Peptide Length |
|---|---|---|---|---|
| 1 | 54 | 64 | NELETGNYAGN | 11 |
| 2 | 88 | 108 | NEIIDENGQTLGVNIDPQTFK | 22 |
| 3 | 122 | 136 | TEAEGAKFNTANLPA | 15 |
| 4 | 150 | 161 | YVGEDGATLTGS | 12 |
| 5 | 180 | 215 | PKNTEAKPKIDKDFKGKANPDTPRVDKDTPVNHQ | 36 |
| 6 | 286 | 297 | LAKVNDQNAEKT | 12 |
| 7 | 312 | 346 | VEVPESNDVTFNYGNNPDHGNTPKPNKPNENGDLT | 35 |
| 8 | 352 | 365 | VDATGAPIPAGAEA | 14 |
| 9 | 409 | 421 | KGYSADYQEITTA | 13 |
| 10 | 428 | 446 | NWKDENPKPLDPTEPKVVT | 19 |
| 11 | 511 | 529 | TAQQQTQQEKEKVDKAQAA | 19 |
| 12 | 598 | 621 | TSYSATGQGIEYTAGSGKDDATKV | 24 |

polyepitope regions, so the protein is immunogenic and can be used as a vaccine candidate [33-35].

Backbone pilin (RrgB) has hydrophilic and stable properties and is a protein with a molecular weight of 71.3 KDa is based on the results of physicochemical tests. In addition to physicochemical properties, antigenicity analysis and epitope mapping also reinforce the presumption that the RrgB protein is immunogenic. Matters that support immunogenicity include hydrophilic properties and have polyantigenic and polyepitope regions

## CONCLUSION

Pilus protein of *S. pneumoniae* 67 kDa is an anti-hemagglutinin adhesion molecule, identifies with chain A backbone pilus *S. pneumoniae,* and is immunogenic, so the protein is a potential candidate vaccine to protect against the infection of *S. pneumoniae*.

## LIST OF ABBREVIATIONS

AA = Amino acid sequence

PCV = Pneumococcal conjugate vaccine

GRAVY = Grand average of hydropathicity

LC-MS/MS = Liquid Chromatography-mass Spectrometry

## ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The study was approved by the Ethics Committee of Jember University (Approval number: 1293/H25.1.11/KE/2019).

## HUMAN AND ANIMAL RIGHTS

No animals/humans were used for studies that are the basis of this research.

## CONSENT FOR PUBLICATION

Not applicable.

## AVAILABILITY OF DATA AND MATERIALS

The data used to support the findings of this study are included in the article.

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## CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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