Original Article

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Cloning and Expression of Mutant and Inactive Form of Bordetella Pertussis Toxin from Iranian Native Strain

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ABSTRACT

BACKGROUND AND OBJECTIVE: Whooping cough is an acute contagious human respiratory disease especially in infants and children and is one of the ten major causes of death from infectious diseases. Despite reducing the risk due to vaccination with killed bacteria pathogen (Bordetella pertussis), increased disease emerged in recent decades indicates re-emerging due caused by genetic diversity of bacteria and the insufficiency of current vaccines. Therefore, the design of new and effective vaccines based on common strains of the population is essential. In this study the inactive recombinant protein of most important bacterial antigens (pertosis toxin, PTX), the most commonly pathogenic strain in Iran, were produced on the basis of the new generation of non-cellular vaccines.

METHODS: Bacterial strain used in this study to determine the genome and gene amplification was selected from pathogenic strains isolated from Iranian patients from the pertussis reference laboratory of Microbiology of the Pasteur Institute of Iran. After bacterial culture and genomic DNA extraction, *ptx- s1*(the enzymatic fragment of PTX) fragments amplified and inserted in pUC18. Site directed mutagenic primers were used for substitution of Arg 9 by Lys and Glu129 by Gly. The confirmed mutant *ptx- s1* (*mptx-s1*) was sub-cloned into expression vector pET15b. The expression of recombinant protein in *E.coli BL21* was induced by 0.5mM IPTG. The expression was analyzed by SDS-PAGE and immuno-blotting. The protein was puifird by Ni-NTA affinity chromatography under denature condition and protein concentration was also determined.

FINDINGS: Glu129Lys, Arg9Gly mutations were confirmed by sequencing. The presence of a 28 kDa band in SDS-PAGE and immunoblot using anti-His antibody showed the expression of mutant PTX. The recombinant protein was purified with amount of 0.36 mg/ml.

CONCLUSION: Based on the results of this study, the inactive form and immunogenic PTX of the dominant pathogen in Iran can be used alone or in combination with other antigens for the design of non-cellular vaccines.

KEY WORDS: *B .Pertussis, Toxin, Acellular Vaccine, Site-Directed Mutagenesis.*

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Introduction

Pertussis is an acute, highly contagious and chronic human respiratory pathogen caused by a gram-negative as Bordetella pertussis (1). All age groups are susceptible to this disease, however, is more common in babies (2). Until 1950 and by vaccination, Pertussis was one of the major causes of death in infants, but with a global vaccination, rate of deaths from the disease fell significantly (3). After the identification of B. pertussis, cellular vaccine as the Whole Cell Vaccines including killed bacteria or detoxification by using methods such as treatment with glutaraldehyde or formaldehyde was designed and built for disease control (4). Cellular vaccines had several side effects such as fever, seizures and encephalopathy (5, 1) that this reduces public confidence and lack of its use in some of countries. Hence a new generation of vaccines as acellular vaccine was introduced (6).

This type of vaccine has far fewer side effects, are very high in efficacy and safety profile more effectively in many developed countries in the vaccination program has been replaced by cell vaccines (8, 7). Acellular vaccines containing one or more common pathogenic factor of B. pertussis, such as Pertussis toxin (PTX), Filamentus Haemaglutinin (FHA), Pertactin (PRN) and Fimbriae (fim) (5, 3). We have more than ten kinds of non-cellular vaccine were produced worldwide in a number of different types of antigens formulated (7).

Most of these vaccines contain Bordetella pertussis antigens 1 to 5 different types of PTX details are fixed, but those who are disabled in different ways (7, 3). PTX complex exotoxin 105kDa and is a family member of two-component AB toxins. This toxin consists of 5 sub-units S1-S5, respectively, toxic Ptx-S1 enzymatic subunit has activity of ADP is Ribosyl transferase (9). Recent research by creating mutations in the active site of pertussis toxin, a deactivated toxin sub-unit (S1 Ptx-) while maintaining immunogenesity properties was produced (11, 10). Pertussis toxin is codes by ptx gene. Genetic changes in pathogenic factor causing different alleles of this gene and this gene has a number of 11 alleles, of them two alleles ptxP1 and ptxP3 is more common among pathogenic strains (12).

