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Immunoproteomic analysis of *Bordetella pertussis* and identification of new immunogenic proteins

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ABSTRACT

Bordetella pertussis is the causative agent of highly communicable respiratory infection whooping cough (pertussis) which remains one of the world's leading causes of vaccine-preventable deaths. In the present study, total soluble proteins extracted from two *B. pertussis* strains, Tohama I and the local isolate Saadet were separated by two-dimensional gel electrophoresis and analyzed by Western blotting for their reactivity with the antisera obtained from the mice immunized with inactivated whole cells as well as those collected from the mice challenged intraperitoneally with live cells of each strain. Of a total of 25 immunogenic proteins identified, 21 were shown to be the novel antigens for *B. pertussis*.

1. Introduction

Whooping cough (pertussis) is a highly contagious, acute respiratory illness of humans that is caused by the gram-negative bacterium *Bordetella pertussis*. This bacterium is a strict human pathogen with no known animal or environmental reservoirs [1]. The introduction of mass vaccination of children in the mid-20th century decreased the incidence of pertussis; however, it is still one of the world's leading causes of vaccine-preventable deaths [2]. As reported by the World Health Organization (WHO), an estimated 50 million cases and 300,000 deaths occur every year; case-fatality rates in developing countries are estimated to be as high as 4% in infants (http://www.who.int/immunization_monitoring/diseases/pertussis_surveillance/en/, as of August 2008).

A new revolution in vaccine design has emerged from the use of post-genomic technologies [3,4]. Much of information about immunogenic components can be derived from proteomics coupled to Western blotting, namely immunoproteomics which has been successfully applied for the discovery of antigens from various bacterial pathogens; examples including *Helicobacter pylori* [5,6], *Staphylococcus aureus* [7], *Bacillus anthracis* [8], *Shigella flexneri* [9], *Francisella tularensis* [10], *Corynebacterium diphtheriae* [11], *Streptococcus pyogenes* [12], *Chlamydia pneumoniae* [13] and *Neisseria meningitidis* [14].

Sequencing of the genomes of *B. pertussis*, *B. parapertussis* and *B. bronchiseptica* [15] provided an essential database for applying proteomics approach to these organisms. First proteomics study on *B. pertussis* involved an analysis of differential protein expression under iron-limitation and iron-excess conditions [16]. Very recently, a comparative surfaceome analysis of the vaccine strain and the clinical strains of this pathogen was reported [17]. In the present study, we report the first immunoproteomic approach to *B. pertussis* Tohama I and Saadet strains which involved the identification of immunogenic proteins of their total soluble proteomes.

2. Material and methods

2.1. Bacterial strains and preparation of total soluble proteomes

The strains of *B. pertussis* used in this study were Tohama I and Saadet, the latter being a local strain. *B. pertussis* Tohama I and Saadet strains were grown on Cohen-Wheeler agar medium [18] for 48 h at 37 °C. For the preparation of whole cell lysates, the cells were collected and suspended in cold TE buffer and centrifuged at 4500 g for 10 min. The pellet was resuspended in 1 ml 0.04 M lysosyme in TE buffer and incubated at 37 °C for 35 min, followed by a centrifugation at 16,000 g for 10 min. The pellet was solubilized in 8 M urea, vortexed at 4 °C for 30 min and centrifuged at 16,000 g for 10 min.

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The supernatant was stored at −20 °C. The protein concentration was determined by Bradford technique [19].

2.2. 2-D gel electrophoresis

IPG strips were passively rehydrated by applying 400 μl of rehydration buffer (8 M urea, 2 M thiourea, 2% w/v CHAPS, 28 mM DTT and 0.5% v/v ampholyte 3-10) containing 350 μg protein sample for 14 h. IEF was performed with commercially available IPG strips (17 cm, pH 3–10, Bio-Rad) and the Protean IEF Cell (Bio-Rad USA). Rehydrated strips were taken to the IEF process. The following voltage profile was used for IEF: 1 h 100 V; 1 h 300 V; 1 h 600 V; 1 h 1000 V; 2 h 3000 V; 2 h 5000 V followed by a linear increase to 8000 V. The final phase of 8000 V was terminated after 50,000 Vh. The IPG strips were equilibrated for 15 min each in 5 mL of solution 1 (6 M urea, 50 mM Tris–HCl (pH 8.8), 30% v/v glycerin, 2% w/v SDS, 50 mg DTT) and then in 5 mL of solution 2 (6 M urea, 50 mM Tris–HCl (pH 8.8), 30% v/v glycerin, 2% w/v SDS, 225 mg iodacetamide) [20]. The isolated proteins were separated in 12% acrylamide/bis-acrylamide gels with a Bio-Rad Cell system (Bio-Rad, USA), applying approximately 25 mA per gel. To visualize the separated proteins, each gel was stained with colloidal Coomassie blue [21]. Coomassie stained gels were digitized by using a scanner (HP Scanjet 4070 Photosmart scanner, USA). Spot pattern analyses were accomplished by using the 2-D image analysis software Delta2D version 3.3 (Decodon, Germany).

