



Investigation of Genetic Variations in Virulence Factor Genes *ptxC*, *tcfA*, and *fhaB* of *Bordetella pertussis* Clinical Isolates and Vaccine Strains in Iran

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ABSTRACT

Aims Pertussis is an important vaccine preventable disease. It is still a major cause of infant morbidity and mortality in the world. Although the incidence of pertussis was successfully reduced after vaccination, the resurgence of pertussis has been reported in many countries even with high vaccination coverage. Genetic variation in virulence factors is one of the important causes for pertussis reemergence. We investigated genetic characteristics and allele types of 3 important virulence associated genes, including *ptxC*, *tcfA*, and *fhaB* in clinical *B. pertussis* isolates collected from different provinces of Iran and vaccine strains.

Materials & Methods Genomic DNA was extracted and *ptxC*, *tcfA*, and *fhaB* gene regions were amplified, using specific PCR primer. DNA sequencing was performed and data were analyzed.

Findings *ptxC2*, *tcfA2*, and *fhaB1* were the dominant alleles with 87.5%, 97.5%, and 97.5% frequencies, respectively. Vaccine strains *B. pertussis* 134 and *B. pertussis* 509 contain the genotypes *ptxC2-tcfA2-fhaB1* and *ptxC2-tcfA2-fhaB1*.

Conclusion Results for dominant alleles in *ptxC2*, *tcfA2*, and *fhaB1* genes in Iran are consistent with dominant alleles of other countries such as Netherland, Finland, and Italy. It seems that *ptxC2*, *tcfA2*, and *fhaB1* are the dominant circulating alleles in many countries after vaccination period, while vaccine strains have different alleles occasionally. More reported cases in recent years despite high coverage vaccination in Iran and genetic distances between clinical and vaccine strains suggest that antigenic changes in virulence factors possibly have an important role in the survival and evolution of the bacteria.

Keywords *Bordetella pertussis*; *fhaB*; *tcfA*; *ptxC*; Genetic Variation

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Introduction

Pertussis is a severe disease of respiratory tract in human. *Bordetella pertussis* gram negative bacterium is the first etiologic agent of the disease. Despite widespread vaccination against pertussis, it is still a major cause of death in infants [1].

Whooping cough can be considered as one of the most common vaccine-preventable diseases in some countries. After more than 50 years of vaccination, pertussis has tremendously reduced, but not completely eradicated. In the 1990s, a resurgence of pertussis was observed in several countries even with high vaccination coverage [2].

Several reasons have been proposed for pertussis resurgence including waning of vaccine-induced immunity, decreased vaccination coverage because of concerns about side effects, improved diagnostic methods, and increased awareness of physicians and people about the disease [3].

One of the factors, which was taught to be involved in pertussis re-emergence, is the adaptation of *Bordetella pertussis* bacterial populations in order to survive in vaccinated hosts. In other words, it is assumed that genetic variation of virulence associated genes in *B. pertussis* population has important role in vaccine efficacy and, thus, differences between vaccine and clinical isolates could be one of the factors, which cause pertussis resurgence over time [4].

Studying *B. pertussis* strains variation in the Netherland showed that antigenic divergence between clinical isolates and vaccine strains may have contributed to the pertussis resurgence for the first time [5].

Bordetella pertussis virulence factors are classified into two types: toxins such as pertussis toxin (PT), adenylate cyclase (CyaA), and tracheal cytotoxin (TCT) and adhesins like pertactin (PRN), fimbriae (FIM), and filamentous hemagglutinin (FHA) [6].

Two types of pertussis vaccine are commercially available: whole-cell pertussis vaccines, which are usually combined with tetanus and diphtheria toxoids (DP_wT) and acellular vaccines composed of purified and detoxified components of the bacterium [7]. Epidemics still occur in areas protected by whole-cell or acellular vaccines [8].

Iran is one of the countries, where still uses whole cell pertussis vaccine for children in its national vaccination program. Despite 99% coverage of pertussis vaccination in Iran, we observed an increase in pertussis incidence since 2004 [9, 10].