Studies have shown that allelic shifts (polymorphism) of strains of B. pertussis causes bacteria to evade the immune system and resistance of bacteria to the safety of the vaccine (14, 13). Polymorphism seems to be associated with recurrent disease whooping cough in the world (15, 13, 8). According to reports in recent years despite vaccination coverage of about 99% in Iran, the disease has increased (17, 16). Cellular vaccine based on standard strain are producing in our country for many years and used. Increase patient reports suggests reducing the immunogenicity of the vaccine against the current strains circulating in the population, therefore, design and production of effective vaccines based on the dominant strains seems necessary.

In this study using epidemiological data to the laboratory reference strains of Pertussis, native strain containing dominant allele was identified and recombinant form of inactive pertussis toxin was produced and verified.

Methods

Bacterial culture and extraction of the genomic content: Strains of bacteria used in this study to determine the genome and gene amplification, were selected from pathogenic strains isolated from Iranian patients based on research conducted at the common polymorphism of antigens of bacteria in (unpublished data) whooping cough reference laboratory of microbiology department of Pasteur Institute of Iran. First, culture was done using bacteria stored in glycerol medium for 72 h at 37°C in Regan Lu medium (18). Genomic content was extracted using Genomic Bacterial extraction kit (Roche) and DNA quality and quantity were evaluated by a 1% agarose gel electrophoresis and spectrophotometer.

Amplification and clonnig of enzymatic active region of pertussis toxin (ptx-s1): The amplification of ptx-s1 that is the the enzymatic active part of toxin was performed using specific primers and Pfu enzyme (Table 1). In forward primers the cutting enzymes KpnI (for cloning in the pUC18 vector) and the status of enzymes NdeI (for cloning of fragment in the expression vector of pET15b) and enzyme cutting sites BamHI in Reverse primer for stronger have been determined.

Cloning of ptx-s1 in pUC18 vector: Amplified fragment was purified with high pure PCR product purification kit (Roche Diagnostic, Germany) and after digestion of Site-specific KpnI / BamHI, Puc18 vector was cloned and ligated with the enzyme of T4 DNA ligase at C4 and then were transformed into E.coli DH5a competent cell with chemical methods. From clones grown in LB agar containing 0/1 Ampicillin

mg/ml several clones randomly selected and Clony-PCR was performed. Positive clones containing pUCptx-s1)) cultivation and extraction of plasmids were carried out and confirmed by enzyme digestion.

Targeted mutations in the ptx-s1: The primer was designed based on the change of amino acid arginine to lysine 9 (R9K) and glutamic acid to glycine 129 (E129G) using application software GeneArt® and then by using Gene Runner, Primer premier softwares secondary structures and temperatures suitable for replication were assessed (table 2). Modified nucleotides are shown highlighted. PUCptx-s1 plasmid was used as template in the first round using specific primers and plasmid mutants R9K do was transformed into E.coli DH5a to create the second jump after confirming this plasmid as a template using primers E129G to react proliferation and then was transformed into bacteria. Screening of clones containing pUCmptx-s1 was performed by enzymatic digestion and sequencing

Table 1. Primer sequences, concentration and temperature program of polymerase chain reaction for amplification of 1ptx-s

Primer	Sequence			
forward PTX-S1-FKN (KpnI,NdeI)	5 TTGGTACCACATATGCGTTGCACTCGGGC 3			
Reverse: PTX-S1-RB (BamHI)	5 CCGGATCCTAGAACGAATACGCGATGCTTTC 3			
The amount of material needed for polymerase chain reaction		temperature program of polymerase chain reaction		
Name of Material	The amount of material needed	Step	Temperature	Time
10X Buffer	2.5 µl	Initial denaturation	95°c	7 min
dNTP (20mM)	2µl	Denaturation	95°c	1 min
Primer F (10pmol)	0.6µl	Annealing	68.1°c	1 min
Primer R(10pmol)	0.6µl	Extension	72°c	1 min
DNA Template	1µl	Final extension	72°c	10min
Pfu Polymerase (2.5U/µl)	1µl	Total cycle : 30		
Water purified	Up to 25µl			

