International Journal of Molecular and Clinical Microbiology 5(1) (2015) 510-515

International Journal of
Molecular and Clinical Microbiology

Cloning of conserved regions of nontypeable *Haemophilus influenzae hmw1* core binding domain

Sepideh Meidaninikjeh[1], Farzam Vaziri[2], Seyed Davar Siadat[2]*

1. Department of Microbiology, Faculty of Advanced Sciences & Technology, Pharmaceutical Sciences Branch, Islamic Azad University, Tehran - Iran (IAUPS)
2. Research Center of Microbiology, Department of Mycobacteriology and Pulmonary Research, Pasteur Institute of Iran, Tehran, Iran.

ARTICLE INFO

Article history:
Received 4 march 2015
Accepted 12 May 2015
Available online 1 June 2015

Keywords:
Nontypeable Haemophilus influenzae, Core binding domain of HMW1, Cloning, Recombinant plasmid.

ABSTRACT

Colonization of nontypeable *Haemophilus influenzae* (NTHi) in nasopharynx causes respiratory tract disease. In 80% of clinical isolates, HMW proteins are the major adhesions and induce protective antibodies in the hosts. Therefore, it can be used as a vaccine candidate. The aim of this study is designing and cloning of the conserved regions of NTHi *hmw1* core binding domain. In this study, the standard strain of NTHi (PTCC1766) was used and cultured on chocolate agar. Genomic DNA was extracted by commercial kit. Then, specific primers were designed for highly conserved regions and PCR product was purified. PCR product was inserted into the PTG19-T plasmid and the recombinant plasmids were transferred to *Escherichia coli* (Top10). For screening the recombinant cells from others, they were cultured on LB agar which had ampicillin and tetracycline antibiotics. Recombinant cells were purified by a specific kit. The presence of conserved regions of NTHi *hmw1* core binding domain was confirmed by colony-PCR, double digest and sequencing. In this study, having analyzed the result of sequencing by Mega6 software, we understood that the sequencing result was same as our target area (Conserved regions of *hmw1* core binding domain), indicating that the cloning process have been performed successfully. It is concluded that, due to the presence of high similarity among some regions of *hmw1* core binding domain among NTHi PTCC1766 and other NTHi strains, core binding domain can be an appropriate candidate for subunit vaccines in case of inducing immune system response.

1. Introduction

Haemophilus influenzae includes encapsulated and non-encapsulated strains and is a reason of respiratory tract infection and about 30% of otitis media in children and infants (Murphy et al., 2009). Based on carefully performed studies, the primary clinical manifestations of infection caused NTHi are otitis media and sinusitis in infants and children (Murphy et al., 2009). The bacterium is the most common cause of exacerbations in adults with chronic obstructive pulmonary disease and also likely contributes to the pathogenesis of disease by chronically colonizing the lower airways (Sethi and Murphy, 2008). Furthermore, NTHi causes community-acquired pneumonia in adults and may cause pneumonia in children in developing countries, although precise estimates are difficult (Hausdorff and Dagan, 2008). Establishment of bacteria on the rhinopharyngeal respiratory mucosa is the fundamental step in the pathogenesis of NTHi and followed by continuous extension of

*Corresponding author: Dr.Seyed Davar Siadat
Tel: +98 21 66405535
E-mail address: d.siadat@gmail.com