Pertussis cases still exist in Iran, while newborns have been persistently immunized with whole-cell vaccine (DP_wT) for more than 40 years. Vaccine program in Iran includes 3 primary doses at 2, 4, and 6 months and 2 booster doses at the age of 18 months and 6 years. These vaccines contain reference strains such as *B. pertussis* 134 and *B.*

pertussis 509.

This study was conducted to investigate genetic characteristics and allele types of 3 important virulence associated genes including *ptxC*, *tcfA*, and *fhaB* in *B. pertussis* vaccine strains and clinical isolates collected from different provinces of Iran, using polymerase chain reaction (PCR) and sequencing methods. Genetic distance and relatedness between vaccine and clinical strains was also analyzed in the study.

Materials and Methods

Strain collection: In this study, 95 *B. pertussis* isolates were analyzed. These isolates were collected through the 6-year period from March 2009 to March 2015. Vaccine strains (*B. pertussis* 509 and *B. pertussis* 134) and Reference strains (ATCC9797 and Tohamal) were also included in the study. Freeze strains were cultured on Regan-Lowe agar medium supplemented with 20% defibrinated sheep blood and 40 µg/ml cephalexin (produced in Pasteur Institute of Iran). They were incubated for 3-4 days at 35°C with humidity.

Strain isolation and identification: All *B. pertussis* isolates were previously confirmed by colony morphology, gram-stain, biochemical tests, and specific antiserum (Difco) [11]. Final validation was performed by Real time PCR assay based on IS 1001, IS 481, and *ptxP* regions [12].

DNA extraction: Microbial suspensions were prepared from single colonies and genomic DNA was extracted according to manufacturer protocol of High Pure PCR Template Preparation kit (Roche Company, Germany).

Gene amplification: Amplification of *ptxC*, *tcfA*, and *fhaB* genes was carried out in a PCR mixture containing 2 µl of template DNA 50-100 ng/ml, 1 µl of Forward, and Reverse primers [Pishgam, Iran] 10 µM and 12.5 µl Red Master Mix2X containing 1.5 mM Mg (Ampliqon Company, Denmark) in a total volume of 25 µl. Primer sequences, product lengths, and melting temperatures are shown in Table 1.

Amplicon sequencing: 40 out of 95 samples with different age, sex, and vaccination history regarding geographical distribution were selected for sequencing. The samples were sequenced by ABI capillary system (Macrogen Research, Korea).

Data analysis: Sequences were checked for their qualification by Chromas program. In order to allele typing of clinical isolates and vaccine strains, reference genotype of the gene was obtained from the NCBI GenBank and aligned with studied sequences by ClustaLW multiple alignment method, using MEGA software ver.4. Genetic distance and relatedness between vaccine, and clinical strains was also studied based on Neighbor joining method, using phylogeny analysis in MEGA.

Table 1) PCR primers used in this study

Primer name with their sequence	Product size	Annealing temperature (°C)	Reference
<i>ptxC</i> F:CTTCCGGAGGTTTCGACGTTTC R:TCTTTCAAGGGATTCATTGCG	814	60-65	19
<i>tcfA</i> F:CTTTCTCCTCCCTCGGCATGG R:AGCGCCGTCGGATTCAAG	549	55	19
<i>fhaB</i> F:GGTTCAGAGCGTCAACAGC R:CTCACCAGCTTCGCAACG	588	51	This study

Findings

During 2009 to 2015, more than 5000 nasopharyngeal Dacron swab samples suspected to pertussis were referred to pertussis reference laboratory in Pasteur Institute of Iran. Almost 1500 samples were identified as positive for *Bordetella pertussis* by Real time PCR. In this study, we analyzed 95 culture positive isolates of *B. pertussis*, which grew up on Regan low medium (Table 2).

