ABSTRACT

Background and Objectives: The re-emergence of pertussis still is being reported all over the world. Pathogen adaptation and antigenic divergence of circulating isolates from vaccine strains are the main reasons of infection resurgence. Waning immunity is also an important factor contributing to resurgence of pertussis.

Materials and Methods: The genetic diversity and evolutionary characteristics of circulating Iranian isolates of *Bordetella pertussis* during February 2015 to October 2018 was investigated by pulsed-field gel electrophoresis (PFGE) and subsequently *ptxA*, *ptxP* and *fim3* alleles were characterized. The next generation genome sequencing was then used to compare the genomics of *ptxP1* and *ptxP3* of selected isolates from PFGE dendrogram.

Results: PFGE differentiated 62 clinical isolates and vaccine and reference strains into 19 PFGE profiles, indicating the higher level of heterogeneity in the population during 2015-2018. The predominant *B. pertussis* genotype harbored pertussis toxin promoter allele, *ptxP3* and the expansion of *ptxA1* isolates, were also observed in our population.

Conclusion: No changes in allelic profile of predominant clone in recent years was observed but antigenic divergence between recently circulating isolates and the vaccine strain has been progressed and significantly was higher than previous studies. The comparative genomic analysis of the *ptxP3* and *ptxP1* isolates indicate that changes in *ptxP3* genome structure including 32 unique SNPs and three unique indels may have contributed to the expansion of the *ptxP3* clone. We compared *ptxP3* and *ptxP1* isolates in pathogenicity-associated genes and found five of them were specific for the *ptxP3* isolates. The polymorphisms in pathogenicity-associated genes suggest structural adaptations for these virulence factors.

Keywords: *Bordetella pertussis*; Genome diversity; Pulsed-field gel electrophoresis; Whole genome sequencing
fants, who are too young to be vaccinated or are not yet fully vaccinated (1).

Introduction of whole cell vaccine (WCV) during the 1950s significantly reduced the morbidity and mortality of pertussis. However, due to the side effect of WCV, acellular vaccine (ACV) was developed in the 1980s (2). Although pertussis is relatively well controlled by extensive vaccination programs, re-emergence of pertussis has been reported in many countries with high vaccination coverage, including the European countries, the United States and Australia (3-6). Pathogen adaptation and antigenic divergence of circulating isolates from vaccine strains are the main reasons of infection resurgence. Waning immunity is also an important factor contributing to resurgence of pertussis (7, 8).

While a vast majority of developed countries switched from WCV to ACV, whole-cell-based combination vaccines is still in use in developing countries (9). In Iran, the whole-cell pertussis vaccine was introduced for children in 1950s and continued until now. Children are immunized with three doses of diphtheria–tetanus, whole-cell pertussis (DTwP) vaccine at 2, 4 and 6 month. Then, young children receive two booster to maintain that protection through in 18 month and 6-years old (10). Despite high pertussis vaccination coverage in Iran, (96% since 2000) pertussis incidence is still the highest amongst all vaccine-preventable diseases. Our population has experienced pertussis resurgence since 2007 and reached its peak in 2012 and 2013 (11).

Recently, antigenic divergence has been reported in different countries. Molecular studies are important for monitoring the spread of antigenic divergence between clinical isolates and vaccine strain (12). B. pertussis has conventionally typed by pulsed-field gel electrophoresis (PFGE). This method has been frequently used worldwide to provide laboratory data for characterization of B. pertussis isolates (13). PFGE achieves some level of resolution that resulted in the high structural dynamics of B. pertussis genome (14, 15). On the other hand, antigenic divergence of B. pertussis can be easily achieved by point mutation. Genotyping of pathogenicity-associated genes is very precious to find the polymorphism of these genes. Comparative analysis of B. pertussis genomes by whole genome sequencing is important to study B. pertussis population diversity (16, 17).

