Immunoinformatics Identifies a Lactoferrin Binding Protein A Peptide as a Promising Vaccine With a Global Protective Prospective Against *Moraxella catarrhalis*

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**Background.** *Moraxella catarrhalis* is an established pathogen that is causing substantial infections to both children and adults. However, so far there is no effective vaccine to halt the spread of these infections.

**Methods.** Immunoinformatics tools were used to predict *M. catarrhalis* epitopes that could offer immunoprotection among major proportions of human populations worldwide. Mice were immunized with the best 3 peptides and then challenged with *M. catarrhalis* in the pulmonary clearance model. Finally, antibodies against these epitopes were detected in humans.

**Results.** Immunoinformatics analyses identified 44 epitopes that are predicted to be good major histocompatibility complex class II binders and at the same time show high population coverage worldwide. After intraperitoneal immunization of mice with the best 3 peptides, peptide A, derived from lactoferrin-binding protein A, showed superior activity in immunogenicity and in clearing *M. catarrhalis* from mouse lungs. Higher clearance was obtained by combining intraperitoneal and intranasal immunization. In the serum samples from children with otitis media infected with *M. catarrhalis*, antibody levels against peptide A were significantly lower than in samples from children without otitis media.

**Conclusions.** Peptide A is the first promising peptide-based vaccine against *M. catarrhalis*. Immunoinformatics predicts that it should have a global protection around the world.

**Keywords.** immunoinformatics; vaccine; *Moraxella catarrhalis*; LbpA; otitis media.

*Moraxella catarrhalis,* a gram-negative, aerobic diplococcus, human-restricted commensal of the upper respiratory tract [1]. However, during the last 3 decades, *M. catarrhalis* has established itself as a primary pathogen in both upper and lower respiratory tract [2]. *M. catarrhalis* is responsible for about 15%–20% of acute otitis media episodes in children [2]. In addition, *M. catarrhalis* is a major concern for adults, especially those with chronic obstructive pulmonary disease causing exacerbations [3]. Moreover, *M. catarrhalis* is involved to a lesser extent in other types of infections such as sinusitis, pneumonia, bacteremia, endocarditis, and meningitis, particularly among immunocompromised individuals [2, 3].

So far, there is no licensed vaccine against *M. catarrhalis*. However, the need to halt *M. catarrhalis* infections with a vaccine is high because of the heavy economic burden worldwide and because almost all *M. catarrhalis* strains are resistant to the widely prescribed β-lactam antibiotics [4]. The development of vaccines against *M. catarrhalis* is progressing at a good pace; it started with using conventional methods for finding vaccine targets that resulted in the analysis of some candidates deemed not appropriate owing to surface epitope heterogeneity or variable expression. At the same time, several *M. catarrhalis* antigens have demonstrated excellent immunogenicity, eliciting functional antibodies and producing protective responses in animal models. However, they have not yet been tested in clinical trials [5, 6].

Interestingly, the combination of reverse vaccinology and immunoinformatics has not been deployed so far in the quest for vaccine candidates against *M. catarrhalis*. The combination of these 2 approaches would cut both time and costs before moving into clinical trials, providing a good estimate of how human populations worldwide would respond to the proposed vaccine candidate(s).

In the current study, microbial bioinformatics, immunoinformatics, and reverse vaccinology were combined with in vivo analyses to predict a protective peptide vaccine against *M. catarrhalis* that is expected to be highly immunogenic in different populations worldwide (Figure 1). One of the selected peptides showed promising efficiency in an animal model, and antibodies against it had significantly higher levels in children without otitis media than in those with otitis media in a randomly selected small-scale pilot screen.
METHODS

Ethics Statement
All animal procedures and human sample collection were approved by the Research Ethics Committee of the Faculty of Pharmacy, Cairo University. Written consent was obtained from subjects’ legal guardians.

Bacterial Strains and Growth Conditions
*M. catarrhalis* strain O35E [7] was used in all the experiments of this study. Bacteria were grown in tryptone soya broth at 37°C with shaking at 180 rpm or on tryptone soya agar at 37°C in a candle jar (approximately 2.3%–3.5% carbon dioxide) [8]. For serum bactericidal assays, *M. catarrhalis* strains O12E [9], SAM71, and SAM77, isolated from 2 otitis media cases in Egypt, were used [10].