Table 2. The sequence of primers with mutation, concentration and temperature program of polymerase chain reaction for amplification of pUCmptx-s1

Mutagenic primers	The nucleotide sequence			
PTX-S1F: R9K	5´ CCGCCACCGTATACAAGTATGACTCCCGCCC3			
PTX-S1 R: R9K	5´GGGCGGGAGTCATACTTGTATACGGTGGCGG3´			
PTX-S1F: E129G	5´ CCACCTACCAGAGCGGG TATCTGGCACACCGG3			
PTX-S1R: E129G	5´ CCGGTGTGCCAGATACCCGCTCTGGTAGGTGG3			
The amount of material needed for polymerase chain reaction		temperature program of polymerase chain reaction		
Name of Material	The amount of material needed	Step	Temperature	Time
10X Buffer	2.5 µl	Ptx-primer 9		
dNTP	2µl	Initial denaturation	95°c	7 min
Primer F(10pmol)	1µl	Denaturation	95°c	1 min
Primer R(10pmol)	1µl	Annealing	64.1°c	1 min
DNA Template	1.5µl	Extension	72°c	9 min
Pfu Polymerase(2.5U/µl)	1µl	Final extension	72°c	10 min
Purified water	Up to 25µl	Total cycle : 30		

Cloning of mptx-s1 fragment in expression vector of pET15b: With enzymatic digestion of NdeI/BamHI plasmid was approved (pUCmptx-s1). mutated fragment was exited from the plasmid and after extraction from the gel in the enzymatic cutting site of NdeI / BamHI of expression vector N-terminal His-tag) pET15b 5708 bp) was cloned. Verifying of cloning was harvested by enzymatic digestion and amplification.

Expression of Mptx-s1, confirm and purification of recombinant protein: Expression of protein in bacteria was performed by inducing bacteria at a

volume of 5ml and concentration of 0.5 mM IPTG 5ml in optical density of 8.5/7 in 37°C in the culture medium of TY2x. Five hours after induction, cells were collected and protein expression by SDS-PAGE and Immunoblotting with Anti His Antibody as the primary antibody and HRP- conjugated antibody and substrate 3'- Diaminobenzidine (DAB) was performed. Purification by affinity chromatography Ni-NTA (Qiagene) and nickel ions adsorbed protein by using 8M urea and gradient pH (8 pH of the lysis solution, 5/6 pH cleaning solution and 5/5 pH of the final solution) for and the concentration was determined by spectrophotometry. All the cloning, expression and purification were performed according to published guidelines (19).

Results

DNA extracted from the native B. pertussis strains led to the preparation of 356 ng/ml genomic content. Ptx-s1 fragment contains 269 amino acid that its amplification results in to the creation of the piece which is 810 bp. In the first stage fragment was amplified and cloned in the puc18 vector based on mentioned details in the method. The result (Fig1) indicates the correct amplified fragment and clone approval of the relevant vector.

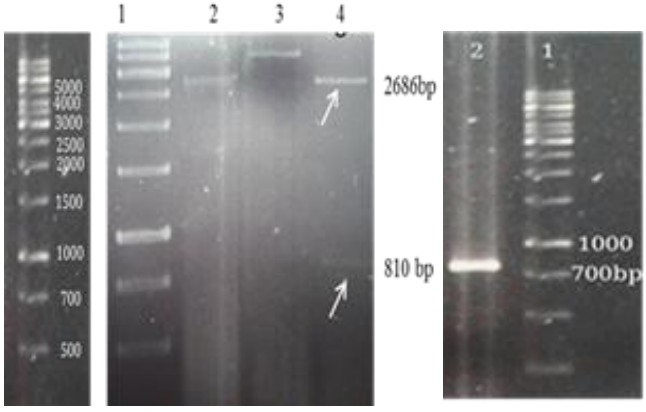

Figure 1. Amplification of ptx-s1 and digestion of pUCptx-s1.