The aims of this study, was to investigate the genetic diversity of recent circulating clinical B. pertussis isolates, using PFGE and genotyping of pathogenicity-associated genes during February 2015 to October 2018. Finally, we selected three ptxP1 isolates and three ptxP3 isolates from PFGE dendrogram for comparative genomics of ptxP1 and ptxP3 isolates, to know the cause of the ptxP3 isolates outbreak in our population using next generation genome sequencing.

MATERIALS AND METHODS

**Bacterial isolates.** In the current study, 62 clinical isolates were obtained from Pertussis Reference Laboratory of Pasteur Institute of Iran during February 2015 to October 2018. Isolates collected from different province of Iran including Tehran, Esfahan, Khorasan, East and West Azarbaijan, Khuzestan. Bp134, Bp509 as vaccine isolates and Bp18323 (ATCC 9797), Tohama I as reference strains, were used for validation.

Bacterial isolates were re-cultured on Regan-Lowe medium containing charcoal agar and 10% defibrinated sheep blood, and incubated at 37 °C for 72 h. B. pertussis isolates were confirmed by a combination of colony morphology, growth rate, Gram stain and conventional biochemical tests such as oxidase and use of specific Bordetella antiserum (Difco B. pertussis Antiserum, Rabbit serum for slide agglutination). DNA extraction was performed using High Pure PCR Template Preparation Kit (Roche Diagnostics GmbH, Mannheim, Germany). Real-time PCR was performed by targeting IS481, ptxP, IS1001 and IS1002 for species confirmation of B. pertussis. Primers and probes sequences are available in Table 1 (18).

**Genotypic analysis.** The polymorphism of pathogenicity-associated genes including ptxA, ptxP and fim3 was determined by DNA sequencing. PCR was performed with specific primers as described previously (19-22). PCR products were purified and sent for Sanger sequencing (Macrogen, South Korea). The sequences were read by using MEGA5 software in conjunction with reference strains Tohama I (23).

**Pulsed-field gel electrophoresis.** PFGE profiles were obtained using the XbaI enzyme (Fermentas, ABI, and Germany). The restriction fragments were separated using a CHEF-DRIII system (Bio-Rad), with initial switch time: 5s, final switch: 45 s and run time: 21 h. Fragments patterns were analyzed with
Table 1. Primers and probes used in real-time PCR for amplification of the \textit{ptxP} and insertion sequences IS481, IS1001, IS1002.

| Gene     | Primer sequences                                                                 |
|----------|-------------------------------------------------------------------------------|
| \textit{ptxP} | PT1a: 5’-GCA TGC GTG CAG ATT CGT C-3’                                |
|          | PT2a: 5’-CTC TGC TGT TTT ATG GTG CCT AT -3’                                |
|          | PT-FAM: 6FAM-AAT CCA ACA CGG CAT GAA CGC TCC TTC--BHQ2                        |
| IS481    | IS481-F 5’-ATAAGACCAGCTTTACC 3’                                            |
|          | IS481-R 5’-TGGGAGTTCTGGTAGGTGTG 3’                                           |
|          | IS481-FAM: 6FAM-AATGGCAAGGCCAAGCTTCA BHQ1                                    |
| IS1001   | IS1001-F 5’-CCA GAG CCG TTT GAG TTC GT 3’                                    |
|          | IS1001-R 5’-AAT TGC TGC AAG CCA ACC A 3’                                     |
|          | IS1001-CY5: CY5-ACA TAG ACC GTCAAGCAG-BHQ-3 3’                               |
| IS1002   | IS1002-F 5’-CTA GGT CGA GCC CTT GTT AAC 3’                                   |
|          | IS1002-R 5’-GCC GCC AAG CCA CTT GTA 3’                                       |
|          | IS1002-FAM: 6FAM-CTA CGT CCA GTT CTG TTG CAT CAC CC-BHQ                      |

Gel Compare II software version 4 (Applied Maths, Belgium). \textit{Salmonella enterica} serotype Braenderup strain H9812 was used as size marker. Dendrogram was drawn by unweighted-pair group method using the arithmetic average algorithm (UPGMA) with 2% band tolerance and 2% optimization settings with the Dice coefficient.