Table 2) Information of strains isolated from patients during 2009-2015

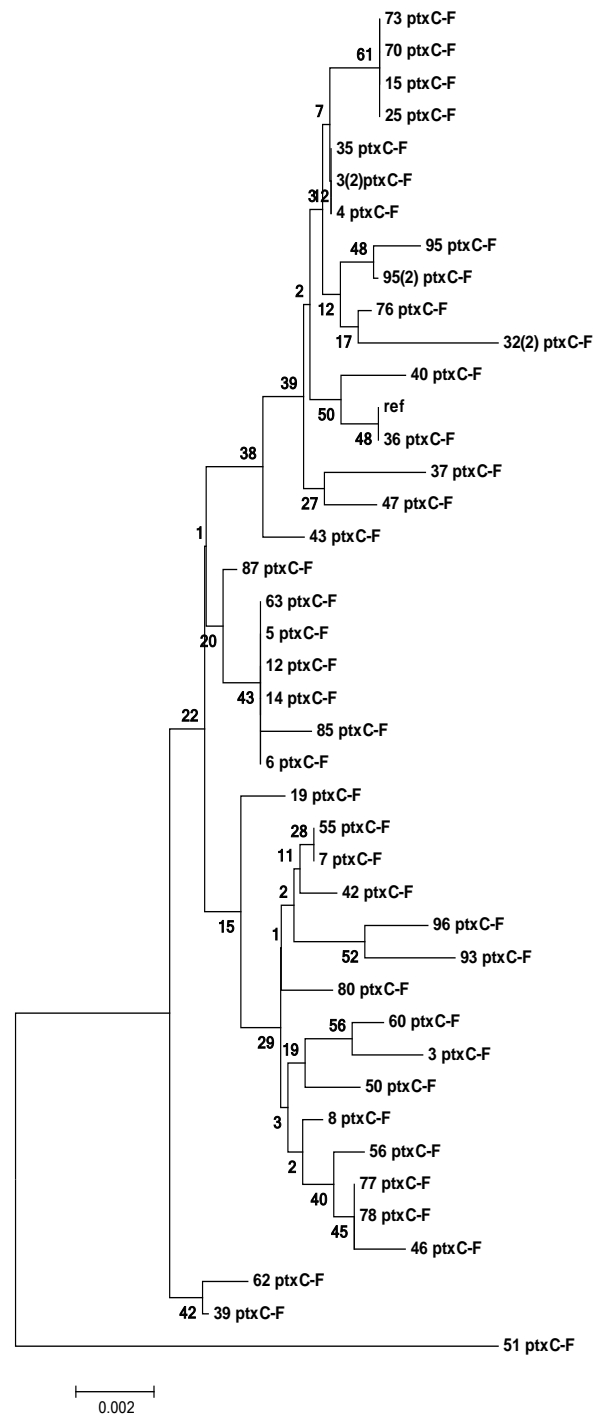
Year of isolation	NO. strains	Positive Vaccine history	Patients' age			
			<6 month	6-24 month	2-6 years	>6 years
2009	7	71%	4	0	2	1
2012	8	62.5%	5	0	1	2
2013	32	62.5%	28	4	0	0
2014	34	50%	23	8	1	2
2015	14	71%	7	1	5	1
Total	95	63.5%	67	13	9	6

***ptxC* gene:** Sequencing results for polymorphic region in *ptxC* gene showed that 87.5% of the circulating strains had *ptxC2* allele and 12.5% had *ptxC1*. Vaccine strains *B. pertussis* 134 and *B. pertussis* 509 and the challenge strain ATCC 9797 contain *ptxC1* similar to reference strain Tohamal. It is clear that dominant allele in Iranian *B. pertussis* population is completely different from alleles in the vaccine strains.

***tcfA* gene:** *tcfA* sequencing results in variable region indicate that *tcfA2* is the dominant allele with 97.5 % frequency and only 2.5% of the clinical isolates contain *tcfA1*. Vaccine strains *B. pertussis* 134 and *B. pertussis* 509 had also *tcfA2* allele, while challenge strain ATCC 9797 harbors *tcfA1*. Allele type of Tohamal is also *tcfA2*.

***fhaB* gene:** Our finding revealed that 97.5 % of the clinical strains as well as vaccine strain *B. pertussis* 134 had *fhaB1* genotype. Tohamal reference strain also contains *fhaB1*, while vaccine strain *B. pertussis* 509 and challenge strain ATCC 9797 contain *fhaB2*.

Phylogenetic trees were also drawn based on Neighbor joining method for *ptxC*, *tcfA* and *fhaB* sequences. Neighbor joining method was used for clustering. Phylogenetic trees were shown in Figures 1-3.