Archive of SID

www.SID.ir

bacteria to sterile sites and respiratory tract. Pilus and nonpilus adhesions have been identified as adherence factors (Rao et al., 1999). Attachment to human epithelial cells is the responsibility of HMW proteins in 80% of NTHi isolates (St Geme et al., 1993). In addition; they are high molecular weight surface-exposed protein that during otitis media are identified as a major target of human serum antibody response (Barenkamp and Bodor, 1990). In one study, one hundred clinical isolates were evaluated for the presence of the genes encoding protein D, Hap, HMW, PilA, and Omp5 by PCR and for their adherence capacity (AC) to Detroit 562 nasopharyngeal cells (D562) and it showed that the presence of *hmw1A* and/or *hmw2A* was associated with increased adherence to D562 cells (Vuong et al., 2013). The core binding domain in HMW1 is 124 amino acids between residues 555- 678 but this region in HMW2 is located between 553-677 and contains 125 amino acids. Moreover; core binding domain is necessary for full level adherence (Dawid et al., 2001). In spite of the fact that core binding domain has diversity, it has conserved amino acid motifs which can help us to realize the relation between binding specificity and protein sequence. Moreover, consideration of HMW proteins in a multicomponent vaccine as vaccine antigens causes that conserved regions of HMW binding domains become important. Other studies are necessary to understand the capability of these regions for inducing immune system response (Giufrè et al., 2006). The main objective of this study is designing and cloning of a recombinant plasmid contained conserved regions of *hmw1* core binding domain gene in an appropriate engineered bacteria such as *Escherichia coli* (Top10).

2. Materials and Methods

2.1. Bacterial strains and plasmids

NTHi PTCC1766 was obtained from the Department of Mycobacteriology and Pulmonary Research, Pasteur Institute of Iran collection and it was grown on the chocolate agar for 18-24 hours in the presence of 5% Co2. *E. coli* (Top10) and pTG19-T plasmid were provided by the Department of Mycobacteriology and Pulmonary Research, Pasteur Institute of Iran, and Vivantis company (Malaysia) respectively. The pTG19-T vector has ampicillin resistance gene and is designed for rapid and efficient cloning of PCR products with 3'-dA overhangs. The linearized pTG19-T vector with 3'-dT overhangs prevent vector recircularization, therefore resulting in high percentage of recombinant clones and low background.

2.2. DNA Extraction

Genomic DNA was extracted by high pure PCR template preparation kit (DNA Technology, Russia).

2.3. Amplification of gene

Primers were designed by Gene Runner software according to the core binding domain of *hmw1* regions which show the most similarity among strains. Tail with 2 nucleotides beyond restriction site was added to the specific sequence of forward and reverse primers.

Highly conserved regions of *hmw1* core binding domain (350 bp) were PCR-amplified using *NcoI* tailed forward (ATC CAT GGT TGA TGT TCA TAA AAA TAT C) and *XhoI*-tailed reverse (ATC TCG AGT TCC AGT AAG TGC GTC) primer pairs and following thermal program was used:

Denaturation at 94°C for 5 min, followed by 35 cycles of: denaturation at 94°C for 30 s, annealing at 57°C for 1 min, and extension at 72°C for 1 min, final extension at 72°C for 5 min. PCR products and negative and positive controls were carried out on the agarose gels (1%). PCR reaction was performed in 20 ul of volume containing 0.4 ul dNTPs (200uM), 2 ul buffer10x, 0.2 ul Taq DNA Polymerase (0.5U/20ul) (SinaClon, Iran), 1ul of each forward and reverse primers (10 pmole) (CinnaGen, Iran), 0.6 ul MgCl2 (1.5mM), 13.8 ul DDW and 1ul extracted DNA (100ng). In this step, NTHi ATCC (49766) and double-distilled water (DDW) were used as positive and negative controls respectively.

2.4. PCR product purification

For purification of the amplified gene from other PCR reagents in the tube, the special kit of gene purification (Vivantis, Malaysia) was used. Firstly, the special region that included

Archive of SID

www.SID.ir

conserved regions of *hmw1* core binding domain was cut and then the other procedures were carried out based on the company protocol and 1ul of sample was utilized for conducting electrophoresis. The above step was taken for verification of the purification, after the solution was transmitted to another tube and kept for ligation with PTG19-T vector in TA cloning.

2.5. Ligation of PCR product to PTG19-T

In accordance with the company instructions, PCR product was ligated into PTG19-T and this process was performed in a single tube which contained PTG19-T, ligation buffer, PCR product, PEG, T4-DNA ligase (Vivantis, Malaysia) and sterile DDW. The ligation product was kept 1 hour at room temperature and after that 12-16 hours at 4-8˚C.

2.6. Competent cells and transformation

After making competent cells, recombinant plasmids were transformed into the appropriate host *E.coli* (Top10) and after transformation, bacteria were grown on a LB agar contained ampicillin and tetracycline antibiotics.

