Original Article

Characterization of a predominant *Bordetella pertussis* strain isolated from Iranian patients

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ABSTRACT

Introduction: Pathogen adaptation is considered as one of the important reasons for the emergence of pertussis (whooping cough). Antigenic divergence between vaccine strains and clinical isolates of *Bordetella pertussis* has been occurred over the years. It is suggested that the predominant genomic profile of *B. pertussis* has an enough capacity to spread among the population. The aim of this study was to characterize a predominant *B. pertussis* strain isolated from Iranian patients during 2008-2015 period. **Methods:** Based on the epidemiologic results of *B. pertussis* circulating strains in Iran, a strain named BPIP91 with predominant genomic and virulence pattern was selected from Biobank of Pasteur Institute of Iran. The antibiotic susceptibility testing was done and the growth rate of this strain was analyzed. The lethal (LD_{50}) and safety dose of infection of BPIP91 was also determined via mice intranasal infection. **Results:** Our results showed that BPIP91 was susceptible to erythromycin, azithromycin, clarithromycin, chloramphenicol, trimethoprim-sulfamethoxazole and rifampin antibiotics. The growth rate of BPIP91 was almost two-fold lower than the vaccine strain. In addition, the LD_{50} and infectious dose of BPIP91 strain were about 2×10^{10} and 4×10^{6} colony forming units, respectively. **Conclusion:** In this study we obtained the growth curve, LD_{50} and intranasal infectious dose of a circulating strain with predominant genomic pattern in Iran. However, further examinations including determination of immunogenicity of this native strain in animal model is needed in order to evaluate its use as a vaccine strain candidate.

KEYWORDS: *Bordetella pertussis*, wP vaccine, Circulating strain, Growth curve, LD_{50}.

INTRODUCTION

Pertussis (also known as whooping cough) is a very contagious respiratory disease that remains as an important life-threatening problem during infancy and childhood [1, 2]. Whole cell (wP) and acellular (aP) vaccines are two kinds of prophylactics against pertussis which are broadly used all around the world. Despite high vaccination coverage, pertussis resurgence has been reported in many developed and developing countries [3] due to reasons such as waning vaccine immunity and pathogen adaptation [4]. The genome of *Bordetella pertussis*, the causative agent of pertussis, has been changed over the years and antigenic divergence between the vaccine strains and the clinical isolates has been reported in many countries even with high vaccination coverage [5-7].

The diversity of virulence factors of *B. pertussis* such as pertussis toxin (PTX), pertactin (PRN), fimbriae (FIM) and filamentous hemagglutinin (FHA) resulted in different alleles among *B. pertussis* population. The antigenic divergence among the circulating pathogens and the vaccine strains might be occurred due to single nucleotide polymorphisms (SNPs). The alleles shifts toward virulence-associated genes has also been documented in many regions [8, 6]. Several different alleles of the virulence factors have been identified so far including 11 alleles of pertussis toxin (*ptxA*), 21 alleles of pertussis toxin promoter (*ptxP*), 17 alleles of pertactin (*prn*), two alleles of fimbriae2 (*fim2*) and six alleles of fimbriae3 (*fim3*) [9]. These changes commonly appear during bacterial genome evolution that increases the adaptation of the bacterial population. Previous studies have shown that genetic diversity is directly or indirectly related to the host immune system and vaccination may result in the evolution of pathogens with non-

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vaccine types of *B. pertussis* antigens. For example, *ptxA* gene polymorphism of *B. pertussis* strains is immunologically relevant to both B and T cell epitopes [6]. Previous studies have also demonstrated that isolates carrying *ptxP3*, the predominant allele in *B. pertussis* stains, show more virulent capacity with increased expression of PTX and PRN than isolates carrying ptxP1 allele (*i.e*. the vaccine allele) [10, 11]. The *ptxP3* lineage with the presence of *ptxP3*, *ptxA1* and *prn2* alleles have gained increased fitness and capacity to spread among the immunized populations [12]. Therefore, it seems that using the predominant antigens of currently circulating strains of *B. pertussis* in wP or aP vaccines formulations, would certainly improve the vaccine efficacy within the communities [13, 14].

