

## Research Article

### New Candidate Antigens for Serodiagnosis of Pertussis

Odanaka K<sup>1</sup> and Watanabe M<sup>1,2\*</sup>

<sup>1</sup>Graduate School of Infection Control Sciences, Kitasato University, Tokyo, Japan

<sup>2</sup>Laboratory of Medical Microbiology, Kitasato Institute for Life Sciences, Kitasato University, Tokyo, Japan

#### Abstract

Pertussis is a contagious respiratory disease caused by *Bordetella pertussis*. Serodiagnosis of pertussis is commonly based on the detection of antibodies to pertussis toxin and anti-filamentous hemagglutinin; however, elevated antibody titers can confuse the disease diagnosis because pertussis vaccination also raises them. Therefore, identification of *B. pertussis* new immunodominant antigens for serodiagnostic testing would increase diagnostic precision and help in epidemiologic studies. Here, we explored immunodominant proteins recognized by sera from culture-positive pertussis patients using immunoproteomics. As a result, GroEL, ATP synthase  $\beta$  subunit, and peptidyl-prolyl cis-trans isomerase were identified as *B. pertussis* immunoreactive antigens by reaction with patient sera. Serum antibody responses to each antigen in patients were significantly higher compared to those in healthy individuals. However, GroEL is highly conserved across species, including infectious bacteria, which may result in low specificity. Therefore, we suggest that *B. pertussis* ATP synthase  $\beta$  subunit and peptidyl-prolyl cis-trans isomerase could be promising candidate antigens for serodiagnosis of pertussis, although they have high similarity with homologous enzymes from *Mycobacterium abscessus* subsp. *abscessus*. Further studies involving larger patient populations are necessary to validate the clinical utility of the identified antigens for pertussis diagnostics.

**Keywords:** Whooping cough; Pertussis vaccine; Serodiagnosis; GroEL; ATP synthase; Peptidyl-prolyl cis-trans isomerase, Par27.

#### Abbreviations

ASB: ATP Synthase  $\beta$  subunit; 2D-PAGE: Two-dimensional Polyacrylamide Gel Electrophoresis; PPIase: Peptidyl-Prolyl cis-trans isomerase; DPBS(-): Dulbecco's modified Phosphate Buffered Saline without calcium and magnesium.

#### Introduction

Pertussis or whooping cough is a highly contagious respiratory infectious disease caused by bacterium *Bordetella pertussis*. The disease is characterized by extended periods of paroxysmal cough and is most dangerous for infants as pneumonia can be a relatively common complication. Diagnosis of pertussis is challenging. Isolation of *B. pertussis* from the nasopharynx is the gold standard for diagnosis, but *B. pertussis* is fastidious, and not all clinical laboratories can successfully culture the bacterium [1,2]. Recently, loop-mediated isothermal amplification (LAMP), which is a simple technique for DNA amplification, eliminating the need for thermal cyclers, has been introduced mainly in Japan [3]. It is a rapid, sensitive, and specific method for *B. pertussis* detection, however, the presence of *B. pertussis* decreases in the course of the disease and/or by antibiotic treatment; therefore, the sensitivity of both culture- and PCR-based detection declines with time [2,4]. Of note, pertussis is a slow-onset disease and therefore infection often predates clinical symptoms by weeks, allowing for antibody development before diagnosis is conducted. Therefore, serum antibody to *B. pertussis* antigens have been used for pertussis diagnosis. Serodiagnosis has a significant advantage over direct *B. pertussis* detection as they can reveal the infection even after

the bacterium has been eliminated by antibiotics. In addition, serological testing is essential for retrospective epidemiologic research. Although a number of enzyme-linked immunosorbent assay (ELISA)-based tests have been developed for serological diagnosis of pertussis, a major problem is that they also measure antibody responses to antigens (such as pertussis toxin and filamentous hemagglutinin) introduced in acellular vaccines. As a result, it is difficult to diagnose pertussis by ELISA in vaccinated individuals who may already have high titers of antibodies to these antigens. In previous study, we tried to find non-vaccine candidates for serodiagnosis from known pathogenic factors of *B. pertussis* and suggested that the catalytic domain of adenylate cyclase toxin and the C-terminal region of filamentous hemagglutinin could be useful for serodiagnosis of pertussis [5]. In this study, we performed immunoproteomics analysis to detect non-vaccine candidate antigens from whole proteins of *B. pertussis*. We identified three *B. pertussis* proteins recognized by sera of culture-positive pertussis patients and evaluated their diagnostic potential by ELISA. Our results indicate that ATP synthase  $\beta$  subunit and peptidyl-prolyl cis-trans isomerase are promising new candidates for serodiagnosis of pertussis and should be validated in further studies.

