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Molecular diversity of *hpd* gene in clinical isolates of *Haemophilus influenzae*

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ABSTRACT

Infections due to *Haemophilus influenzae* result in tremendous global morbidity. The conjugated vaccines against *H. influenzae* type b (Hib) have dramatically reduced the incidence of invasive Hib disease in the routine immunization of infants. The several proteins used as vaccine candidates for this pathogen, but they don't produce efficient immune in animal models against all strains of *H. influenzae*. This study aimed to determine the diversity of *hpd* gene nucleotide sequences of Iranian native clinical isolates of *H. influenzae* as a native vaccine candidate compared to standard strains.

Twenty isolates of *H. influenzae* recovered from different clinical specimens of patients admitted to Milad and Imam Khomeini hospitals, Tehran, Iran. Then, isolates detected and identified as *H. influenzae* using biochemical tests, and further confirmation through *omp6* gene PCR. The *hpd* gene was amplified by PCR using gene-specific primers, and the amplicons digested with *EcoR1*. For four isolates, the Amplicon of *hpd* gene sequenced, and the sequences aligned with sequences harbored in GenBank. Subsequently, sequences were submitted to the EMBL site (http://www.ebi.ac.uk/embl/).

EcoR1 restriction enzyme pattern was the same among the 19 clinical isolates, and only one isolate was different. That different one with 3 out of 19 isolates were sequenced. The results showed that the nucleotide sequences and the deduced amino acid sequences for protein D in clinical isolates were highly conserved with similarities > 95%.

In conclusion, regarding high similarity up to 99% in clinical isolates, protein D can be a novel vaccine candidate against all types of *H. influenza* from Iran. This finding should be proved with more isolates, and also, evaluate the immunological features of protein D in animal models.

1. Introduction

Haemophilus influenzae is a pleomorphic Gram-negative bacillus found in the human upper respiratory tract. It causes invasive infections, such as bronchitis, otitis, pneumonia, meningitis, septicemia, and epiglottitis (Fink and Geme, 2006). Isolates of *H. influenzae* can subdivide into two important forms; encapsulated and non-encapsulated that serotype b causes meningitis, mainly in children below four years of age (Resman et al., 2011). Nontypeable *H. influenzae* (*NTHi*) usually is a commensal with the capability of producing infections of the upper and lower respiratory tracts, such as sinusitis, bronchitis acute otitis media, as well as, more infrequently, severe invasive infections such as pneumonia, bacteremia, and meningitis (Clementi and Murphy, 2011; Yamanaka, 2011).

Protein D (PD) is a highly conserved Lipoprotein in the outer membrane of both typeable and non-typeable (NTHi) strains of *H. influenzae*. PD is a feasible vaccine candidate against NTHi strains (Cripps and Otczyk, 2006). Although PD is not an adhesion, it indirectly promotes bacterial adhesion and invasion due to glycerophosphodiesterase (GlpQ) activity and enhances bacterial colonization into human monocytes (Clarke et al., 2017).

The studies showed that > 97% of the strains antigenic similarity and 67% with glycerophosphodiesterase in *Escherichia coli*, and this shows the PD has a domain periplasmic (1, 2). In addition, nucleotide,

Abbreviations: Hib, *H. influenzae* type b; *NTHi*, Nontypeable *H. influenzae*; PD, Protein D; RFLP, restriction fragment pattern; CSF, cerebral spinal fluid

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and deduced amino acid sequences were found to be highly homologous *hpd* gene from NTHi (Behrouzi et al., 2017). The C-terminal region, due to more selective pressure by the immune system of the host can be variable than the N-terminal part (Behrouzi et al., 2017).

So, this study aimed to determine the diversity of *hpd* gene nucleotide sequences of Iranian native clinical isolates of *H. influenzae* as a native vaccine candidate compared to standard strains.

2. Methods

2.1. Study population

Patients admitted to Milad, Imam Khomeini hospitals, Tehran, Iran included in the present study. The samples gathered from suspected infection cases.

2.2. Source of isolates

A total of 20 isolates of *H. influenzae* originated from different samples, including blood, eyes, nasal secretions, throat, CSF collected during a year (*from* August 2010 to 2011). All samples were cultured on Stuart transport medium and shipped to the Department of Bacteriology, Pasteur Institute of Iran, Tehran. Afterward with the samples were cultured using by swabs on chocolate agar medium containing 10% sterile sheep blood and then incubated for 24 to 48 h at 37 °C in a 5% CO2 incubator. Organisms were identified as *H. influenzae* if they were small, pleomorphic, gram-negative rods on Gram stain, and required supplemented with Vitox (Oxoid Ltd., Basingstoke, Hampshire) for growth on Trypticase soy agar. The *H. influenzae* isolates were serotyped by Hib-specific antiserum as described previously (Kim et al., 2011; Satola et al., 2007). Stock cultures were maintained at −70 °C in Trypticase Soy Broth with 20% Glycerol.