Peptides
Three peptides—peptide A (RLNIKITPNLVSKLL), B (GRI FNQFTASYPLLR), and C (RIFNQFTASYPLLRS)—were chemically synthesized by Life-Tein. Each peptide was dissolved in

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**Figure 1.** Schematic diagram of the workflow of experiments performed in this study. Abbreviations: IC₅₀, median inhibitory concentration; IEDB, Immune Epitope Database; *M. catarrhalis*, Moraxella catarrhalis.
dimethylsulfoxide, diluted in sterile phosphate-buffered saline (PBS) to a final volume of 1 mL, and stored at −20°C.

Human Blood Samples
Serum samples were obtained from 9 children with and 10 without otitis media, as described elsewhere [10]. Briefly, children were aged 2–6 years; those with otitis media had a recent acute otitis media episode diagnosed by an ear, nose, and throat specialist, and those without otitis media had noninfected ears. All 19 subjects yielded positive *M. catarrhalis* nasal culture.

Bioinformatics Analyses

Subcellular Localization
The amino acid sequences of all the proteins encoded by the *M. catarrhalis* O35E genome were retrieved from the National Center for Biotechnology Information Genome Project (http://www.ncbi.nlm.nih.gov/nuccore/416257007) and analyzed using the PSORTb (version 3.0) server (http://db.psort.org/) [11]. The amino acid sequences of those proteins that are predicted to be located either in the outer membrane or extracellular were further analyzed using the SignalP 4.1 server (http://www.cbs.dtu.dk/services/SignalP/) [12] to predict the signal peptides and their cleavage site.

Major Histocompatibility Complex Type II Receptor Binding
The sequences of the mature polypeptides obtained from the SignalP 4.1 server were then analyzed using the MHC-II Binding Prediction tool available at the Immune Epitope Database (IEDB) Analysis Resource tool (http://tools.immuneepitope.org/mhcii/) [13]. The sequences were scanned for binding peptides to a list of human HLA-DR, HLA-DQ, and HLA-DR alleles (936 alleles), available with the NetMHCpan-3.0 prediction method for each protein (IEDB) Analysis Resource tool (http://tools.immuneepitope.org/mhcii/)[13]. The restricted alleles MHC population coverage tool (http://tools.immuneepitope.org/)

Population Coverage
The selected epitopes and their binding major histocompatibility complex class II (MHC II) alleles were used as an entry in a population coverage tool (http://tools.immuneepitope.org/tools/population/iedb_input) [15]. The restricted alleles MHC II population coverage calculation for each epitope was done for 78 population grouped into 11 geographic areas. The top 3 peptides with the highest population coverage score were selected for chemical synthesis and animal testing.

Transmembrane Topology and Sequence Conservation
The sequences of the proteins containing the selected epitopes were subjected to transmembrane topology prediction using TMRPres2D software, which predicts transmembrane beta strands of the outer membrane proteins based on hidden Markov model [16] and then detects the surface exposure of each of the high-scoring epitopes. The conservation of the amino acid sequence of each epitope was checked by performing multiple sequence alignments using the CLC Main Workbench 7 software (Qiagen) to compare the selected epitopes sequences from multiple *M. catarrhalis* strains.

Mice Immunization
Three groups of 6–8-week-old female BALB/c mice (Theodor Bilharz Research Institute) were immunized intraperitoneally with 100 µg of each peptide emulsified with incomplete Freund adjuvant (IFA) in 100-µL volumes. Another 2 groups were immunized with the 3-peptide mixture (33.3 µg of each peptide mixed, emulsified with IFA). Finally, another group was immunized with sterile endotoxin-free PBS emulsified with IFA to serve as a negative control. Injections were repeated after 14 days with 50 µg of a single peptide or 17 µg of each peptide for the mixture. One of the groups, which received the 3 peptides, was immunized 3 times intranasally with 50 µg (17 µg of each peptide) in 25 µL of sterile endotoxin-free PBS, with 4-week intervals in between. The last intranasal dose was received on the day of the bacterial challenge in the other groups.