2.7. Confirmative tests
2.7.1. Colony PCR

The presence of the DNA insert determined by screening bacterial colonies by PCR. In this stage, 14 colonies were selected and individual colonies were subjected directly to the PCR master mix using thermo scientific reagents, then matrix plate was prepared and the remaining portion of the colony were inoculated on the culture plate or in LB media with tetracycline and ampicillin antibiotic for downstream application. Finally, the colonies with positive colony PCR were cultured in LB broth containing ampicillin and tetracycline antibiotics and will be used for recombinant DNA extraction. Commercial kit (Vivantis, Malaysia) was used to extract recombinant plasmids. In this study, ATCC (49766) and double-distilled water (DDW) were used as positive and negative controls respectively.

2.7.2. Double digest

The digestion solution was prepared with recombinant plasmid, Tango buffer 10X, *NcoI*, *XhoI* (Fermentase, United states) and sterile H20. After incubation at 37˚C for 16-20 hours, 1ul of this product was used for electrophoresis on 1% agarose gel.

2.7.3. PCR on extracted recombinant plasmids

After extraction of recombinant plasmids by commercial kit (Vivantis, Malaysia), PCR reaction was performed on the recombinant plasmids again.

2.7.4. Sequencing

The recombinant plasmid was sent to sequencing (Takapouzist company, Iran), which was ordered by Universal primers (M13 forward and reverse), and the result was subsequently examined by Mega6 software.

3. Results

3.1. PCR product electrophoresis

Electrophoresis of the PCR products was carried out on the agarose gel 1%, and as expected, the size of the amplified products for the highly conserved regions of NTHi *hmw1* core binding domain was 350 *bp* (Figure1).

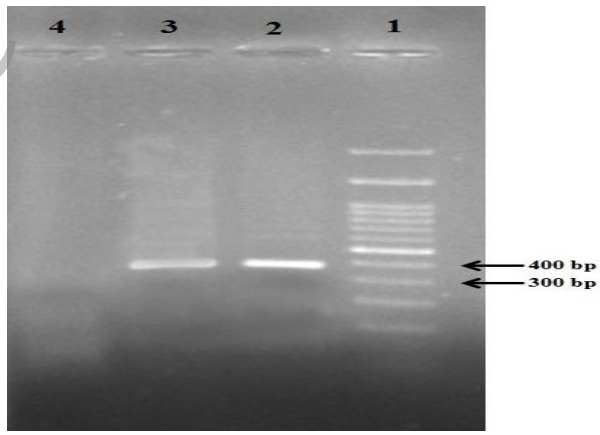

Figure 1. Electrophoresis of PCR product on 1% agarose gel. Lane 1:100 bp DNA ladder (size marker); Lane 2: Conserved regions of NTHi *hmw1* core binding domain (PCR product 350 bp); Lane 3: Positive control; Lane 4: Negative control.

3.2. DNA purification

The purification product was run in 1% agarose and the band of 350 bp appeared again (Figure 2).

Archive of SID

www.SID.ir

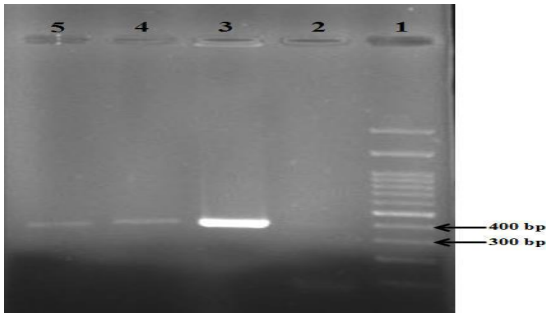

Figure 2. Electrophoresis of purification product on 1% agarose gel. Lane1: 100 bp DNA ladder (size marker); Lane 2: Negative control; Lane 3: Positive control; Lane 4 and 5 DNA purification product (Conserved regions of NTHi *hmw1* core binding domain 350 bp).

3.3. Colony PCR

Among 14 selected colonies, 12 of them showed amplified 350 bp gene obviously on gel agarose electrophoresis 1% (Figure3).