2.2.1. DNA extraction

The DNA of isolates was purified by QIAamp DNA mini kit (Qiagen, Valencia, CA) according to manufacturer's instructions. Extraction of bacteria harvested in the exponential growth phase after culturing on chocolate agar at 37 °C in 5% CO2. The concentration of purified DNA used as the standard for quantification measured in a NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE).

2.2.2. PCR amplification of the P6 gene

PCR amplification was performed to detect the *omp6* gene to confirm isolates as described by Saiki et al. performed (Lerner et al., 2007). The length of the amplified products expected to size 351 bp. The sequences of oligonucleotides for the primers were as follows: Primer F; 5′-AAC TTT TGG CGG TTACTC TG-3′, and primer R; 5′-CTA ACA CTGCAC GAC GGT TT-3′. The oligonucleotide sequences were synthesized by Kurabo (Osaka, Japan).

2.2.2.1. PCR amplification of the hpd gene. The primer set, *hpd* -F and *hpd* -R, was designed based on the published DNA sequences in GenBank (L15200.1) for the *hpd* gene, using Vector NTI Advanced 11.0. The length of the amplified products was expected to be 1095 bp. The sequences of oligonucleotides for the primer set and probes were as follows: Primer *hpd* -F, 204- ATG AAA CTT AAA ACT TTA GCC CTT TC-230 (melting temperature, 56.89C); and primer R-1299 TTA TTT TAT TCC TTT TAA GAA TTC CAC G-1272 (melting temperature, 55.71C). The oligonucleotides were synthesized by Macrogen (Seoul, Korea).

2.2.2.1.1. Restriction fragment length analysis (RFLP). First, 6.5 mL of samples was added to microtubes, then, 5 mL deionized distilled water, 6 mL product PCR, 1.5 mL restriction enzyme buffer, and 1 μL restriction *EcoR1* added and mix well (do not vortex). Next, Enzymes incubated for 4 h at 37 °C (Del Rio et al., 2006). Finally, electrophoresis was done on 2% agarose gel using low voltage 60 V.

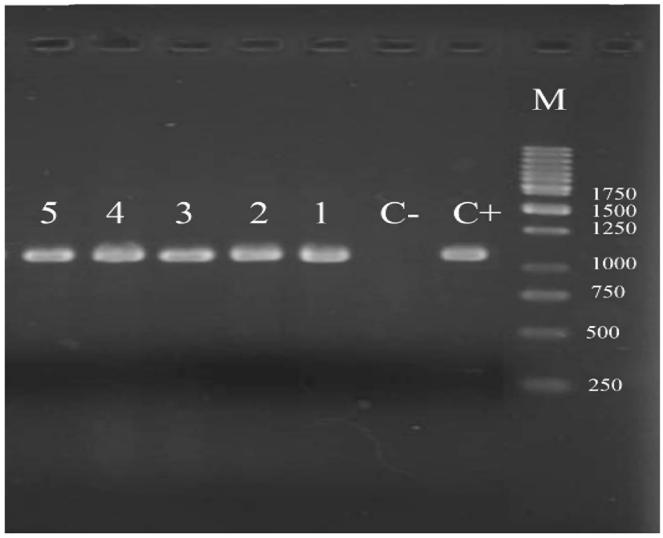

Fig. 1. PCR amplification of *hpd* gene *H. influenzae* from clinical isolates.

2.3. Nucleotide sequencing

The PCR product of each restriction fragment pattern (RFLP) was randomly chosen and was sequenced. Sequence analysis performed with the Applied Biosystems Hitachi 3130 Genetic analyzer.

3. Results

Of the total clinical isolates, 30% (n = 6) were isolated from cerebral spinal fluid (CSF) and Blood, 30% (n = 6) from eye, 40% (n = 8) from throat, and nasopharynx. The amplification of the *hpd* gene using PCR produced a DNA fragment of approximately 1095 bp (Fig. 1).

Digestion products were resolved on 2% agarose gels and visualized on a UV transilluminator after ethidium bromide staining. Molecular weight markers were run to estimate fragment sizes (100-bp ladders). Digestion of these fragments produced with *EcoR1* Enzyme. All the isolates except one produced a restriction pattern with two types of fragments, one with 653 bp and another with a fragment of approximately 443 bp (Fig. 2).