**Bacterial selection and whole genome sequencing.** Six isolates were selected based on the hospitalized patients, mortality and the results of PFGE dendrogram in present and our previous study (24). We choose three \textit{ptxP1} isolates and three \textit{ptxP3} isolates for comparative genomics analysis. Details of clinical isolates are shown in Table 3.

Genomic DNA was extracted and purified from pure culture using phenol-chloroform method (25). DNA libraries were constructed using Nextera XT kit (Illumina, San Diego, USA) according to manufacturer’s protocol and sequenced on the Illumina NextSeq instrument using 2 × 150 bp paired-end protocol. The contigs were aligned to the reference \textit{B. pertussis} strain Tohama I (Gene Bank accession number BX470248) using progressive Mauve (version 2.3.1) and the de novo assembly of the raw reads were performed using SPAdes version 3.13.0 (26, 27). To extract Single nucleotide polymorphism (SNPs) a combination of Burrows-Wheeler Alignment (BWA) tools (version 0.7.5), Samtools (version 0.1.19) and progressive Mauve (28, 29) were used. Briefly, the filtered SNPs from mapping were compared to the SNPs exported by progressive Mauve and final SNPs were selected for further analysis. Distribution of short insertions/deletions (indels), which are less than 100 bp, were also identified using SAMtools. The nucleotide sequences have been deposited under bio sample accession number PRJNA600651. The version described in this paper is the first version.

**RESULTS**

**Clinical samples characterization.** In this research, a total of 62 currently circulating Iranian \textit{B. pertussis} isolates were selected based on year and state of isolation focusing on the states with more isolates including Tehran, Azarbaijan, Khorasan and Mazandaran. Around half of the isolates were collected from infants younger than 3 months of age 56% (n=35). The general characteristics of \textit{Bordetella pertussis} cases are shown in Table 2. Twenty-five (40%) of the children (under 3 month) were unvaccinated and 34 (55%) were vaccinated. The results

| Characteristic | Total patients N | (%) |
|---------------|------------------|-----|
| Sex distribution |                  |     |
| Female        | 35               | 56% |
| Male          | 27               | 44% |
| Age           |                  |     |
| < 3 month     | 35               | 56% |
| > 3 month     | 27               | 44% |
of the real time PCR showed the presence of ptxP, IS481, IS1002 among pertussis isolates. IS1001 was not detected among 62 clinical isolates.

**Genotypic analysis.** The pertussis-toxin subunit A (ptxA), fim3 and pertussis toxin promoter (ptxP) were genotyped. Four ptxA (ptxA1, ptxA2 and ptxA4, ptxA5), four ptxP (ptxP1, ptxP2, ptxP3 and ptxP4) three fim3 (fim3-1, fim3-2, fim3-3) alleles were detected among clinical isolates, vaccine isolates and the reference genomes (Bp134, Bp509, Bp18323, Tohama I). The frequencies of the alleles were analyzed the reference genomes (Bp134, Bp509, Bp18323, Tohama I). The frequencies of the alleles were analyzed in our phylogenetic tree (Fig. 1). Results revealed that ptxP3 allele were predominant among local isolates with frequencies of 90.3% (n=56) and it has been a significant expansion compared to years before 2015. ptxA1 allele was predominant with frequencies of 96.6% (n=60). Data on fim3 sequencing showed that, the predominant allele is fim3-2 with frequencies of 83.8% among clinical and vaccine isolates. Only one isolate had ptxA2 (IR133) meaning that there is an allelic mismatch between the currently circulating strain and the vaccine strain. Out of the 56 ptxP3 isolates, 52 isolates carried fim3-2. Four isolates were ptxP1 which were further differentiated by ptxA allele into ptxP1/ptxA1/fim3-1 (IR136, IR138, IR146) and ptxP1/ptxA2/fim3-1 (one isolate). ptxA1/ptxP3/ fim3-2 were determined as the predominant allelic profile circulating in Iran (85%) while vaccine seed strain Bp134 showed ptxA2/ptxP1/fim3-1 and vaccine seed isolates Bp509 showed ptxA4/ptxP2/fim3-1 allelic profile.