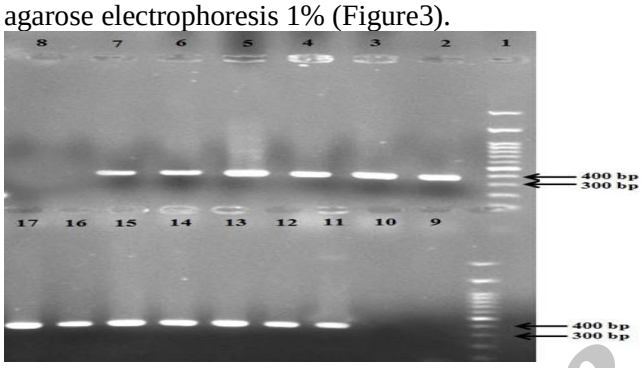

Figure3. Electrophoresis of the colony PCR on 1% agarose gel. Lane1: 100 *bp* DNA ladder (size marker); Lane number 2 to 6 from top row and Lane number 11 to 17 from bottom row are positive Colony PCR products; Lane 7: Positive control; Lane 9: Negative control.

3.4. PCR on Extracted recombinant plasmids

PCR was done on the extracted recombinant plasmid and the band of 350 bp is shown in figure below (Figure 4).

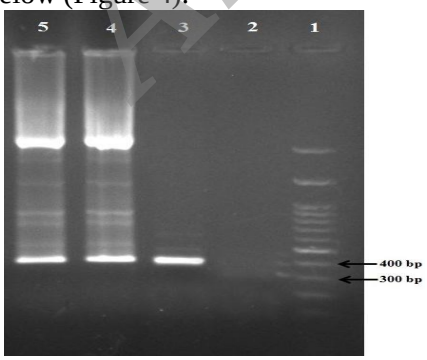

Figure 4. Electrophoresis of the recombinant plasmid PCR product on the 1% agarose gel. Lane1: 100 bp DNA ladder (size marker); Lane 2: Negative control; Lane 3: Positive control; Lane 4 and 5: Recombinant plasmid PCR product.

3.5. Double digest

Recombinant plasmids were digested by *NcoI* and *XhoI* enzymes, afterwards the digestion product and recombinant plasmids were run on the agarose gel 1%.The release of insert was another confirmative step for this test (Figure 5).

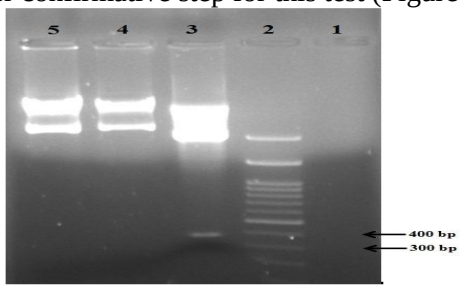

Figure 5. Electrophoresis of the double digest product and recombinant plasmids on 1% agarose gel. Lane 1: Negative control; Lane 2: 100 bp DNA ladder (size marker); Lane 3: Digestion product (The release of 350 bp fragment during digestion process); Lane 4and 5: Recombinant plasmids.

3.6. Sequencing

The result was analyzed by Mega6 softwere. The sequencing result was the same as the target area (Conserved regions of NTHi *hmw1* core binding domain).

4. Discussion

NTHi cause respiratory tract disease such as otitis media, conjunctivitis, sinusitis, pneumonia, bronchitis, and conjunctivitis (Turk, 1984). Colonization of the nasopharynx is the first step in the pathogenesis of disease (Murphy et al., 1987). Persisting of colonization with a particular strain may last for weeks to month, usually without any symptoms (Spinola et al., 1986; Trottier et al., 1991). However, in allergic and viral diseases NTHi contiguously outspread the sinuses, conjunctiva, lungs and middle ear middle ear. For successful colonization, overcoming of organism to the mucociliary escalator is needed. During the first 3 years old of life, the outbreak of otitis media has enormous effect on intellectual ability, school achievement speech, and languages (Teele et al., 1990). More than 100% of one year old children in developing countries will have their first episode of otitis media (Teele et al., 1989; Smith-Vaughan et al., 1997) and NTHi is also responsible for 27 to 37% of these episodes (Murphy and Apicella, 1987; Harabuchi et al., 1994; Geme, 1996). Since the vaccine of