#### Materials and Methods

##### Bacterial strains and culture conditions

*B. pertussis* Tohama I [6] was used in this study. For routine propagation, bacteria were grown on Bordet-Gengou agar containing 1% (v/v) glycerol and 15% (v/v) sheep

defibrinated blood at 36.5°C for 4 days. *Escherichia coli* DH5α and Rosetta2 (DE3) (Merck, Darmstadt, Germany) used for cloning and expression of recombinant proteins were grown in Luria-Bertani broth at 37°C.

### Serum samples

We used serum samples described in previous report [5]. In brief, the serum samples were obtained from 20 culture-positive children in Cincinnati Children’s Hospital Medical Center and 6 healthy adult volunteers in University of Cincinnati after approval by the institutional review boards of Cincinnati Children’s Hospital Medical Center and University of Cincinnati. Mean and standard deviation (SD) of age of the patients were 12.2 ± 2.6 (range 7-17) years. Mean and SD of interval between cough start and hospital visit were 49.5 ± 19.9 (range 32-107) days. The information on all serum donors was anonymized.

### Depletion of antibodies against pertussis toxin and filamentous hemagglutinin from patient sera

Pertussis toxin and filamentous hemagglutinin were purified as described previously [7] and chemically conjugated to Dynabeads M-450 Tosylactivated (Thermo Fisher Scientific, Waltham, MA, USA) following the manufacturer’s protocol. Then, 20 μL of each sample of conjugated Dynabeads was added to 1 mL of diluted human serum (1:500), and the mixture was incubated overnight at 4°C, centrifuged, and the supernatant was analyzed by western blotting to confirm depletion of the antibodies.

### Two-dimensional polyacrylamide gel electrophoresis and western blotting

*B. pertussis* was inoculated in Stainer-Scholte medium at 2 × 10<sup>9</sup> cells/mL and cultured for 4 days at 36.5°C. The culture was centrifuged at 13,400 g for 30 min at 4°C, bacterial cells were collected, and proteins were extracted using ReadyPrep Protein extraction kit (Bio-Rad, Hercules,

CA, USA) according to the manufacturer’s recommendations. Protein samples were then subjected to two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) using the PROTEAN i12 IEF system and Mini-PROTEAN TGX gel system (isoelectric focusing range, pH 3–10; gel gradient, 5–20%; Bio-Rad). Fractionated proteins were either stained by SYPRO Ruby Gel Stain (Bio-Rad) or transferred to a polyvinylidene difluoride membrane, which was incubated with diluted serum samples depleted of anti-pertussis toxin and anti-filamentous hemagglutinin antibodies for 2 h at room temperature. After washing with Dulbecco’s modified phosphate buffered saline without calcium and magnesium [DPBS(-)] containing 0.05% Tween 20, the membrane was incubated with horseradish peroxidase (HRP) -conjugated goat anti-human IgG (1:5,000; Jackson ImmunoResearch Laboratory, West Grove, PA, USA) and proteins recognized by human sera were detected using the Immobilon Western Chemiluminescent HRP substrate (Merck) according to the manufacturer’s instructions.

### Identification of proteins

Proteins recognized by patient sera were subject to in-gel digestion by trypsin, and then analyzed by electrospray ionization-tandem mass spectrometry (ESI-MS/MS). The protein was identified by using MASCOT (Matrix Science, Boston, MA, USA) and NCBI Blast databases. The analysis was performed by Nihon Techno Service (Tsukuba, Japan).