**PFGE analysis of Iranian B. pertussis isolates from 2015 to 2018.** PFGE was highly discriminatory between our isolates, and identified 19 distinct PFGE profiles, named BP1 – BP19 (Fig. 1). All profiles were closely related to each other and based on similarity higher than 95%, they were classified into 14 major clades (A-N). Four profiles consisted of closely related isolates (BP1 - BP4). The major profile was clade A, which consisted of 41 isolates and characterized by ptxA1/ptxP3/fim3-2. BP2 as a major group among isolates, comprised 31 isolates that classified in clade A. Clade H indicated the same profile as the Iranian vaccine seed strain Bp134 with a little diversity in ptxA antigen (due to point mutation). In clade I, only one isolate, grouped with reference strain BP18323 (9797) with the same PFGE pattern. This strain (strain no. IR142) was isolated in 2015 with the similar allelic profile to B. pertussis 18323 (ptxA5/ptxP4/ fim3-1) and had low similarity with all clinical isolates tested. Finally, we found no correlation between year and state of isolation in PFGE dendrogram.

**Whole genome sequencing and identification of single nucleotide polymorphisms.** In this study, we illustrated comparative genomics of ptxP1 and ptxP3 isolates (Table 3). We calculated an approximate genome size for each isolate using qualimap. The average genome size of our clinical isolates is 3, 885, 151 bp. A total of 366 SNPs was identified, of which 102 SNPs were in intergenic (IG) region and 264 located in genes, while 149 SNPs were non- synonymous (nsSNPs), 115 SNPs were synonymous (ssSNPs).

Twenty SNPs were common in all isolates, among them, eight were intergenic (IG) and 15 located in genes. The nsSNPs located in BP1660 (sphB2), found in all isolates, is an auto transporter and involved in pertussis pathogenicity. PtxP3 isolates differentiated from ptxP1 isolates by 32 unique SNPs, among them seven were intergenic and 24 SNPs were in coding regions (Table 4). There is one ptxP3 isolate (IR175), carrying prn9 allele. Thirteen unique SNPs were detected related to prn9 isolate with ptxA1/ptxP3/fim3-2/fim2-1 antigenic profile. The main nsSNPs mutations located in BP1226 (conserved hypothetical), BP2548 (regulation) and BP3405, nsSNPs in BP3405 (ompQ) were only in prn9 Iranian isolates.

We compared ptxP3 and ptxP1 isolates in pathogenicity-associated genes, we found five of them were specific for the ptxP3 isolates (Fig. 2). Polymorphisms were found in pathogenicity-associated genes suggesting structural adaptations for these virulence factors.

On the other hand, the SNP density (the number of SNPs per bp of gene in each category) of ptxP3 and ptxP1 isolates was calculated. The overall SNP density of the whole genomes were, 0.0008 SNPs/bp with uses of functional categories defined by Parkhill et al. (30). The highest SNP density were in ribosome constituents (0.002SNPs/bp), phage-related or transposon-related (0.0014SNPs/bp) and regulation (0.0011SNPs/bp). The main SNP density differences between ptxP3 and ptxP1 isolates in cell processes and ribosome constituents that shows in Fig. 3.