To confirm the validity of the RFLP analysis and to explore sequence differences in RFLP pattern, we chose 1 different (KC608170) with

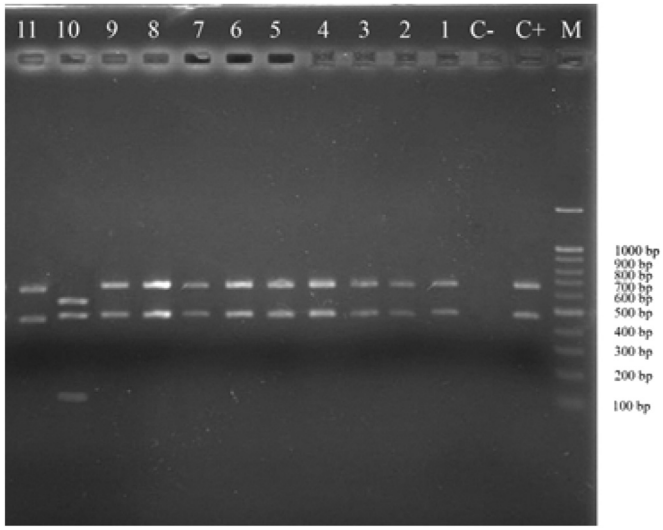

Fig. 2. RFLP analysis of PCR amplified *hpd* gene from the genomic DNA of clinical isolates of *H. influenzae*.

Table 1
Comparison of nucleotide sequences of clinically isolated with Gene Bank databases.

Sample ID	Accession number	Max ident	Accession Gene Bank
3	KC608167	98%	GQ402004 GQ402006
6	KC608168	100%	GQ402007 GQ402006
9	KC608169	99%	GQ402004 GQ402007
10	KC608170	92% 98%	GQ402000 GQ402008

three other representative *hpd* genes sequenced. Sequence Alignment *hpd* isolated from KC608170 with cases recorded in GeneBank (GQ402000.1, GQ402008.1, and Z35656); respectively 92%, 98%, and 93% similarity were observed. In comparison, the mutant isolate with isolates recorded in NCBI was found that have the difference in11 amino acids (Table 1).

4. Discussion

H. influenzae serotype b (Hib) is a significant cause of bacterial meningitis and other invasive infections among children younger than four years. Since the vaccination against Hib, other stereotypes and nontypeable strains have emerged as a significant cause of *H. influenzae* associated diseases (Bottomley et al., 2016).

It is transmitted by the respiratory tract from infected to susceptible people. Although this microorganism exists globally the burden of Hib disease was significantly higher in developing countries (Izadnegahdar et al., 2013).

Several effective Hib vaccines against strains made, but these vaccines can protect children against infections associated with NTHi (Bresee et al., 2004). A large study on several proteins, such as cell surface and outer membrane proteins (OMP) has been conducted to develop an effective vaccine against Hib and NTHi (Cripps et al., 2002; Green et al., 1993). In previous studies, a high homology between the nucleotide and deduced amino acid sequences of *Eco*RI sites within the *hpd* gene from *H. influenzae* strains was determined; so, they concluded that there was only a limited diversity within the *hpd* gene. The C-terminal end of protein D due to expose on the outside of the bacterium is more variable than the N-terminal part due to avoid the immune system (Janson et al., 1993; Song et al., 1995).

In this study, we determined the DNA sequence of the structural *hpd* gene, and amino acids in four clinical isolates and compared with the previously reported *hpd* sequences from NTHi strains included in NCBI.

These findings suggest that the *hpd* gene is conserved in terms of antigenic and functionality, so it has high homology to stereotypes and biotypes strains of *H. influence* belonging to various other countries. Amino acids from KC608167, KC608168, and KC608168 strains were similar together up to 99%, but the KC608170 strain was different in thirty-four amino acid from others. At 81 position, cysteine amino acid converted proline and against it, proline in 85 position converted to cysteine. Restriction site polymorphism was observed in KC608170 isolates. Hence, it reflects this isolate be different from other native strains. All of the mutations were non-synonymous and altered amino acids in cutting enzyme sites.

The results showed that the nucleotide sequences and the deduced amino acid sequences for protein D in clinical isolates were highly conserved with similarities > 95%.

5. Conclusion

In conclusion, regarding high similarity up to 99% in clinical isolates, protein D can be a novel vaccine candidate against all types of *H. influenza* from Iran. This finding should be proved with more isolates, and also, evaluate the immunological features of protein D in animal models.

Declaration of competing interest

None declared.

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