**Figure 1)** Dendrogram based on *ptxC* sequences of clinical and vaccine strains

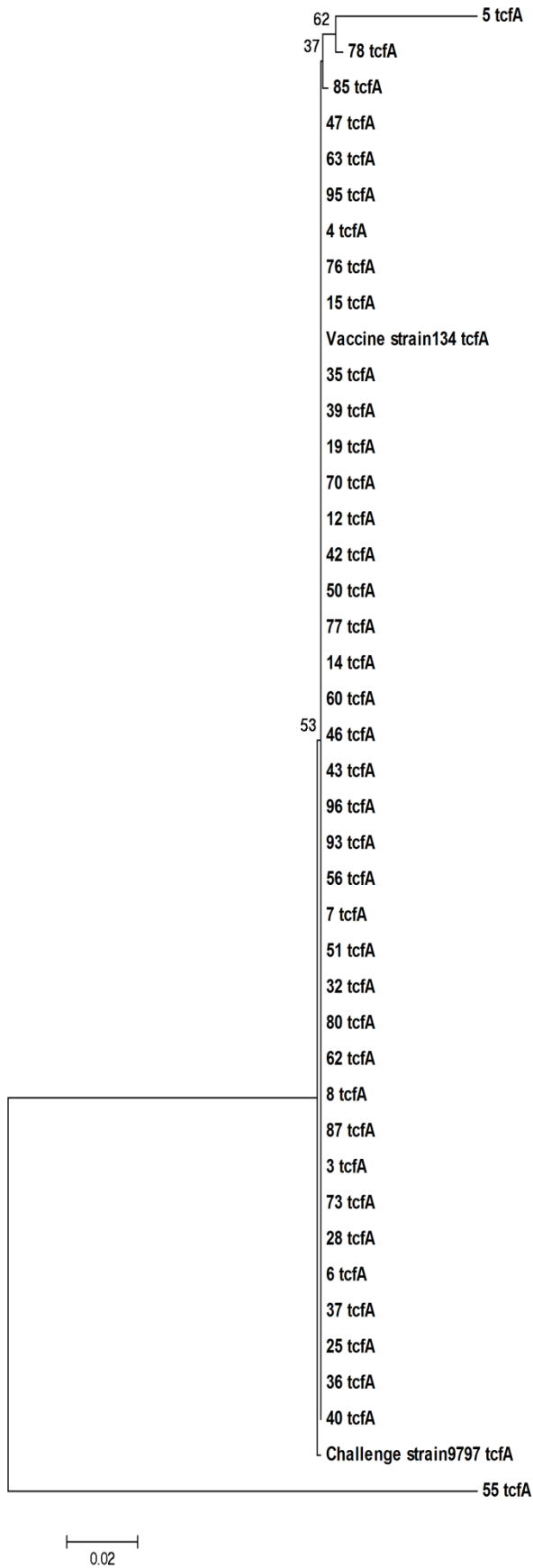


Figure 2) Dendrogram based on *tcfA* sequences of clinical and vaccine strains

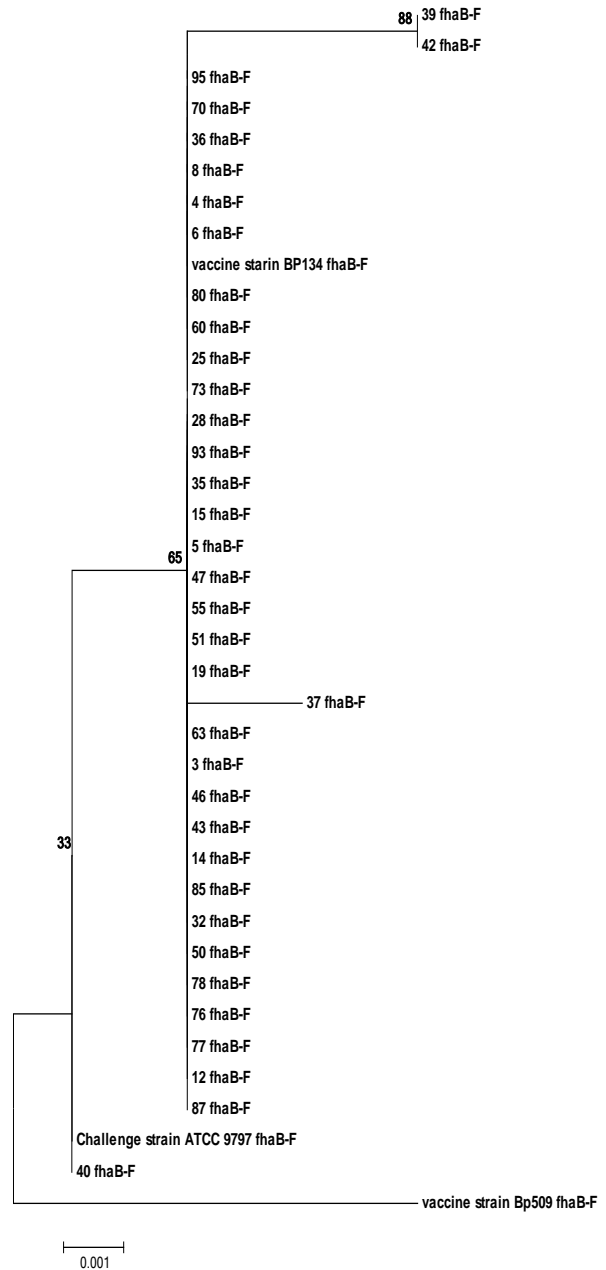


Figure 3) Dendrogram based on *fhaB* sequences of clinical and vaccine strains

Discussion

Although pertussis is a disease with high morbidity and mortality, vaccination programs have been highly successful in preventing severe conditions in infants worldwide. DP_wT combined vaccines are today still the most widely childhood formulation to protect against pertussis [1].

Pertussis prevention has also been achieved in Iran by vaccination, even though the disease has not been eradicated yet. Increase in pertussis incidence was observed since 2004 and pertussis cases were diagnosed and reported yearly. Some reasons such as waning of vaccine-induced immunity, decreased vaccination coverage, improved diagnostic methods,

and increased awareness could be suggested for pertussis resurgence [10, 12].

Most of the positive cases were confirmed by Real time PCR and bacterial culture belongs to the 4 recent years. More reported cases in recent years is possibly because of increased awareness among people and physicians and improved surveillance system in Iran after foundation of the pertussis reference laboratory in Pasteur Institute of Iran.

Majority of the reported pertussis patients are expectedly infants below 6 month that are the most sensitive age group for pertussis. Patients' information also showed that vaccination history is positive in almost 65% of the cases. It should be also noted that DP_wT vaccine normally cause not 100% protection against pertussis and the immunity will be decreased over time.