### Construction of plasmids and purification of recombinant *B. pertussis* proteins

*B. pertussis* genes encoding GroEL, ATP synthase β subunit (ASB), and peptidyl-prolyl cis-trans isomerase (PPIase) were amplified by PCR using primers containing *Nde*I and *Bam*HI sites at the 5’ and 3’ ends, respectively (Table 1), inserted into pCRBlunt II-Topo (Invitrogen, Carlsbad, CA, USA), and cloned in *E. coli* DH5α. After cloning, the regions were sequenced for verification and inserted into the pET22b expression plasmids (Merck), which were used to transform *E. coli* Rosetta2 (DE3).

Target gene	Sequence
GroEL (BP3495)	Forward 5'-GGGCATATGATGGCTGCCAAGCAAGTTCTGTTTGCC-3' Reverse 5'-GGGATCCCCGCTACAGCTTCTGAGCGAGCTCC-3'
ATP synthase β subunit (BP3288)	Forward 5'-GGGCATATGGACATGAGCAACGGAA-3' Reverse 5'-GGGATCCCGGATCCTTATTGGAGT-3'
Peptidyl-prolyl cis-trans isomerase (BP3561)	Forward 5'-GGCATATGATGAAACGCATCGCCATGCT-3' Reverse 5'-GGGATCCGCTTGCGCTTACTGGATCT-3'

**Table 1:** Primers for PCR.

Proteins were expressed using the Overnight Expression System (Merck) and purified using the B-PER II Bacterial Protein Extraction Reagent (Thermo Fisher Scientific) according to the manufacturer’s recommendations. The recombinant proteins expressed as inclusion bodies were solubilized in Inclusion Body Solubilization Buffer (Thermo

Fisher Scientific) and loaded onto a HiTrap-Ni column (GE Healthcare, Little Chalfont, UK). The column was then washed with 10 mM Tris-HCl (pH 8.0) containing 0.5 M NaCl and 6 M urea, and proteins were eluted with a linear gradient of imidazole (0–0.5 M) and dialyzed against 10 mM Tris-HCl (pH 8.0) with 0.5 M NaCl and 2 M urea.

## Antibody quantification by ELISA

Wells of 96-well microtiter plates were coated with 100  $\mu$ L of each recombinant antigen (1  $\mu$ g/mL in 50 mM carbonate buffer, pH 9.6) for 16 h at room temperature and washed three times with DPBS(-) containing 0.05% Tween 20. Then, 100  $\mu$ L/well of each serum sample diluted 1:50 in incubation buffer [DPBS(-), 0.05% Tween 20, and 10% nonfat dry milk] was added for 2 h at room temperature, followed by 100  $\mu$ L/well of secondary HRP-conjugated goat anti-human IgG (1:5,000 in incubation buffer) for 2 h. After washing, 100  $\mu$ L/well of substrate (PIERCE TMB substrate kit; Thermo Fisher Scientific) was added for 30 min at room temperature, followed by 100  $\mu$ L of 2 M sulfuric acid, and the optical density was measured at 450 nm (OD<sub>450</sub>).

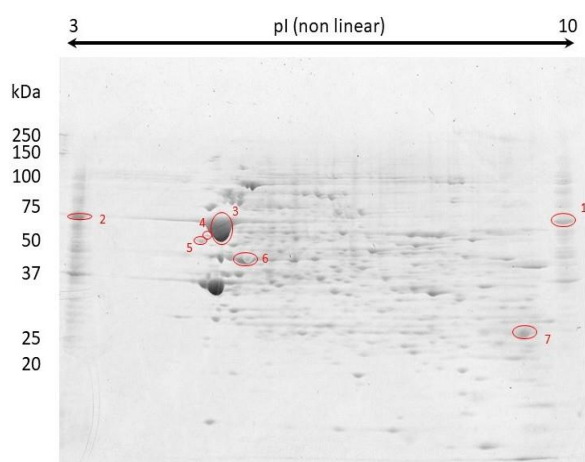
## Statistical analysis

Statistical significance of differences between groups was determined by t-test; P<0.05 was considered significant. All statistical analyses were performed using the GraphPad Prism software, version 6 (GraphPad Software, La Jolla, CA, USA).