Enzyme-Linked Immunosorbent Assay
Mice blood, collected on the day of the intranasal challenge with live *M. catarrhalis*, was allowed to clot at room temperature, and serum was collected by centrifugation. Antibody levels were determined by means of enzyme-linked immunosorbent assay (ELISA). Briefly, flat-bottom ELISA 96-well microplates (Greiner Bio-One) were coated with 1 µg of peptide in 50 µL of sodium carbonate buffer (pH 9.5). After blocking and washing, mouse serum samples diluted 1:10 were incubated with the antigens. Anti-mouse anti–immunoglobulin (Ig) G–alkaline phosphatase–linked antibody (KBL), diluted 1:1000, was used as a secondary antibody, and the antibody levels were determined by measuring the absorbance of the color produced after the addition of p-nitrophenyl phosphate at 405 nm. To determine the exact titer end point, serum samples were serially diluted in 10-fold dilutions, and the end point was defined as the highest dilution giving an absorbance >3 times that of the wells that received all treatments except the serum samples after the addition of p-nitrophenyl phosphate at 405 nm. To determine the exact titer end point, serum samples were serially diluted in 10-fold dilutions, and the end point was defined as the highest dilution giving an absorbance >3 times that of the wells that received all treatments except the serum samples [17]. Irrelevant polypeptide derived from the *Staphylococcus aureus* universal stress protein 2 [18] was used as coating antigen to test for antibody specificity.

Similar procedures were performed with human serum samples, except that the secondary antibody used was anti-human anti-IgG–alkaline phosphatase–linked antibody (Sigma) diluted 1:5000. To detect immunity, lung homogenates (in 1 mL of saline) from both immunized and naïve mice were tested with ELISA, using a mixture of anti-mouse anti-IgG and anti-IgA–alkaline phosphatase–linked antibody (Sigma), diluted 1:500.

Complement-Dependent Bactericidal Assay
Pooled normal human serum collected from healthy volunteers was depleted from *M. catarrhalis*–specific antibodies, as described elsewhere [19]. Briefly, 2 mL of O35E overnight culture
were spun down, and then the pellet was washed with PBS, resuspended in 0.5 mL of fresh normal human serum, and incubated at 4°C for 1 hour with mild agitation. Bacteria were spun down, and the supernatant was filter sterilized through a 0.22-µm filter and served as a source of complement.

Bactericidal assay was performed as described elsewhere, with slight modifications [20]. Briefly, serum samples from 3 mice, showing the highest titer against the respective peptides, were pooled and then heat deactivated (56°C for 30 minutes). Bacteria were grown to midlogarithmic phase (optical density at 600 nm, approximately 0.6) and resuspended in PBS containing 0.1% gelatin. A 10-µL aliquot containing ~1 × 10^3 to 2 × 10^3 colony-forming units (CFUs) was mixed with 40 µL of PBS containing 1.5 mmol/L calcium chloride and 5 mmol/L magnesium chloride (PBS⁺). To this mixture was added 25 µL of the heat-inactivated mouse serum and 25 µL of the complement source, followed by incubation at 37°C for 60 minutes. Next, 10-µL aliquots were removed and plated, and CFUs were counted after overnight incubation. As a control, bacteria were incubated with the same mixture except that the complement source was heat-inactivated. The percentage of killing was calculated as follows: [(CFUs from control − CFUs from sample)/CFUs from control] × 100. Antibodies were considered bactericidal if the percentage was >50% [20].

Murine Pulmonary Clearance Model

The model described by Unhanand and coworkers [21] was used, with slight modification. Briefly, 21 or 10 days after the intraperitoneal and intranasal last boost, respectively, mice were infected intranasally with approximately 5 × 10^6 CFUs in 40 µL, under anesthesia. At 4.5 hours after inoculation, mice were euthanized by anesthesia overdose, followed by cervical dislocation. The lungs were excised, homogenized, serially diluted, and plated for colony counts.

Statistical Analysis

Statistical analyses were performed using GraphPad Prism 5 software. For comparison between different groups, 1-way analysis of variance was applied, followed by Bonferroni multiple comparison test. Differences were considered significant at P ≤ .05.

RESULTS

Immunoinformatics Analyses of the M. catarrhalis Strain O35E Proteome

Subcellular localization of 1719 M. catarrhalis proteins predicted 44 proteins in the outer membrane and only 10 extracellular (Supplementary Table 1). Forty-four peptides, derived from those proteins, were predicted to have an IC₅₀ ≤ 50 nmol/L and at the same time be able to bind to >400 HLA alleles distributed worldwide. A summary of the top 10 scoring epitopes is presented in Table 1.