Archive of SID

www.SID.ir

H.influenzae type b has no effect on NTHi infections, designing vaccines for preventing infectious diseases caused by NTHi become a great of interest. (Foxwell et al., 1998). Among diverse strains of NTHi, both pilus and nonpilus adhesions have been defined (Dawid et al., 2001). In 80% of NTHi isolates, the major adhesions are related proteins called HMW1 and HMW2 (Geme et al., 1998). In 1990, HMW1 and HMW2 were identified as a high molecular weight protein and the major targets of humane antibodies during otitis media (Barenkamp and Bodor, 1990). The hmw1A gene is 4.6 Kb and *hmw1B* and *hmw1C* is located downstream of *hmw1A* gene. The HMW1 preprotein contains 1536 amino acids and according to analysis of a series of complementary HMW1-HMW2 chimeric proteins and a set of fusion proteins containing fragments of HMW1 or HMW2, the domains responsible for interacting with epithelial cells receptors have localized to the region defined by amino acids 555-914 in HMW1 and the region defined by amino acids 553-916 in HMW2. These domains correspond to the areas of the maximal sequence dissimilarity between HMW1 and HMW2 (Dawid et al., 2001). Chimeric proteins showed, the 124 amino acids between 555 - 678 in HMW1 and the 125 amino acids between residues 553 - 678 in HMW2 ,which are called core binding domain, play a fundamental role in efficient attachment. These results illustrate that these regions can be used in a subcomponent vaccine against NTHi. In 2006 Maria Giufre *et al* showed that there is a great diversity among core binding domains of NTHi invasive isolates. However, there are some conserved amino acid motifs within core binding domain of both HMW1 and HMW2 proteins which may be used as a vaccine candidate against NTHi strains. Furthermore, the main goal of some studies is to measure the capability of these regions to induce cross reactive antibodies in the human immune system (Giufrè et al., 2006). In this study we designed specific primers for highly conserved regions of NTHi *hmw1* core binding domain gene and the strain that used in this project was NTHi PTCC1766. The aim of this study was designing and cloning of recombinant plasmid contained conserved regions of NTHi *hmw1* core binding domain. In addition, the accuracy of our study was confirmed by confirmative tests such as colony PCR, double digest, and sequencing.

Acknowledgement

This work was financially supported by the Grant No. 571 from Pasteur Institute of Iran, Tehran, Iran. We thank all members of the Department of Mycobacteriology and Pulmonary Research, Pasteur Institute of Iran.

Refereces

Barenkamp, S.J., and Bodor, B.B., 1990. "Development of serum bactericidal activity following nontypable Haemophilus influenzae acute otitis media." The Pediatric infectious disease journal. 9(5): 333-338.

Dawid, S., Grass, S., and Geme, J.W.S., 2001. "Mapping of Binding Domains of NontypeableHaemophilus influenzae HMW1 and HMW2 Adhesins." Infection and immunity. 69(1): 307-314.

Foxwell, A.R., Kyd, J.M., and Cripps, A.W., 1998. "Nontypeable Haemophilus influenzae: pathogenesis and prevention." Microbiology and molecular biology reviews. 62(2): 294-308.

Geme, J.W.S. 1996. "Progress towards a vaccine for nontypable Haemophilus influenzae." Annals of medicine. 28(1): 31-37.

Geme, J.W.S., Kumar, V.V., Cutter, D., and Barenkamp, S.J., 1998. "Prevalence and Distribution of the hmwand hia Genes and the HMW and Hia Adhesins among Genetically Diverse Strains of Nontypeable Haemophilus influenzae." Infection and immunity. 66(1): 364-368.

Giufrè, M., Muscillo, M., Spigaglia, P., Cardines, R., Mastrantonio, P., and Cerquetti, M., 2006. "Conservation and diversity of HMW1 and HMW2 adhesin binding domains among invasive nontypeable Haemophilus influenzae isolates." Infection and immunity. 74(2): 1161-1170.