## Results and Discussion

### Identification of proteins recognized by patient sera

Since anti-pertussis toxin and anti-filamentous hemagglutinin antibodies could interfere with detection of other immunoreactive *B. pertussis* proteins, we removed these antibodies from patient sera by immunoprecipitation prior to use; the depletion was confirmed by western blotting (data not shown). After fractionation of the *B. pertussis* proteome by 2D-PAGE, seven specific protein spots were recognized by all patient sera tested (Figure 1).



**Figure 1:** Profiling of *B. pertussis* proteome by 2D-PAGE.

Proteins extracted from *B. pertussis* Tohama I cells were separated by 2D-PAGE and stained by SYPRO Ruby Gel

Stain. Seven spots corresponding to proteins recognized by patient sera are circled and numbered; they were subjected to trypsin digestion and ESI-MS/MS for identification.

The seven immunoreactive spots were then subjected to trypsin digestion and ESI-MS/MS and three *B. pertussis* proteins, GroEL (encoding gene, BP3495), ASB (encoding gene, BP3288), and PPIase (encoding gene, BP3561) were identified (Table 2).

Spot	Protein (encoding gene)
1	GroEL (BP3495)
2	GroEL (BP3495)
3	GroEL (BP3495)
4	Not identified
5	ATP synthase $\beta$ subunit (BP3288)
6	Not identified
7	Peptidyl-prolyl cis-trans isomerase (BP3561)

**Table 2:** Proteins identified by immunoproteomics using pertussis patient sera.

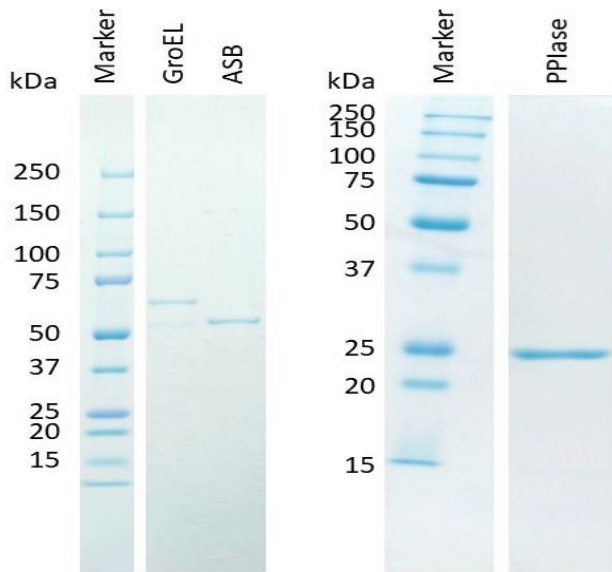
No consistent peptide sequence data were obtained from spot 4, probably due to insufficient amount of sample. Several sequences of peptides were recovered from spot 6, however, MASCOT search failed to identify the protein. The spot might be unknown protein coded in unidentified mobile genetic elements. Further study is required for characterization of the proteins.

### Antibody response to *B. pertussis* GroEL

Recombinant GroEL, ASB, and PPIase expressed in *E. coli* were purified (Figure 2) and used to test the immunoreactivity of patient sera (n=20) and healthy control sera (n=6), which was expressed as OD<sub>450</sub> units (Figure 3).

The mean IgG response to GroEL of sera from culture-positive patients was  $2.37 \pm 0.73$ , which was significantly higher than that of control sera ( $0.73 \pm 0.53$ ; P<0.0001, Figure 3a), suggesting a possibility of using GroEL for pertussis diagnostics.

GroEL, also known as Cpn60, is a cytosolic chaperon playing an important role in protein folding [8]; it is also present in outer membrane vesicles of *B. pertussis* [9]. Interestingly, Gebara et al. [10] reported that GroEL acted as an adjuvant when added to an acellular pertussis vaccine, suggesting that GroEL could itself activate host immune response. However, GroEL is highly conserved in a variety of species, including infectious agents [11,12]. For example, identity of amino acid sequences between *B. pertussis* and *E. coli* and *Pseudomonas aeruginosa* proteins is 77% and 75%, respectively. High similarity among GroEL proteins from different bacteria should be an issue of concern because GroEL as a diagnostic antigen of *B. pertussis* may have poor specificity. Therefore, it is important to check the specificity of antibody response to *B. pertussis* GroEL compared to its homologues from other species.