The wP vaccine has been used for more than 60 years in Iran as three doses and two boosters with approximately 95% vaccination coverage. However, a pertussis surveillance system has been started recently to monitor pertussis among Iranian populations. For this purpose, nasopharyngeal specimens from the suspected patients are sent to Pertussis Reference Laboratory of Pasteur Institute of Iran to investigate and identify the Bordetella spp. among the samples of circulating *B. pertussis* strains [15-18]. The presence of different allelic profiles of recent circulating strains in Iran compared to the vaccine strains has been reported by Pertussis Reference Laboratory of Pasteur Institute of Iran [19, 17, 20]. The predominant antigenic profile among the isolated strains has been *ptxP3*, *ptxA1*, *prn2*, *fim2-1* and *fim3-2* which were similar to the other studies all over the world like The Netherlands, Tunisia and Mexico [6, 21, 7, 11]. However, genomic pattern analysis of Iranian patients' isolates by pulsed field gel electrophoresis (PFGE) has shown low heterogeneity among the isolates which indicates clonal spread of *B. pertussis* over the past decade. It means dissemination of a clone including approximately 50% of *B. pertussis* isolates in Iran (pattern No. 10). The genomic pattern of this unique clone was similar to that of the most common profile of BpSR11 clone in European countries [22].

Since *B. pertussis* evolves by time, recent studies have suggested that the use of circulating strains in improved wP or aP vaccines might be an appropriate mean to decrease and eventually eradicate *B. pertussis* from the globe [23, 24, 13]. It is also envisaged that data gathered from monitoring and characterization of *B. pertussis* circulating isolates would lead to a new generation of wP vaccines with better efficacy than today's vaccines. Accordingly, considering that Iran is a country with high vaccination coverage, here our aim was to characterize a dominant circulating strain of *B. pertussis* in Iran, called "BPIP91", which belongs to the foremost PFGE pattern containing about 50% of *B. pertussis* strains with predominant alleles of virulence factors. This PFGE profile was collected from different provinces of Iran and the growth rate, the lethal dose and the safety dose of strain BPIP91 were evaluated. Altogether, it seems that the strains showing this profile have enough capacity to infect and spread throughout the country.

MATERIALS and METHODS

Strain selection

B. pertussis BPIP91 was selected as the dominant circulating strain in Iran, based on a previous study on 100 circulating strains of *B. pertussis* from Pertussis Reference Laboratory of Pasteur Institute of Iran, isolated during 2008-2015 [15]. Based on a genomic analysis of *B. pertussis* strains, BPIP91 strain was located in the cluster of predominant PFGE pattern (No. 10) and was isolated in 2012 from a 4-month- old female baby. The virulence allele profile of this strain was *ptxP3*, *ptxA1*, *prn2*, fim2-1, fim3-2 [15] which is a common profile in Iran.

Culture and media

In order to characterize BPIP91 strain, it was revived by sub-culturing on Regan-Lowe (RG) agar (Difco) containing 10% defibrinated sheep blood. After 72 h incubation, the colonies were collected and assessed with biochemical tests and agglutination by *B. pertussis* antiserum (Difco). Molecular analysis of the strain was performed by Real-Time PCR (Light cycler) for the presence of *IS481*, *IS1002* and ptxP targets in the genome [17].

Antibiotic susceptibility test

Antibiotic susceptibility tests of BPIP91 were performed using the disks of erythromycin (15 µg), azithromycin (15 µg), clarithromycin (15 µg), chloramphenicol (30 µg), trimethoprim-sulfamethoxazole (25 µg) and rifampin (5 µg) (MAST, Bootle, United Kingdom). A bacterial suspension in PBS equal to 0.5 McFarland turbidity standard was grown on RG agar and analyzed after 72 h incubation at 35ºC [25].

Growth rate determination

The growth of *B. pertussis* BPIP91 strain compared to *B. pertussis* 134 (the vaccine strain) were plotted based on the optical density (OD) at 600 nm in Stainer-Scholte (SS) broth medium as described previously [26]. A loopful of fresh hemolytic (Bvg+) colonies from Bordet-Gengou (BG) agar, supplemented with 10% defibrinated sheep blood were inoculated in SS medium [27]. After 16 h incubation at 35ºC and 150 rpm, the OD^{600} was adjusted to 0.05 and were inoculated to a fresh sterile SS media. Then, OD^{600} of the samples were recorded continuously for 70 h. Each sample was tested in triplicate culture.

In vivo determination of 50% lethal dose of infection (LD_{50})

For determination of the colony forming unit (CFU) of BPIP91, this strain was cultivated on RG agar. Following 72 h incubation at 35ºC, the Bvg+ colonies were suspended in PBS and adjusted to OD = 1 at 600 nm. A tenfold serial dilution was cultured in triplicates on RG agar and after 4 days of incubation at 35-37 ºC, the colonies were counted to determine the CFUs at turbidity of OD = 1.