Finally, a total of 40 indels were found in our isolates, 24 deletion and 16 insertion. Twenty-four indels were in genes and 16 were intergenic (IG). Among indels located in genes, 19 were frameshifts,
Fig. 1. Classification of the 62 Iranian pertussis isolates, including the two reference and two vaccine strains. Dendrogram analysis of 19 PFGE profiles of *B. pertussis* isolates circulating in Iran during 2015-2018. The unweighted pair group method using arithmetic averages (UPGMA) with 2% band tolerance, 2% optimization settings and cut off 94% was used as the clustering method.
of which 10 resulted from single base pair (bp) indels, two from three bp indels and six each from 4, 7, 8 and 31 bp indels. Two frameshift indels located in BP2232, BP2928 had a stop codon resulting in proteins that were shorter than expected which were considered as pseudogenes (30). Three indels were unique in ptxP3, all as frameshift mutations. BP0880 was a pseudogene encoding for a putative exported protein and BP1054 codes prn precursor that cause prn new allele production (Table 5).

### DISCUSSION

When the first pertussis surveillance system has started monitoring whooping cough in our country, predominant profile expresses ptxP3/ptxA1/fim3-2. In previous study carried out by our team analyzed the allele typing and PFGE profile of clinical B. pertussis isolates collected during 2008-2015. In this study we continued PFGE typing of new isolates collected during 2015-2018. In previous study, Heravi
et al. showed that the majority of current circulating *B. pertussis* isolates in Iran are *ptxP3* strains following the worldwide pattern (24). In the current study, there was no changes in allelic profile of predominant clone in recent years but antigenic divergence between recently circulating isolates and the vaccine strain has progressed and significantly is higher than previous years. We found that almost all circulating

![Fig. 3. Comparative SNP densities between *ptxP3* and *ptxP1* strains based on functional category.](image)

**Table 5.** List of Indels in *PTXP3* isolates

| Gene ID | Position | Name | Function | Deletion/Insertion |
|---------|----------|------|----------|--------------------|
| BP0880  | 919013   | prn  | Putative exported protein | Deletion (frameshift mutation occurs) |
| BP1054  | 1098926  | prn  | Prn precursor | Insertion |
| BP3224  | 3436723  |      | Putative cytochrome oxidase | Insertion |
isolates harbored \textit{ptxP3}, \textit{ptxA1} and \textit{fim3-2} alleles, while our vaccine seed isolates Bp134 shows \textit{ptxA2/ptxP1/fim3-1} allelic profile and vaccine seed isolates Bp509 shows \textit{ptxA4/ptxP2/fim3-1}. Divergences between the vaccine isolates and PFGE predominant clade are clear and may have effect on vaccine efficacy.

In the present study, we used PFGE, as a tool to monitor the pertussis population. Nineteen distinct PFGE profiles from phylogenetic analysis of Iranian pertussis population indicated the higher level of heterogeneity in the population during 2015-2018. Some PFGE patterns of currently predominant \textit{B. pertussis} clones were found within PFGE groups BP2 that classified in clade A, this predominant clonal type represent a single profile identical to the European profile BpSR11, which is part of PFGE group IV (31, 32). This profile comprised 10 to 50% of the isolates, from all European countries (except Poland) and characterized to possess \textit{ptxA1/ptxP3/fim3-2} (32-34).

Similar PFGE profiles were observed in Canada with 82% of the isolates represented, \textit{ptxA1/ptxP3/fim3-2} and belonged to PFGE group IV (35). In these countries vaccinations with WCV were started 50 to 60 years ago and switched to ACV 10 to 20 couple of years ago (32, 35, 36). Iranian predominant profiles are different from other countries using whole cell vaccines such as Poland. The replacement of the \textit{B. pertussis} population in Iran by Clade A with \textit{ptxP3/ptxA1} profile, where only WCV has been used, suggests that the expansion of \textit{ptxP3} strains was not driven by ACV selection alone. In Poland where WCV has also been in use for decades, in 2000-2013, \textit{ptxP1} isolates predominated with 77.5%. However, in a recent study in Poland during 2010-2016, 61.5% of the isolates were found to be \textit{ptxP3} and were attributed to the increasing use of ACV since 2013 for primary immunization. (37, 38). We conclude that, the rate of predominant allelic profiles not necessarily related to the type of population vaccine.