The first peptide, peptide A, is derived from the lactoferrin-binding protein A (LbpA), whereas both peptides B and C were derived from the lipopolysaccharide assembly protein precursor with overlapping core sequences. DNA sequence conservations analysis indicated that the 3 peptides are very well conserved (100%) throughout 11 M. catarrhalis strains whose sequences are publicly available (Figure 2). To confirm the accessibility of these epitopes to targeting antibodies, membrane topology prediction was applied, which indicated that for peptide A 7 of 15 amino acids are predicted to be extracellular (Figure 2A), compared with only 6 and 5, respectively, for peptides B and C (Figure 2B).

Peptide A Induction of Systemic and Mucosal Immune Response in Mice

Analysis of the serum samples obtained from mice after immunization with ELISA indicated that peptide A is immunogenic in mice (Figure 3). Peptide A produced significantly higher antibodies titers in immunized mice than in sham-immunized mice (Figure 3). On serial dilution of mice serum samples, the titer turned out to be 7 × 10^4, significantly higher than in the sham-immunized mice (P ≤ .05). Peptides B and C were able to elicit the production of some antibodies in mice, but their titers (10^3 and 10^2, respectively) did not significantly differ

Table 1. Highest Scoring Epitopes Based on Immunoinformatics Analyses Performed in This Study

<table>
<thead>
<tr>
<th>Peptide Designation</th>
<th>Amino Acid Sequence</th>
<th>Start Position</th>
<th>End Position</th>
<th>Source Protein</th>
<th>Restricted Alleles, No.</th>
<th>Population Coverage, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>RLNIKTPLNSKLL</td>
<td>332</td>
<td>346</td>
<td>LbpA</td>
<td>512</td>
<td>79.42</td>
</tr>
<tr>
<td>B</td>
<td>GRIKQGASYPYRLL</td>
<td>546</td>
<td>560</td>
<td>LPS assembly protein precursor</td>
<td>469</td>
<td>78.42</td>
</tr>
<tr>
<td>C</td>
<td>RIFQGASYPYRLLS</td>
<td>547</td>
<td>561</td>
<td>LPS assembly protein precursor</td>
<td>486</td>
<td>78.12</td>
</tr>
<tr>
<td>D</td>
<td>GVRWVSQGRK</td>
<td>201</td>
<td>215</td>
<td>Hypothetical protein EA1_01094</td>
<td>498</td>
<td>78.81</td>
</tr>
<tr>
<td>E</td>
<td>KNLVAKARHMD</td>
<td>461</td>
<td>475</td>
<td>D15 surface antigen family protein</td>
<td>504</td>
<td>75.56</td>
</tr>
<tr>
<td>F</td>
<td>ARNKITPLNSKLL</td>
<td>331</td>
<td>346</td>
<td>LbpA</td>
<td>460</td>
<td>79.29</td>
</tr>
<tr>
<td>G</td>
<td>ASNQKIQSLRMLR</td>
<td>355</td>
<td>386</td>
<td>Outer membrane protein assembly complex protein (YaeT)</td>
<td>505</td>
<td>73.73</td>
</tr>
<tr>
<td>H</td>
<td>NQKQSLRSLRML</td>
<td>357</td>
<td>385</td>
<td>YaeT</td>
<td>509</td>
<td>73.73</td>
</tr>
<tr>
<td>I</td>
<td>AILPQFQAFRFLS</td>
<td>174</td>
<td>188</td>
<td>Moraxella catarrhalis FhaC-like protein (MhaC)</td>
<td>518</td>
<td>73.73</td>
</tr>
<tr>
<td>J</td>
<td>SNQKIQSLRMR</td>
<td>356</td>
<td>370</td>
<td>Outer membrane protein assembly complex protein YaeT</td>
<td>521</td>
<td>73.73</td>
</tr>
</tbody>
</table>

Abbreviations: LbpA, lactoferrin-binding protein A; LPS, lipopolysaccharide.
from those detected in the control group (Figure 3A). The levels of the antibodies detected against any of the 3 peptides were significantly higher than those detected when an irrelevant polypeptide was used as the coating antigen (Figure 3A). When IgG and IgA levels were analyzed in lung homogenates from mice immunized intranasally with the 3 peptides, only peptide A, again, was able to produce antibodies at levels significantly higher than in naive nonimmunized mice (Figure 3B).