As mentioned before, it seems that *B. pertussis* adaptation is an important factor, which affects the survival of bacteria in vaccinated population. The hypothesis of bacterial adaptation because of vaccination pressure was first proposed by Mooi *et al.* by observation of moderate nucleotide changes in some virulence factor genes [5].

Studying *B. pertussis* population structure shows that antigenic type of bacterial strains have been changed a lot during vaccination period and many adaptive mutations were occurred, using whole cell vaccines [13, 14]. Antigenic changes in virulence factors possibly are vital in the survival and evolution of the bacteria, since they have an important role in pathogenicity by involving in adhesion, invasion, and sometimes interference with host immune cells.

The genes encoding some virulence factors including *ptxA*, *ptxP*, *cyaA*, *prn*, *fim2*, and *fim3* have been studied before on Iranian strains [11, 15]. Here, we investigated other important genes *ptxC*, *tcfA*, and *fhaB* to monitor the bacterial populations and vaccine strains. These genes encode pertussis toxin subunit3, tracheal colonization factor, and filamentous hemagglutinin, respectively.

Pertussis toxin increases cAMP and ultimately bacterial toxicity in host cells [16]. Tracheal colonization factor has an effective role in cell adhesion and filamentous hemagglutinin involves in the attachment of bacteria to integrins in epithelial cells [17, 18].

The first study about *ptxC* gene polymorphism was conducted by Van Loo *et al.* on 196 isolates collected from Netherlands, Finland, Italy, Japan, and The United States in 1949 to 1999. Two alleles were reported and dominant allele was *ptxC1* with 78% frequency [19].