Left: Column 1 DNA marker and column 2 bands belong to the PTX-S1 810 bp on the right: Column 1 DNA marker, columns 2 and 3 vectors pUC18 linearized with the enzyme BamHI did not have fragment (2686 bp (and a fragment (3496 bp) and column 4 digestion with two enzymes KpnI / BamHI fragment was cloned in and out. the location is marked with an arrow placement.

Mutation and approved pUCmptx-s: pUCptx-s1 as a template and use of mutagenic primers for mutagenesis targeted in positions 9 and 129 resulted in formation of a bond with size of about 3500 bp (total base pairs of vector and cloned fragment was 2686+810) and plasmid containing mutant fragment pUCmptx-s was obtained (Fig 2, part a). The nucleotide sequencing was approved the accuracy of targeted mutations. graphs of sequencing by examining them with Chromas software confirms the replacement of arginine (CGC) position 9 to lysine (AAG) R9K and glutamic acid (GAA) position 129 with glycine E129G (GGG) (Fig 2 part B and C). After confirming the mutations and digestion, pUCmptx-s1 was cloned with enzymes BamHI and NdeI in equivalent positions in the expression vector of pET15b. Cloning checking using digestion with BamHI/NdeI and exit of 810 bp fragment was confirmed the authenticity of cloning (Fig 2 part D).

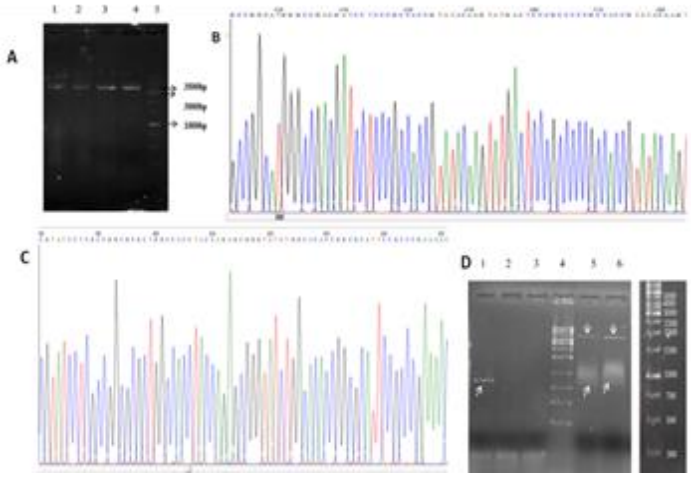

Figure 2. Mutations and confirmation of pUCmptx-s and confirmation of the pETmptx-s1 expression vector.

Part A: Results of amplification of pUCptx-s1 vector using R9K primers, columns 1 and 2 indicated the first stage and columns 3 and 4 indicates results of amplification with E129G primers in the second round that at both the fragment of about 3500 bp can be seen, Column 5 DNA 1Kb. Part B: Confirmation of mutation of arginine (CGC) position 9 to lysine (AAG) R9K 150-153 nucleotides. Part C: Confirmation of mutation at position 129 glutamic acid (GAA) with glycine E129G (GGG) 616-618 nucleotides. Part D: Confirmation of pETmptx-s1. Column 1 results of gene amplification with pETmptx-s1 vector and presence of 810 base pairs fragment , columns 2 and 3 negative control, column 4 DNA 1Kb, column 5 and 6 results of enzymatic digestion of vector containing the pETptx-s1 with the enzymes NdeI/BamHI and presence of the 810 and 5708 bp fragments of the vector. Placement locations marked with arrows.

Expression of recombinant MPTX-S1 protein: After confirmation of cloning of MPTX-S1 in expression vector, amplified vector after extraction and purification of the proliferating bacteria E.coli DH5a was transformed into the expression host E.coli BL21. 28 (Fig 3). Expression in different concentrations of IPTG and culture showed that IPTG with a concentration of 5.0 mM and induction in the optical density of 7.5 to 8 and maintenance of cells in culture

for three to four hours in TY2x medium results in optimal expression of the desired protein with a molecular weight of 28 (Fig. 3).