Harabuchi, Y., Faden, H., Yamanaka, N., Duffy, L., Wolf, J., and Krystofik, D., 1994. "Nasopharyngeal colonization with nontypeable Haemophilus influenzae and recurrent otitis media."Journal of infectious diseases. 170(4): 862-866.

Hausdorff, W.P., and Dagan, R., 2008. "Serotypes and pathogens in paediatric pneumonia." Vaccine. 26: B19-B23.

Murphy, T.F., 2009. "Current and future prospects for a vaccine for nontypeable Haemophilus influenzae." Current infectious disease reports. 11(3): 177-182.

Archive of SID

www.SID.ir

Murphy, T.F., and Apicella, M.A., 1987. "Nontypable Haemophilus influenzae: a review of clinical aspects, surface antigens, and the human immune response to infection." Review of Infectious Diseases. 9(1): 1-15.

Murphy, T.F., Bernstein, J.M., Dryja, D.M., Campagnari, A.A., and Apicella, M.A., 1987. "Outer membrane protein and lipooligosaccharide analysis of paired nasopharyngeal and middle ear isolates in otitis media due to nontypable Haemophilus influenzae: pathogenetic and epidemiological observations." Journal of Infectious Diseases. 156(5): 723-731.

Murphy, T.F., Faden, H., Bakaletz, L.O., Kyd, J.M., Forsgren, A., Campos, J., Virji, M., and Pelton, S.I., 2009. "Nontypeable Haemophilus influenzae as a pathogen in children." The Pediatric infectious disease journal. 28(1): 43-48.

Rao, V.K., Krasan, G.P., Hendrixson, D.R., Dawid, S., and Geme, J.W.S., 1999. "Molecular determinants of the pathogenesis of disease due to non-typable Haemophilus influenzae." FEMS microbiology reviews. 23(2): 99-129.

Sethi, S., and Murphy, T.F., 2008. "Infection in the pathogenesis and course of chronic obstructive pulmonary disease." New England Journal of Medicine. 359(22): 2355-2365.

Smith-Vaughan, H., Sriprakash, K., Mathews, J., and Kemp, D., 1997. "Nonencapsulated Haemophilus influenzae in Aboriginal infants with otitis media: prolonged carriage of P2 porin variants and evidence for horizontal P2 gene transfer." Infection and immunity. 65(4): 1468-1474.

Spinola, S.M., Peacock, J., Denny, F.W., Smith, D.L., and Cannon, J.G., 1986. "Epidemiology of colonization by nontypable Haemophilus influenzae in children: a longitudinal study." Journal of Infectious Diseases. 154(1): 100-109.

St Geme, J.W., Falkow, S., and Barenkamp, S.J., 1993. "High-molecular-weight proteins of nontypable Haemophilus influenzae mediate attachment to human epithelial cells." Proceedings of the National Academy of Sciences. 90(7): 2875-2879.

Teele, D.W., Klein, J.O., Chase, C., Menyuk, P., and Rosner, B.A., 1990. "Otitis media in infancy and intellectual ability, school achievement, speech, and language at age 7 years." Journal of Infectious Diseases. 162(3): 685-694.

Teele, D.W., Klein, J.O., and Rosner, B., 1989. "Epidemiology of otitis media during the first seven years of life in children in greater Boston: a prospective, cohort study." Journal of infectious diseases. 160(1): 83-94.

Trottier, S., Stenberg, I., Von Rosen, A., and Svanborg, C., 1991. "Haemophilus influenzae causing conjunctivitis in day-care children." The Pediatric infectious disease journal. 10(8): 578-583.

Turk, D., 1984. "The pathogenicity of Haemophilus influenzae." Journal of medical microbiology. 18(1): 1-16.

Vuong, J., Wang, X., Theodore, J.M., Whitmon, J., de Leon, P.G., Mayer, L.W., Carlone, G.M., and Romero-Steiner, S., 2013. "Absence of high molecular weight proteins 1 and/or 2 is associated with decreased adherence among non-typeable Haemophilus influenzae clinical isolates." Journal of medical microbiology. 62(Pt 11): 1649-1656.

Archive of SID

www.SID.ir