The 50% lethal dose (LD_{50}) in mice was determined using female BALB/c mice (8 weeks old). All the procedures involving animals were conducted under Pasteur Institute of Iran's Ethics Committee under project approval number TP-9127. Briefly, the mice were intranasally infected by approximately 10^8, 10^9 and 10^{10} bacteria in 30 µl PBS, according to the previous colony counting on RG agar under aseptic conditions. Mice survival was recorded daily, up to one week.

Toxicity mouse weight gain test (MWGT)

The toxicity due to BPIP91 strain was assessed by mouse weight gain test (MWGT), according to the WHO guidelines [28]. Briefly, 6 groups of mice (10 mice per group) were anaesthetized with intraperitoneal injection of 120 µl of a solution containing 200 mg/kg ketamine and 100 mg/kg xylazine. The mice were then intranasally dropped with a sub-lethal dose of a bacterial suspension (10^5, 10^6, 10^7, 10^8, 10^9) of BPIP91 in 30 µl PBS and PBS as negative control. The mice weights were daily measured and recorded up to a week (Table 1). The weight gain average was compared with the control

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group at the end of the 7th day which should not be less than 60% of the control group, based on the WHO guidelines.

Table 1. The *in vivo* toxicity test (MWGT). The average weight gain per mouse was determined after day 3 and day 7 of the intranasal infection.

Dose of infection (CFU)	Average weight gain per mouse (g)		
	Day 3	Day 7	Weight gain compared to the control (%)
~10^5	0.7	0.9	72.5
~10^6	0.8	0.9	72.5
~10^7	0.1	0.5	38.5
~10^8	-1.2	-0.39	-31.5
~10^9	-1.2	-0.38	-30.6
PBS	0.5	1.24	-

RESULTS

B. pertussis strain BPIP91 was sub-cultured and confirmed by biochemical and molecular diagnostic tests. The *IS481*, *IS1002* and *ptxP* targets in the genome were examined by PCR to confirm the molecular profile. The virulence allele profile of this strain was *ptxP3*, *ptxA1*, *prn2*, *fim2-1* and *fim3-2*.

Antibiotic susceptibility test

Antibiotic susceptibility testing showed that this strain was susceptible to all 5 studied antibiotics and no resistance was observed.

Growth curve

In vitro liquid culture of BPIP91 strain showed a lower growth rate, compared to B. pertussis 134 (the vaccine strain). However, the growth profiles of BPIP91 and B. pertussis 134 were similar which indicated that the growth kinetics of *B. pertussis* rose to exponential and stationary phases, after approximately 14 and 40 h, respectively (Fig. 1).

***In vivo* LD_{50}**

The results of the colony counting for BPIP91 strain on RG agar showed that OD = 1 contains approximately 4×10^9 CFU/ml in PBS. According to our results, from 10 mice that were infected in each group, only in 1 group (2×10^{10} CFU) 6 mice died after 72 h of the infection. Thus, the 50% lethal dose of the infection was determined as approximately 2×10^{10} CFU.

MWGT results

The toxicity test results of BPIP91 strain in the tested mice demonstrated that this strain was infectious in sub-lethal dose and was toxic at the concentrations of about 10^9, 10^8 and 10^7 CFU. However, the concentrations of 106 and 105 CFU did not show any weight-loss within 7 days, based on the WHO criteria for MWGT [28]. According to Table 1, these observations showed that the safety dose of BPIP91 strain was about 4×10^6 CFU and therefore this number of bacteria were reasonable for further mouse examination.

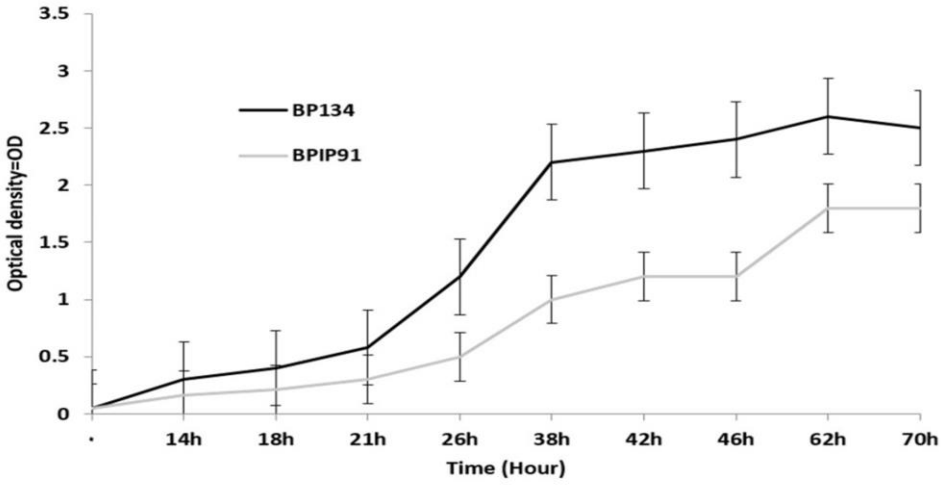


Fig. 1. The growth curve of the native circulating strain BPIP91 and vaccine strain *B. pertussis* 134 in the same experiment. Inoculations were done in triplicate and the OD was recorded at 600 nm.