2.3. Preparation of antisera against B. pertussis

B. pertussis Tohama and Saadet strains were grown on Cohen-Wheeler agar media for 48 h. The cells were suspended in 0.85% saline solution to contain ca. 4×10^{10} bacteria/ml. For subcutaneous immunization, the suspension was inactivated at 56 °C for 30 min. Inactivated bacterial cells were used as the antigen and anti-*B. pertussis* polyclonal antibodies were raised by immunizing mice. For each strain, 10 mice received two subcutaneous injections of 0.5 ml per animal at two week intervals between the first and second injections. Their sera were collected and pooled 14 days after the second injection. For intraperitoneal challenge, live cells were suspended in 5% sodium glutamate containing 1% casamino acid (pH: 7.4) solution containing approximately 4×10^{10} cells/ml. For each strain, 10 mice received one intraperitoneal injection of 50 μl per animal and after 14 days their sera were collected and pooled.

In Western Blot analysis, the antisera Th (sc), Sa (sc), Th (ip) and Sa (ip) were used. The antisera Th (sc) and Sa (sc) were obtained from the mice which were subcutaneously immunized with inactivated whole cells of *B. pertussis* Tohama I and Saadet strains, respectively. The antisera Th (ip) and Sa (ip) were obtained from the mice which were intraperitoneally challenged with live whole cells of *B. pertussis* Tohama I and Saadet strains, respectively.

2.4. Western blotting of 2-DE gels

Proteins from the identical, but non-stained gels were transferred to a NC membrane for 1 h at 400 mA in transfer buffer (25 mM Tris, 192 mM glycine, 2% w/v SDS and 20% v/v methanol) by using semi dry blotter (Cleaver Scientific Ltd.). After the transfer, the membrane was blocked for 2 h with 10% skim milk in TBS solution (20 mM Tris and 5 M NaCl) at 37 °C. After rinsing for 10 min with TBS-Tween 20, the membrane was incubated with primary antibody, mouse anti-*B. pertussis*, at a dilution of 1:300 in 0.05% Tween-20 in TBS (TTBS) containing 5% skim milk for 1 h at room temperature on a gentle shaker. The membrane was rinsed for 10 min and incubated with rabbit anti-mouse IgG-alkaline phosphatase (Sigma), at a dilution of 1:15,000 in TTBS containing 5% skim milk for 1 h. The membrane was then washed with TBS for 10 min and developed with substrate (AP Conjugate Substrate Kit, Bio-Rad) until optimum color was developed. The immunoreactivity of each spot that gave positive signal in Western blot analysis was verified through their excision from 2-D gels followed by dot-blotting.

2.5. Protein identification

The identifications were accomplished by mass spectrometry according to established protocols. Briefly, protein spots were excised from stained 2-D gels, destained and digested with trypsin (Promega, Madison, WI, USA). For the extraction of peptides, the gel pieces were covered with 60 μl 0.1% trifluoroacetic acid in 50% CH3CN and incubated for 30 min at 40°C. Peptide solutions were mixed with an equal volume of saturated α-cyano-3-hydroxycinnamic acid solution in 50% acetonitrile-0.1% trifluoroacetic acid (v/v) and applied to a sample plate for MALDI-TOF-MS. Mass analyses were carried out on the Proteome-Analyzer 4800 (Applied Biosystems). The three most abundant peptides in each MS spectrum were chosen for MS/MS experiment. The resulting sequence data were included for the database search to increase the reliability of protein identification. Mass accuracy was usually in the range between 10 and 30 ppm.

2.6. Database searches

Amino acid sequences for *B. pertussis* proteins were obtained from Sanger Institute organism's genome project web site (http://www.sanger.ac.uk/Projects/B_pertussis/). The peak lists of each protein spot were analyzed with the aid of "PMF" and "MS/MS Ion Search" engines of MASCOT software (http://www.matrixscience.com/). The searches considered oxidation of methionine and modification of cysteine by carbamidomethylation as well as partial cleavage leaving one internal cleavage site. Of the results given by the MASCOT software, those having a probability score value higher than 53 were considered for successful protein identification. To find out putative functions, protein accession numbers of the identified spots were searched in the website for *B. pertussis*. To predict epitopes of the identified immunogenic proteins of *B. pertussis*, the artficial network based B-cell epitope prediction server ABCpred [22] was used. The PSORTb version 2.0.4 [23] was used for prediction of subcellular localization of the identified immunogenic proteins. The SignalP webserver (http://www.cbs.dtu.dk/services/signalp/) was employed for signal peptide prediction.

3. Results

3.1. Total soluble proteome

Total soluble proteomes of *B. pertussis* Tahoma I and Saadet strains were resolved by 2-DE. Three technical replicates of 2-DE gels for each of the three independent biological samples were run for each strain. 350 μg of extracted proteins were applied to the IPG strips. After being stained with colloidal CBB G250, over 600 spots could be detected on the gels within the pI range 3–10 (Fig. 1).

3.2. Identification of immunoreactive proteins

Western blotting of the 2-D gels using antisera designated as Th (sc), Sa (sc), Th (ip) and Sa (ip), respectively as primary antibody and anti-mouse IgG as secondary antibody revealed a total of 45 immunoreactive protein spots. These spots matched well to