In Netherlands, whole cell vaccines containing *ptxC1* were introduced in 1953. Another study on circulating strains in the post-vaccination era showed that dominant allele have been switched to *ptxC2*, which is different from allele type in vaccine strain [20].

Frequency of the *ptxC2* allele has also been increased in England since 1920 and was determined as the dominant allele during 2000 to 2002 [20]. Dominant alleles in France and Germany were also *ptxC2* in 1998 to 2001, while main vaccine used in 1955 in Germany was based on Tohamal harboring *ptxC1* [21].

ptxC2 was also dominant allele with 87.5% frequency in Iran after vaccination, while our vaccine strains *B. pertussis* 134 and *B. pertussis* 509 contain *ptxC1*. It shows that allele type of the gene is different between the most of clinical isolates and vaccine strains.

The *tcfA* gene polymorphism has been studied in several cases. The first study discovered 4 different alleles for *tcfA* and showed that *tcfA2* is dominant in Netherlands, Finland, Italy, Japan, and The United States since 1949 to 1999 [19]. Next study in Netherlands indicated that *tcfA2* again predominates during 1998 to 2001 [22].

Further studies in France, Germany, Sweden, Korea, and Poland were also verified *tcfA2* as the dominant allele. Polish vaccine strains also contain *tcfA2* allele [21-24]. *tcfA2* was also observed in 90% of clinical isolates and considered as dominant allele in England during 1920 to 2002. Vaccine strains harbor *tcfA2* similarly [25].

Results of *tcfA* gene polymorphism in Iran are in concordance with previous surveys in other countries. It seems that *tcfA2* is usually the dominant allele in clinical strains before and after vaccination period and circulating isolates in post-vaccination era harbor the same *tcfA2* allele, which also exists in vaccine strains. Mooi *et al.* suggested that the *tcfA* gene as an adhesion is rarely undergo immune pressures in comparison to other virulence factors [26].

There are only limited studies on *fhaB* gene sequence, because of its big size and many repeats inside the gene [26]. Two different alleles were reported for *fhaB*. *fhaB1* was dominant allele in Netherlands, Finland, Italy, Japan, and The United States in 1949 to 1999, in Korea during 2000 to 2009, and in Alberta of Canada in 2012 [19, 24, 27]. *fhaB1* also exists in the majority of circulating strains in Iran and vaccine strain *B. pertussis* 134.

Phylogenetic trees represented based on *ptxC*, *tcfA*, and *fhaB* gene sequences show that the most of clinical isolates are classified in a same cluster, so they have genetic similarity. Vaccine strains in *ptxC* dendrogram are clustered separately from the most clinical isolates, which indicate that there is a genetic distance between vaccine and clinical strains. Vaccine strains are situated in a same cluster with clinical strains in *tcfA* sequence based-dendrogram that verified there is a genetic similarity between vaccine and clinical strains. Phylogenetic tree based on *fhaB* sequences clustered vaccine strain *B. pertussis* 134 near the majority of clinical isolates, but *B. pertussis* 509 was completely

separated, which shows the most genetic distance between *B. pertussis* 509 and clinical isolates. It has been suggested that *B. pertussis* 134 is genetically more likely related to clinical isolates and maybe is still a suitable strain for use in vaccination.

Results for *ptxC*, *tcfA*, and *fhaB* dominant alleles in Iran are in concordance with other countries such as Netherlands, Finland, and Italy. It seems that *ptxC2*, *tcfA2*, and *fhaB1* are the dominant circulating alleles in many countries after vaccination period, while vaccine strains have different alleles occasionally. More reported cases in recent years despite high coverage vaccination in Iran and genetic distances between clinical and vaccine strains suggest that antigenic changes in virulence factors possibly have an important role in the survival and evolution of the bacteria.

Conclusion

Variation in *B. pertussis* genome is potentially important because it can affect vaccine potency and efficacy. The question that whether genetic differences occur between strains, gives some strains an advantage in fighting against host defense mechanisms, or in overcoming immunity, still remains without a clear answer. It could only be stated that genetic characteristics and allele types in present circulating strains differed from vaccine strains and clinical isolates of pre vaccination era.

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