**Bactericidal Antibodies Against Peptide A**

When the bactericidal activity of serum samples obtained from mice immunized with either peptide A or B were tested, only those from mice immunized with peptide A caused >50% killing in strain O35E. This effect was observed in other *M. catarrhalis* strains, such as O12E, which has been shown to be genetically distinct from O35E [22, 23], in addition to another 2 isolates from Egypt, SAM71 and SAM77. The killing percentages for the 4 strains (O35E, O12E, SAM71, and SAM77) were 96%, 87%, 93%, and 96%, respectively, compared with 24%, 0%, 15%, and 6% for samples from mice immunized with peptide B.

**Enhancement by Selected Peptides of *M. catarrhalis* Pulmonary Clearance in Mice**

Immunized mice were challenged with *M. catarrhalis* intranasally. Then, 4.5 hours after infection, mice were euthanized, their lungs were removed, and bacterial loads in homogenates were
determined (Figure 4). Both peptides A and B individually caused significant clearance of *M. catarrhalis* from mouse lungs compared with the control mice, which were mock-immunized with PBS. However, peptide C was not successful in that. On the other hand, when the 3 peptides were used in combination for intraperitoneal immunization (using one-third the dose used in the individual peptide immunizations), *M. catarrhalis* was very efficiently cleared from the lungs of the mice (Figure 4). Finally, combining intraperitoneal immunization with intranasal immunization using the 3-peptide mixture resulted in the highest and most significant clearance of *M. catarrhalis* from the lungs (Figure 4).

**Higher Antibody Levels Against Peptide A in Children Without Otitis Media**

Analysis of serum samples obtained from children with or without otitis media showed that children without otitis media have significantly higher levels of antibodies against peptide A than those with otitis media (Figure 5). The mean level of antibodies against peptide B was slightly higher in the non–otitis media group, but the difference was not statistically significant. On the other hand, levels of antibodies against peptide C were very low in both otitis media and non–otitis media groups.

**DISCUSSION**

Reverse vaccinology integrates genome information to rationally design vaccines, saving time and costs that used to be spent in conventional screening of vaccine candidates [24]. Previous in silico analysis identified some proteins that are highly conserved among different strains and are expressed during human infection with *M. catarrhalis* [25]. *Moraxella* surface protein (Msp) 22, Msp75, and oligopeptide permease A (OppA) were later shown to be effective in clearing *M. catarrhalis* from mouse lungs [26–28]. More recently, it was reported that serum antibody to both OppA and Msp22 increased with age in naturally immunized children after nasopharyngeal colonization and acute otitis media [5]. In addition, reduced *M. catarrhalis* carriage correlated well with high antibody titers against OppA and Msp22 [5].

In the current study, reverse vaccinology was adopted to look for vaccine candidates within the genomic context of *M. catarrhalis*. Moreover, immunoinformatics was deployed to test how much potential vaccine candidates would induce the immune system of individuals from different population all over the world, enabling maximum population coverage. Peptide-based vaccines come with many advantages, such as absence of...

![Figure 4](image1.png)

*Figure 4.* Immunization with the selected peptides efficiently enhances *Moraxella catarrhalis* pulmonary clearance. After intraperitoneal immunization with peptides A, B, and C individually, a mix of the 3 peptides, the 3-peptide mix in combined intraperitoneal (IP) and intranasal (IN) form, and just phosphate-buffered saline (control), mice were infected intranasally with approximately $5 \times 10^6$ colony-forming units (CFUs) of strain O35E. Then, 4.5 hours after infection, lungs were harvested, homogenized, serially diluted and plated. Each mouse is represented by a data point in the figure, and the horizontal bar represents the mean of the log$_{10}$ CFU count. Statistical analysis was performed by applying analysis of variance (P value shown), followed by Bonferroni multiple comparison test. * $P \leq .05$; † $P \leq .001$.