**Figure 2:** SDS-PAGE analysis of purified recombinant proteins.

GroEL, ATP synthase  $\beta$  subunit (ASB), and peptidyl-prolyl cis-trans isomerase (PPIase) were expressed in *E. coli* Rosetta2 (DE3), purified by metal-chelate affinity chromatography, separated by SDS-PAGE, and stained by Biosafe Coomassie Blue.

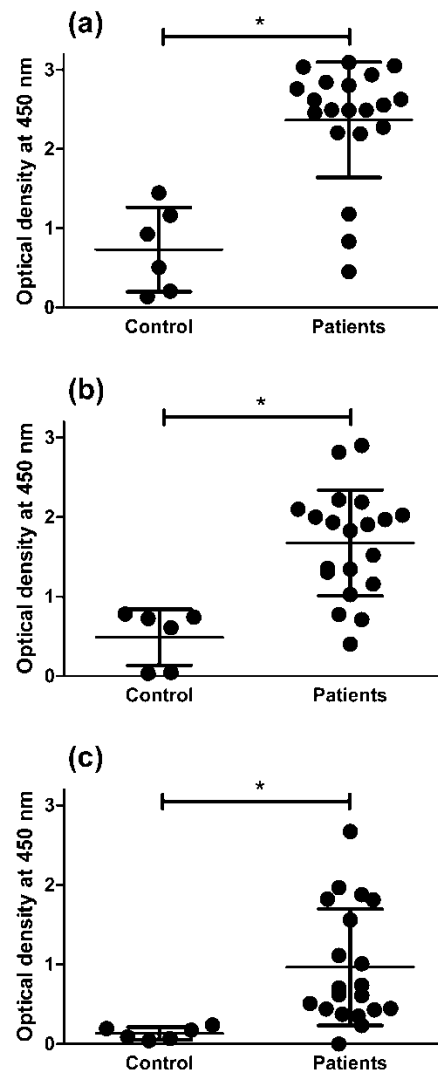
### Antibody response to *B. pertussis* ASB

The mean IgG responses to ASB in pertussis patients and healthy individuals were significantly different:  $1.68 \pm 0.67$  and  $0.49 \pm 0.35$ , respectively ( $P=0.0004$ ; Figure 3b), suggesting ASB as a new candidate diagnostic antigen.

In general, bacterial F-type ATP synthases consist of eight subunits ( $\alpha$ ,  $\beta$ ,  $\gamma$ ,  $\delta$ ,  $\epsilon$ , a, b, and c) [13]. ATP synthase of *B. pertussis* has not yet been studied; however, eight corresponding genes (*atpA-atpH*) were identified in the *B. pertussis* Tohama I genome. The enzyme is located on the cell membrane or in periplasmic space [13], and is also found in outer membrane vesicles [14]. ATP synthase is a protein complex essentially representing a proton-driven mechanism for the production of ATP from ADP and inorganic phosphate, thus playing a major role in ATP generation in the process of cell respiration. Therefore, ATP synthase has recently been focused on as a target of anti-microbial agents [15].

Although ATP synthases are common essential enzymes in many organisms, they have species-specific variations in amino acid sequences [16]. Nuyttens et al. [16] reported that an ATP synthase of *Mycoplasma pneumoniae* did not show cross-reactivity with sera from culture-positive pertussis patients. We performed BLAST search to identify proteins homologous to *B. pertussis* ASB. Although various bacteria were found to contain ASB homologues, they were mostly non-pathogenic environmental species such as *Achromobacter* and *Burkholderia* spp. Human pathogens do not have enzymes highly homologous to *B. pertussis* ASB, except *Mycobacterium abscessus* subsp. *abscessus* which expresses