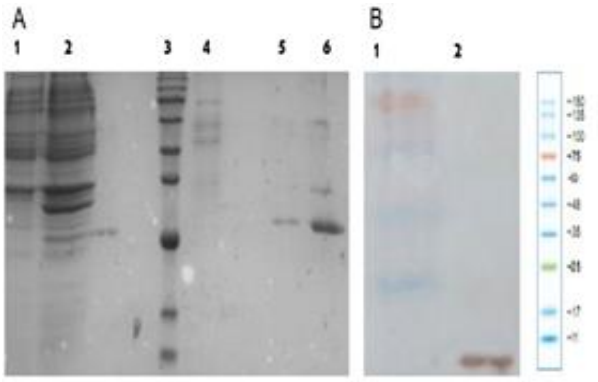

Figure 3. Evaluation of the expression and purification of mPTX-S1 and its confirmation using immunoblotting

Part A columns 1: bacterial protein content before induction, column 2: bacterial protein content after induction Column 3: protein marker Column 4: protein content in the purified washing solution without band of MPTX-S1. Columns 5 and 6: band of MPTX-S1 with molecular weight of 28 kDa Part B: identification and confirmation of recombinant protein and the mutant MPTX-S1 using immunoblotting: Column 1 protein marker 10 to 170 kDa. Column 2: 28 kDa band of the MPTX-S1 protein

Discussion

This study has been successfully made gene structures of inactive form of bacterial toxin of B. pertussis. Nucleotide sequence analysis showed the accuracy of mutations in the active site of an enzyme toxin is considered. Protein expression in E.coli BL21-pET15b represents an appropriate choice of expression system and host structures mutant recombinant expression in prokaryotic system maintenance and mass production in future studies to have delivered the toxin.

Optimization of the purification method allows early purification by affinity chromatography. Vaccination against pertussis has an old history and studies suggest increased risk, especially in populations younger than 6 months and more than 10 years, and there are several reasons such as decreased immunity after vaccination with time, flaws in immunization compatibility pathogens (20). For the compatibility of pathogens, the best known example is the emergence of resistant strains after prolonged use of antibiotics and less-known compatibility of pathogens is vaccinations (20, 13). In other words, due to antigenic changes, different immune responses will not be able to control and destruction of microorganisms (21, 15).

About Bordetella pertussis genetic changes in bacteria virulence factors, including fha, ptx, prn, fim leads to the emergence of multiple alleles of these genes (6). Several studies has shown a direct connection between the polymorphism and recurrent of whooping cough disease in different parts of the world (15, 4). In Iran, according to the World Health Organization WHO, the incidence of pertussis in 2004, 98 in 2005, 1250 and 2011, 650 cases have been reported (17, 16). But in recent years in Iran, like other countries, the incidence of whooping cough patient is observed that one of the main reasons can be genetically modified strains in circulation. Studies conducted by the Reference Laboratory of whooping cough in Pasteur Institute of Microbiology confirming these investigations (16).

In fact, using a standard non-native species for more than half a century has led to ineffective vaccination program against whooping cough. Despite the side effects of vaccination, population tolerance of impunity is also not satisfactory. The new generation of vaccines in controlling pertussis containing one or two and sometimes a combination of Bordetella pertussis antigens, including PTX and FHA and PRN is important that these types in some developing countries have been replaced by cell vaccines (6).

In this study, by use of native strains selected based on epidemiological and molecular evaluations, design of new generation vaccines based on recombinant non-cell pertussis toxin have been performed. Studies show that pertussis toxin alone also able to create passive safety which is effective against bacteria. Expressive structures designed and approved can be used as a tool to produce the antigen non-cellular vaccine. Since the native strain of the study was used as a template, therefore the structure of expression and recombinant protein obtained in this study, the possibility of designing of acellular vaccines based on significant and prevalent antigens from Iran has provided for future studies.

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References

1.Guiso N. Bordetella pertussis and pertussis vaccines. Clin Infec Dis. 2009;49(10):1565-9.