In the last ten years two \textit{ptxP} alleles, \textit{ptxP1} and \textit{ptxP3}, were predominated among the Iranian populations of \textit{B. pertussis}. The \textit{ptxP3} isolates were observed in 2005 for the first time and then gradually increased in frequency and now in recent years has been replaced by \textit{ptxP1} isolates, while we had only 16.7% \textit{ptxP1} among our isolates. Predominant \textit{ptxP3} profile appears to have infection-associated biologic characteristics such as higher Ptx production and enhanced respiratory colonization which contributed to the pertussis epidemic that differ from those of \textit{ptxP1} isolates (21, 39, 40).

Recently, whole-genome sequencing has been used to identify genetic changes in the \textit{B. pertussis} population. For better understanding of \textit{ptxP3} and \textit{ptxP1} genomic diversity, we compared whole genome sequences of \textit{ptxP3} genomes and \textit{ptxP1} isolates. We confirmed that \textit{ptxP3} isolates differed from \textit{ptxP1} isolates in genomics. There are 32 unique SNPs that differentiated the \textit{ptxP3} isolates from \textit{ptxP1} isolates. The most important mutations in the virulence genes unique for \textit{ptxP3} strains that occurs in \textit{prn}, \textit{ptxC} and \textit{fim3}, responsible for changes in alleles of the genes encoding vaccine antigens and may be the cause of \textit{ptxP3} lineage expansion in our population. Our results showed that \textit{ptxP3} genomic profile is similar to those collected worldwild due to the better genome fitness of \textit{ptxP3} isolates, the most important mutations in the virulence genes unique for \textit{ptxP3} isolates that occurs in \textit{sphB1}, \textit{prn}, \textit{ptxC}, \textit{bseI} and \textit{fim3}, responsible for changes in alleles of the genes encoding vaccine antigens and may be the cause of \textit{ptxP3} lineage expansion (40, 41). BP2249 (\textit{bseI}), BP1568 (\textit{fim3}), that maybe adaptive and due to the emergence of a new allele, nSNPs in \textit{fim3} was involved in antigenic shift from \textit{fim3-1} to \textit{fim3-2} (5).

On the other hand, \textit{ptxP1}, contains isolates which represent \textit{ptxA1/ptxP1} and \textit{ptxA2/ptxP1}. \textit{ptxA1/ptxP1} was distinguished by 35 unique SNPs from \textit{ptxA2/ptxP1} isolates. The main non-synonymous mutations found in \textit{ptxP1/ptxA1} profile located in BP3812 and BP3869 are involved in cell surface, BP1568 encoding \textit{fim3} involve in pathogenicity, and BP3137, putative two-component system sensor protein involved in regulation.

In conclusion, currently in Iran the surveillance system is affected by many limitations. The underestimation of pertussis in adolescents and adults is mainly related to the atypical clinical characteristics of cases and lack of lab confirmation, the age group is considered as the reservoir of infection. Epidemiological results of sequencing and PFGE is very helpful to control and prevention of pertussis. However, Iran has not experienced pertussis epidemic after 2013, but our finding suggest the fact that the presence of non-vaccine \textit{ptxP3/ptxA1}, may associate with rising trend of pertussis among populations since 2007. The comparative genomic analysis of \textit{ptxP3} and \textit{ptxP1} isolates provided new insights into the evolution of pertussis isolates in Iran.
Our findings illustrate that changes in ptxP3 genome structure including 32 unique SNPs and three unique indels may have contributed to the expansion of the ptxP3 clone. Furthermore, this is the first study about comparative genome analysis of ptxP1 and ptxP3 allele type with next generation sequencing, in Iran. These results could be impressive in controlling of infection and help policy makers in the healthcare and community settings but there is a need to perform more sequence of ptxP3 isolates in order to obtain a better picture of the pathogen adaptation under vaccine pressure.

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