ASB showing 99% amino acid sequence identity with *B. pertussis* ASB with 100% coverage. *M. abscessus* subsp. *abscessus* is a non-tuberculous mycobacterium usually isolated from water sources [17]; however, the bacterium can cause pulmonary disease resembling tuberculosis, and the number of such cases in Japan has increased recently [17,18]. Furthermore, the clinical importance of *M. abscessus* subsp. *abscessus* has been growing because the bacterium is intrinsically resistant to most currently used antibiotics [17, 19]. Since clinical manifestations such as long-lasting cough could be similar between pertussis and pulmonary disease due to *M. abscessus* subsp. *abscessus* infection, serodiagnosis based on ASB detection should be supplemented with X-ray examination and/or isolation of rapidly growing mycobacteria from sputum. Further studies should be performed to determine cross-reactivity of sera from *M. abscessus* subsp. *abscessus*-infected patients with *B. pertussis* ASB.



**Figure 3:** Serum antibody responses to the identified non-vaccine antigens in culture-positive patients and healthy individuals. (a) anti-GroEL IgG, (b) anti-ATP synthase  $\beta$



subunit IgG, (c) anti-peptidyl-prolyl cis-trans isomerase IgG. Mean values (middle line) and two-sided standard deviation (top and bottom lines) for each group are indicated; \*P<0.05 compared to control.

### Antibody response to *B. pertussis* PPIase

Similar to GroEL and ASB, IgG response to *B. pertussis* PPIase was significantly higher in the culture-positive group ( $0.97 \pm 0.73$ ) compared to the control group ( $0.14 \pm 0.08$ ; P=0.0116; Figure 3c).

PPIase catalyzes cis-trans isomerization of peptide bonds preceding prolyl residues and assists protein folding [20]. Bacterial PPIases have been identified as virulence factors and suggested as potential drug targets in infectious diseases [20]. Thus, Basak et al. [21] reported that PPIase secreted by *Helicobacter pylori* was responsible for damage of gastric epithelial cells. Furthermore, *B. pertussis* PPIase identified here has been previously characterized as a virulence-contributing factor by Hodak et al. [22]. The authors showed that PPIase, which they named Par27, had both peptidyl-prolyl cis-trans isomerase and chaperone activities, probably exerting pleiotropic effects on *B. pertussis* virulence by facilitating secretion of filamentous hemagglutinin in the periplasmic space [22].

We next performed BLAST search to find proteins homologous to *B. pertussis* PPIase and found that the enzyme had high amino acid sequence similarity to PPIases of *M. abscessus* subsp. *abscessus* and *Achromobacter* spp. (99% and 83–90%, respectively), the bacteria which also express ASB. To our knowledge, evolutionary relationship among *B. pertussis*, *M. abscessus* subsp. *abscessus*, and *Achromobacter* spp. has not been studied; however, it is possible that gene transmission could have occurred among their ancestors. It was also reported that PEB4 of *Campylobacter jejuni* had structural similarity to PPIase of *B. pertussis* [23]; however, the similarity of amino acid sequences is relatively low (13.5%) and should not lead to immune cross-reactivity.

The results of sequence similarity search indicate that sera from patients infected with *M. abscessus* subsp. *abscessus* should be examined for cross-reactivity with *B. pertussis* PPIase.

### Conclusion

We identified three *B. pertussis* antigens, GroEL, ASB, and PPIase, as new candidates for serodiagnosis of pertussis. Among them, ASB and PPIase are the most promising, as GroEL is highly conserved across species. Since the antigens are not included in the current acellular pertussis vaccines, the specificity of diagnostic tests based on the identified antigens may be higher than that of conventional serodiagnosis based on pertussis toxin and filamentous hemagglutinin. However, this study was performed with a relatively small number of serum samples and it is important to repeat the investigation in a larger patient population including individuals carrying other infectious agents such as *M. abscessus* subsp. *abscessus*, which expresses homologous enzymes.

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**\*Corresponding author:** Mineo Watanabe, Laboratory of Medical Microbiology, Kitasato Institute for Life Sciences, Kitasato University, 5-9-1 Shirokane, Minato-ku, Tokyo 108-8641, Japan, Tel: +81-3-5791-6122; Email: [watanam@lisci.kitasato-u.ac.jp](mailto:watanam@lisci.kitasato-u.ac.jp)

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