![Figure 5](image2.png)

*Figure 5.* Antibodies against peptide A are higher in healthy individuals than in children with otitis media. Blood was obtained from children with (closed circles) or without (open circles) otitis media. Serum samples were analyzed for antibodies against the selected peptides using enzyme-linked immunosorbent assay; plates were coated with the respective peptide (coating antigen; peptide A, B, or C), and human antibodies were detected using anti-human immunoglobulin G–alkaline phosphatase–linked antibody. Y-axis represents the absorbance at 405 nm of the color developed after the addition of the alkaline phosphatase substrate. Each subject is represented by a data point in the figure; horizontal bar represent the mean, and ticks represent standard errors. Statistical analysis was performed by applying analysis of variance (P value shown), followed by Bonferroni multiple comparison test. * $P \leq .05$. 

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infectious material, ability to exclude deleterious sequences from full-length antigens, ease of introducing chemical modifications, low costs of large-scale production, feasibility of storage in a dried frozen form (eliminating the need for costly cold-chain transport), and ability to include multiple epitopes either from different pathogens or from the same pathogen [29]. However, they have poor immunogenicity, which can be overcome using modified formulations of the vaccine [29]. To avoid a vaccine candidate with ethnically biased population coverage, we selected only candidate epitopes predicted to successfully bind to >400 alleles distributed in 78 population grouped into 11 geographic areas.

The most promising peptide, peptide A, is derived from LbpA, one of two lactoferrin-binding proteins expressed by M. catarrhalis [30]. Interestingly, it was reported that whole LbpA, both native and recombinant, is not immunogenic [30, 31]. In our hands, the peptide derived from LbpA is highly conserved across all the tested strains; it is highly immunogenic in mice, and it effectively cleared M. catarrhalis from mouse lungs. In addition, antibodies against peptide A are bactericidal against heterogeneous M. catarrhalis strains, could be readily detected in human serum samples, and had significantly higher levels in healthy children than in those with otitis media. The reasons for the discrepancy between the previous results and ours regarding the immunogenicity of LbpA are not clear at this point, but this might highlight the power of vaccines based on carefully selected peptides rather than whole proteins.

The next 2 peptides in the ranking were derived from the lipopolysaccharide assembly protein precursor, on which very little is reported on in the M. catarrhalis literature. The gene encoding this protein has been reported to show slightly down-regulated expression on cold shock (shift from 37°C to 26°C) [32]. It is interesting that these 2 peptides differ only in the first and last amino acids, yet they also differ in their ability to enhance pulmonary clearance as peptide C is more predicted to be less exposed on the surface of M. catarrhalis. However, this agrees with the membrane topology prediction of these peptides. Although both peptides B and C are predicted to be highly immunogenic based on amino acid sequence and MHC-II reactivity, biological testing confirmed their low value as good immunogens.

The murine pulmonary clearance model remains a widely accepted model for testing vaccine candidates [28, 33–35]. In the current study, intraperitoneal vaccination with either peptide A or B alone or a mix of the 3 peptides enhanced clearance significantly, by 0.5–1 log, in agreement with findings of similar studies performed with other vaccine candidates [27, 33, 36]. However, the most dramatic effect was observed by combining intraperitoneal with intranasal vaccination, which reduced the CFU counts by almost 2 logs compared with the mock-treated-mice. There was a synergistic effect in the pulmonary clearance of M. catarrhalis when the 3 peptides were mixed, which would argue that even though there were no significant differences in immunogenicity between peptides B and C and the sham control in the mouse serum samples, the mean value of the absorbance reading was still increased (Figure 3A).

These small differences could be behind the synergistic effect seen in the pulmonary clearance, but the more synergistic effect seen when combining intraperitoneal and intranasal immunization could be attributed to the mucosal immunity acquired against peptide A, because this was the only peptide to show a response in this setting (Figure 3B). However, the almost 2-log reduction seen in this group could be partly due to the fact that the mice in this group were older than in other groups with about 10 days on the day of challenge. Finally, serum samples from healthy children showed higher levels of only antipeptide A antibodies than those from children with otitis media. This observation is indicative of a potential protective role for antibodies against peptide A in human, but more extensive screening studies are needed to support this conclusion.

The overall conclusion of the current study is that peptide A derived from LbpA is a very promising vaccine candidate against M. catarrhalis. Efforts would continue to test other peptides predicted using immunoinformatics analyses conducted in this study, in order to add other peptides to the proposed vaccine and maximize the protection.

Supplementary Data
Supplementary materials are available at http://jid.oxfordjournals.org. Consisting of data provided by the author to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the author, so questions or comments should be addressed to the author.

Notes
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