DISCUSSION

By studying the circulating strains of *B. pertussis* in the community from 2008 to 2015, we found strains with the predominant genomic and antigenic profile which were isolated from about 50% of the Iranian patients [15]. This antigenic profile has also spread broadly in different provinces in Iran and was even found after 2015 (data not published). Interestingly, this has also been observed as a predominant circulating profile in other parts of the world, especially in the European countries [22]. This finding encouraged us to characterize BPIP91 as a typical strain of this profile among Iranian populations by *in vitro* and *in vivo* experiments, in order to find its antibiotic susceptibility, *in vitro* growth rate, infectious, lethal and safety doses.

Based on the antibiotic susceptibility testing, BPIP91 strain was a drug sensitive strain that was susceptible to all the antibiotics examined in this study which are routinely used against pertussis infection. In pertussis cases, the use of antibiotics is to prevent dissemination of this contagious respiratory illness. The antimicrobial treatments prevent the progression of the disease and reduce the severity of the associated symptoms. Furthermore, antimicrobial therapies decrease person-to-person spread of most pathogens. The results of this study showed that

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fortunately, the infection with this predominant circulating strain can be easily treated with antibiotic therapy.

The optical density at 600 nm started to increase, 12–14 hours after the incubation (exponential phase) and reached to the highest level after 60 hours and then entered to the stationary phase. The comparison of the growth curve for BPIP91 and the vaccine strain (*i.e*. *B. pertussis* 134) revealed that the growth pattern was the same for both; however, the growth rate of BPIP91 was approximately two-fold lower than *B. pertussis* 134. As many factors affect the growth of *B. pertussis* [29], it is clear that the vaccine strains have been fully characterized to be used in the vaccine combinations. As for the content of wP vaccines, they are standardized to Opacity Units using a WHO reference preparation (IU) [30].

In this study, Balb/C mice were infected intranasally to determine 50% lethal dose and also safety dose of infection with BPIP91, as a predominant circulating strain isolated from Iranian patients. The result of LD_{50} determination indicated that BPIP91 strain was able to cause a lethal infection at a dose of approximately 2×10^{10} CFU. This lethal dose was obviously higher than the previously observed doses for other standard strains, such as Tohama I and BP 18323 [31] or the clinical strains examined in Belgrade [32]. Our study also showed that the selected strain can infect the mice at the concentration of 4×10^{6} CFU in the safety dose, according to the WHO guidelines. This means that this number of bacteria can infect the mice without any weight loss or sever toxicity. These findings indicate that safety dose of the infection by this strain is the same as *B. pertussis* mutant strain BPZE1 [33] and this strain is less virulent than the challenge strain, BP 18323 [31]. Since *B. pertussis* is a human exclusive pathogen, often large infectious doses are required to colonize the animals, compared to humans [34].

According to *B. pertussis* mutant strains studies, it has been shown that *B. pertussis* cannot be lethal without pertussis toxin and adenylate toxin production which are the two main virulence factors of this bacterium [31]. Obviously, BPIP91 circulating strain in Iran has the main virulent and invasive factors; however, further studies are needed in order to confirm the presence of other important antigens of *B. pertussis* in the dominant strain. A lethal infection by *B. pertussis* needs the interaction of several virulence factors. In human, after attaching to the respiratory tract, the bacteria grow, evade the host defense, and finally produce enough toxin(s) to damage the host. We determined the lethal infection dose on the experimental mice as a convenient model, although some of the aspects of this system differ from the human form of the disease.

In conclusion, *B. pertussis* is evolving and the recent expansion of clones, carrying variants of genes encoding *B. pertussis* antigens in response to the vaccination pressure has occurred [35]. The predominant *B. pertussis* circulating strain in Iran is invasive and has a potential to be further studied for the future vaccine combination plans. The presence of the required antigens in a strain in one hand and the human immune response against a pertussis vaccine, on the other hand, make it necessary to fully characterize the strains used in the vaccine combinations. Next generation sequencing (NGS) has been conducted recently in our laboratory to fully characterize the genome of the selected BPIP91 strain. It would be suggested that such studies be expanded and repeated with more predominant *B. pertussis* strains.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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