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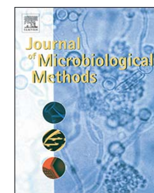

Production of a chimeric protein and its potential application in sero-diagnosis of *Mycoplasma hominis* infection

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ABSTRACT

Introduction: *Mycoplasma hominis* is an opportunistic pathogen of the human genital tract. Detection of antibodies against this organism in human serum or plasma is theoretically unreliable because of high variation in bacterial surface antigens. In this study, we applied the bioinformatics tools to design a chimeric protein constructed of specific, conserved and predicted immuno-dominant epitopes from two different membrane proteins, P120 and P80.

Material and methods: Linear B-cell epitopes of P120 and P80 were predicted and evaluated by bioinformatics tools and the designed chimeric protein was expressed in *Escherichia coli*. The chimeric protein, Mh128, was further analyzed in terms of immuno-reactivity by western blotting and enzyme immuno-sorbent assay (ELISA).

Results: We found eight specific, conserved and immuno-dominant epitopes within P120 and P80 based on the bioinformatic studies. The constructed chimeric protein showed immuno-reaction in both western-blotting and ELISA tests.

Discussion: Because of extensive variation of genomic and antigenic structure, diagnosis of *M. hominis* infection is difficult. Mh128 as a predicted specific and conserved recombinant protein can be potentially used for sero-diagnosis of *M. hominis* infection. We plan to develop an immuno-assay based on Mh128 and further evaluate the clinical specificity and sensitivity of the method.

1. Introduction

Molecular and conventional detection of infection caused by *Mycoplasma hominis*, an opportunistic pathogen in the genital tracts of 20–50% sexually mature females (Hosny et al., 2017; Taylor-Robinson, 2017), are complicated due to bacterial variation in genome and antigenic structure (Ferandon et al., 2013; Nyvold et al., 1997) and its fastidious requirements for cultivation (Miranda et al., 2005). Sero-diagnosis of *M. hominis* infection, as a non-invasive and simple method, can be used to determine the sero-prevalence of the organism. Multiple specific proteins such as P50, P60, P80 and P100 on the surface of this cell wall-free organism, play an important role in attachment of bacteria to the host cells, an essential step for colonization and survival of bacteria in human genital tracts (Henrich et al., 1993; Kitzerow et al., 1999; Boesen et al., 2004). It has been shown that *M. hominis* genetically diversifies its adhesion proteins by truncation (Henrich et al., 1998) and/or alters the coding gene expression by reversible frame-shift mutation (Zhang and Wise, 1997) to probably provide a better condition for attachment to different receptors in human body (Zhang and Wise, 1996) and causing extra-genital infection such as arthritis (Ataee et al., 2015), brain abscess (Bergin et al., 2017), endocarditis (Gagneux-Brunon et al., 2015), pneumonia (Garcia et al., 2007) and wound infection (Marini et al., 2008). In addition, phase variation is another strategy to spread infection and escape from host immune responses (Zhang and Wise, 2001). Such variable surface antigens are unreliable targets for designing of immunoassays (Chernov et al., 2005). On the other hand, intact peripheral proteins have potentially common and nonspecific regions resulting in false positive reactions with related or even unrelated organisms (Brown et al., 1987). Therefore, immunoassay methods for detection of antibodies against *M. hominis* in humans, which are usually based on surface antigens, may show significantly different results (Constans et al., 1991). Altogether antigenic variation plays an important role in different results between DNA amplification assays and the immunoassays which are based on

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surface antigens (Baczynska et al., 2008). One method of mitigating the above-mentioned problems is to target constant and specific sequences of more than one surface protein and using these fragments in a chimeric protein to increase the probability of detection of specific antibodies against *M. hominis* in human serum or plasma. In this study we used a combination of immunoinformatics tools for prediction of B-cell epitopes of two antigenic proteins of *M. hominis*, P120 and P80. According to our computational data we designed a chimeric protein composed of specific, constant and antigenic epitopes of P120 and P80 that can be potentially used in immunoassay methods. Then we created a synthetic construct of the chimeric protein and after cloning and expression we purified the desired recombinant protein, Mh128. We then showed that Mh128 is immuno-reactive against patient's pooled serum containing *M. hominis* antibodies.

2. Material and method

2.1. B-cell linear epitope prediction

The online tools of Immune Epitope Database Analysis Resource (IEDB) (Vita et al., 2010) were used for prediction of B-cell linear epitopes of P120 [GenBank accession no: CAX37501.1] and P80 [GenBank: CAX37485.1] derived from the *M. hominis* type strain PG21 (ATCC 23114).

The Kolaskar & Tongaonkar Antigenicity method (Saha and Raghava, 2006) by window size of 20 amino acids was used as the first prediction step. The predicted epitopes were searched for sequence similarity against UniProtKB database with the BLAST program to exclude non-specific sequences. To find the most conserved sequences, all available full length protein sequences of P120 and P80 were extracted from NCBI Protein Database and the sequences of each protein aligned by M-Coffee multiple sequence alignment tool (http://tcoffee.crg.cat/apps/tcoffee/do:mcoffee) and the conserved epitopes were selected for further evaluations. Final selection of predicted epitopes was based on their physicochemical properties. The Karplus & Schulz Flexibility Prediction (Karplus and Schulz, 1985) and Parker Hydrophilicity Prediction (Parker et al., 1986) methods were used for evaluation of flexibility and hydrophilicity of the predicted epitopes, respectively. SWISS-MODEL server (Biasini et al., 2014) and Phyre2 web portal (Kelley et al., 2015) were used to model the three dimensional structure of P120 and P80 amino acid sequences. The modeled 3D structures were used to determine the probable location of the selected epitopes in each protein.