2.Rodríguez ME, Hellwig SM, Vidakovics MLP, Berbers GA, Van De Winkel JG. Bordetella pertussis attachment to respiratory epithelial cells can be impaired by fimbriae-specific antibodies. FEMS ImmunolMed Microbiol. 2006;46(1):39-47.

3.Tozzi AE, Celentano LP, degli Atti MLC, Salmaso S. Diagnosis and management of pertussis. Canad Med Associa J. 2005;172(4):509-15.

4.Berbers GA, de Greeff SC, Mooi FR. Improving pertussis vaccination. Human Vaccine. 2009;5(7):497-503.

5.Marzouqi I, Richmond P, Fry S, Wetherall J, Mukkur T. Development of improved vaccines against whooping cough: current status. Hum vaccin. 2010;6(7):543-53.

6.Keitel WA. Cellular and acellular pertussis vaccines in adults. Clin Infect Dis. 1999;28 Suppl 2:S118-23.

7.Locht C. A common vaccination strategy to solve unsolved problems of tuberculosis and pertussis?. Microb Infect. 2008;10(9):1051-6.

8.Cherry J, The epidemiology of pertussis: a comparison of the epidemiology of the disease pertussis with the epidemiology of Bordetella pertussis infection. Pediatrics. 2005 May;115(5):1422-7.

9.Locht C, Coutte L, Mielcarek N. The ins and outs of pertussis toxin. FEBS J. 2011;278(23):4668-82.

10.Hijnen M, Mooi FR, van Gageldonk PG, Hoogerhout P, King AJ, Berbers GA. Epitope structure of the Bordetella pertussis protein P. 69 pertactin, a major vaccine component and protective antigen. Infect Immunit. 2004;72(7):3716-23.

11.Stein PE, Boodhoo A, Armstrong GD, Cockle SA, Klein MH, Read RJ. The crystal structure of pertussis toxin. Structure. 1994;2(1):45-57.

12.Willems RJ, Mooi FR. From whole cell to acellular pertussis vaccines. Rev Med Microbiol. 1996;7(1):13-22.

13.Mooi FR, Van Loo I, Gent Mv, He Q, Bart MJ, Heuvelman KJ, et al. Bordetella pertussis strains with increased toxin production associated with pertussis resurgence. 2009;15(8):

14.He Q, Mertsola J. Factors contributing to pertussis resurgence. Future Microbiol. 2008;3(3):329-39.

15.Mooi F, Van Der Maas N, De Melker H. Pertussis resurgence: waning immunity and pathogen adaptation–two sides of the same coin. Epidemiol Infect. 2014;142(04):685-94.

16.Shahcheraghi F, Lotfi MN, Parzadeh M, Nikbin VS, Shouraj F, Zahraei SM. Isolation of bordetella Pertussis and bordetella parapertussis of clinical specimens from different provinces of Iran. J Mazandaran Univ Med Sci. 2012;22(88):2-8.[In Persian].

17.Izadi M, Afsharpaiman S, Jafari NJ, Ranjbar R, Gooya MM, Sarpooshi JR, et al. Immunization status of Iranian military recruits against Bordetella pertussis infection (whooping cough). J Infect Dev Count. 2011;5(03):224-6.

18.Levinson W, Jawetz E. Medical microbiology and immunology: examination and board review: Appleton & Lange; 1996.Availabe from: http://trove.nla.gov.au/work/6754015

19.Maniatis T, Fritsch EF, Sambrook J. Molecular cloning: a laboratory manual: Cold Spring Harbor Laboratory Cold Spring Harbor, NY; 1982.

20.Cardeñosa N, Romero M, Quesada M, Oviedo M, Carmona G, Codina G, et al. Is the vaccination coverage established enough to control pertussis, or it is a re-emerging disease?. Vaccine. 2009;27(25):3489-91.

21.Gerlach G, von Wintzingerode F, Middendorf B, Gross R. Evolutionary trends in the genus Bordetella. Microbes and Infection. 2001;3(1):61-72.