2.2. Construction of chimeric recombinant protein (Mh128)

As two single amino acid variations were present in two selected epitopes (p4 and p15) and in order to cover all the probable variants, the two sequences including both variants were included in the chimeric protein. The theoretical isoelectric point (pI) of each selected epitope was computed by pI/MW program at the ExPASy portal and the peptides were arranged based on uniform distribution of their pI to make the chimeric protein with minimum polarity. The sequence of the chimeric protein was reverse translated and the nucleic acid sequence was codon optimized by Biomatik Corporation (Ontario, Canada) according to the preferable codon usage of *Escherichia coli*, GC content adjustment and secondary structure removal. The sequence was synthesized by Biomatik Corporation (Ontario, Canada) with *Bam*HI and *Xho*I restriction sites at the N and C-termini to clone into pET28a vector (Biomatik Corporation-Ontario, Canada). The Codon Adaptation Index (CAI) of Mh128 nucleic acid sequence was calculated relative to the codon usage table of highly expressed genes in *E. coli* by the online tool available at http://www.biologicscorp.com.

2.3. Bioinformatics evaluation of Mh128

The theoretical pI, molecular weight, estimated half-life, instability index and grand average of hydropathicity of the final chimeric protein along with two histidine tags at both ends (Mh128), were computed by ProtParam tool at the ExPASy portal. The secondary structure of Mh128, P120 and P80 proteins were predicted using PSSpred server at https://zhanglab.ccmb.med.umich.edu/PSSpred/.

2.4. Cloning of designed construct

The synthesized nucleic acid sequence was cloned into pET28a vector, without any signal sequence, to generate pMh128. To evaluate if the synthesized gene is consistent with target, the inserted gene in pMh128 was sequenced. The *E. coli* DH5α strain was used for transformation of pMh128. The transformed bacteria were selected by screening the colonies on Luria Bertani (LB) agar medium containing kanamycin at 30 μg/ml final concentration.

2.5. Expression and purification of Mh128

For expression of recombinant protein, pMh128 was transformed into *E. coli* BL21 (DE3) expression host. LB broth medium supplemented with kanamycin was inoculated with a single colony of transformed *E. coli* BL21 (DE3) with pMh128 and was incubated in a shaking incubator at 37 °C with constant agitation (250 rpm), to reach the logarithmic phase culture (OD_{600}: 0.7–0.9). To induce the expression of recombinant Mh128, Isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM and incubation period continued for another 3 h at 37 °C with shaking at 250 rpm. The induced bacteria were then harvested by 30 min centrifugation at 9000 × *g*. Bacterial pellet was re-suspended in urea buffer containing 100 mM NaH_2PO_4, 10 mM Tris.Cl, and 8 M urea (pH 8), by stirring the cells for 60 min at room temperature. His-tagged Mh128 was allowed to bind to Ni-NTA agarose (Qiagen, Hilden, Germany) for 1 h at room temperature. Lysate–resin mixture was loaded into a 100-ml purification column (Econo-Column® Chromatography Columns, 2.5 × 10 cm) followed by a washing step with urea buffer pH 6.5. Finally, the protein content was eluted with urea buffer pH 4. Washing and elution flow-through were monitored by Bio-Rad's Model EM-1 Econo UV monitor. The quality and quantity of purified recombinant Mh128 were evaluated by Coomassie Blue stained 12% SDS-PAGE (Invitrogen, NuPAGE® 4–12% Bis-Tris Gel) and BCA method (Thermo Scientific, Pierce® BCA Protein Assay), respectively.

2.6. Human sera used for analysis

A pooled serum composed of at least 15 *M. hominis* positive sera and another pooled serum containing of at least 15 *M. hominis* negative sera were used as positive and negative controls. Each individual serum was tested previously for detection of specific IgG against organism by indirect immunofluorescence assay (EUROIMMUN AG, FI 2201–1005 G, Germany). Presence or absence of *M. hominis* in the endocervical specimen of each patients was confirmed by PCR targeting 16S rRNA.

2.7. Evaluation of recombinant Mh128 immuno-reactivity

The immuno-reactivity of recombinant Mh128 was evaluated by western blotting and enzyme immuno-sorbent assay (ELISA).

2.7.1. Western blotting

The purified recombinant Mh128 was run on a 12% SDS-PAGE and later electrotransferred to a nitrocellulose membrane with a transblot apparatus (Invitrogen, XCell II™ Blot Madule) at 30 V for 60 min. Following overnight blocking in Blotto buffer (5 M NaCl; 1 M Tris pH 8; 10% non-fat dry milk Omniblock; 0.1% NP40 IGEPAL®) the membrane

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