

Introducing two new loci of VNTR9 and VNTR10 for *Bordetella pertussis* genotyping

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Abstract

Background & Aims: *Bordetella pertussis*, the causative agent of whooping cough, continues to infect human hosts even in those populations where infants and children are routinely vaccinated. Causes of pertussis epidemiology are not fully identified unless strains of the pathogen are characterized by molecular means. Globally, Multi Locus Variable Number of Tandem Repeats analysis (MLVA) has proved very useful in inter-laboratory surveillance of majority of world most important bacterial diseases. This work was conducted to improve the current MLVA typing method developed by Schouls in 2004.

Materials & Methods: An in silico search was comparatively conducted on the whole genomes of 5 laboratory/vaccine strains of *B. pertussis* deposited in the NCBI genome database by Tandem Repeat Finder software. PCR protocols were then adopted to enable simultaneous amplification found loci. A further comparative genomic analysis of 20 world-known *B. pertussis* strains from diverse spatial and temporal origins was performed using the detected new VNTR loci.

Results: Two polymorphic loci carrying tandem repeats (TRs) with 6 (AAGCCC) and 9 (GGCTGGCCG) nucleotides were detected and designated as VNTR9 and VNTR10, respectively. Application of these on genomic templates from *B. pertussis* 107 and *B. pertussis* 509 vaccine strains used by Razi institute in manufacturing the pertussis vaccine resulted in successful production of PCR amplicons from both strains. Nei's diversity indices of 0.38 and 0.1 were achieved by these loci, respectively in comparative genomic analysis of *B. pertussis* strains from across the world.

Conclusion: We assume inclusion of VNTR9 and VNTR10 in MLVA analysis of clinical isolates of *B. pertussis* is useful in improving current understanding of pertussis in Iran.

Keywords: whooping cough, Epidemiology, Strains

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Introduction

Pertussis is a contagious disease of the respiratory tract which is caused by *Bordetella pertussis*. Based on

available epidemiological evidences, the incidence of the disease is not confined to geographical, ethnic and age borders. Baha-O-doleh Razi in Chapter 13 of his 28-

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chapter book in 1502 AD, argued about pertussis epidemic in the cities of Herat and Rey- Iran (1). Five hundred years later, in 2013, more than 23 people per hundred thousand citizens were living with the disease despite the almost full vaccination coverage (2).

The prevalence of many infectious diseases such as polio, diphtheria and measles has decreased until eradication in the years after World War II, using the nation-wide vaccination strategy in many parts of the world, particularly in industrialized countries (3). However, countries such as United States of America (3), England (4) Austria (5), Japan (6) and Belgium (7) have been encountered with the emergence and the resurgence of whooping cough epidemics in recent years. The incidence of genetic changes in global and regional population of *Bordetella Pertussis*, failure in the implementation of vaccination coverage as well as insufficient immunogenicity of the vaccine available have been identified among the main reasons explaining the contemporary global epidemiology of pertussis (3). Current vaccines against pertussis evolved and introduced in the 50s and 60s, thus it is important to analyze whether the level of safety is at the expected stage in terms of health management after half-century of the introduction. Pertussis bacteria population changes over time based on the genitival evidences that identifying the bacteria and stimulating the host immune system to deal with them is affected by modifying the antigenic characteristics (8). Besides, intensifying or reducing the activity of certain genes as a result of events such as genetic mutations over time may affect the extent and virulence of this pathogen (9). For example, the mutation of ptxP3-allele increases toxin production in *Bordetella Pertussis* or mutations in the target protein encoding genes, including pertussis (10) will be effective in the clinical efficacy of existing vaccines (11).

Due to the structure of *Bordetella pertussis* homogeneous, the developed genotyping methods to

study genetic differences in the population of the pathogens face with difficulties. RFLP-IS1002 (12, 13), PFGE (14), and MLST (15, 16) and MAST (17) are among such techniques for typing these pathogens which have demonstrated the degrees of genetic diversity among the isolates of the pathogen. In 2004, Scholes introduced multiple-locus variable-number tandem repeat analysis (MLVA) typing system in the pertussis bacteria (18). In the last two decades MLVA typing system has become as public health standard genotyping methods for many important bacterial pathogens. The technique in the complexes of *Mycobacterium Tuberculosis* *Bacillus cereus* (including *Bacillus anthracis* causing anthrax in humans and animals) and *Mycobacteria Avium* (including *Mycobacterium Paratuberculosis* causing Johne's disease in ruminants) relies on 24, 12 and 8 VNTR loci, respectively. The results of our study in wide international scale show an increase in the number of loci examined in this method, regardless of the type of organism and the population under study increase the discrimination power although the genetic variation and the frequencies of alleles per locus and proportionate to the population may vary in a single organism.

6 Variable Number Tandem repeat (VNTR) Locus namely as VNTR1 VNTR2 VNTR3 VNTR4 VNTR5 VNTR6 have been used in the study of Scholes. Currently, the only *Bordetella Pertussis*- based international system genotyping is according to MLVA which is developed by the National Institute for Public Health and the Environment in Netherlands and acts based on the 5 loci of loci proposed by Scholes (VNTRs1,3, 4, 5 & 6). Information on the reported genetic type of the pathogenic isolates from around the world are kept in the database (accessible through the website of <http://www.mlva.net/bpertussis/default.asp>). By March 2017, 237 types have been registered in this database. Eight VNTR loci usable in the genotyping *Bordetella Pertussis* loci have been identified since

2004, including sextets loci of Scholes and two loci (Vntr7 and Vntr8) introduced by Kurniawan (Table 1). The genome of Bordetella Pertussis is investigated in order to examine the possibility of increasing the strength of the discrimination power of conventional MLVA Taiping system and two new luxury VNTR9 and VNTR10 have been introduced.

Materials and Methods

A- Bacteria Culture: B pertussis 134 and B pertussis 509 strains used in the Bordetella Pertussis vaccine in Iran are selected from microbial archives of Razi Institute and cultured on Bordet-Gengou agar containing 10% defibrinated sheep blood and were incubated at 35 ° C for 4-5 days until observing the bacteria colonies. Genetic material of bacteria was prepared using boiling method (19). To this end, the volume of the bacterial culture was taken by a standard loop (10 µl) and then after preparation of a bacterial suspension with 400 µl of TE buffer, was heated in a micro tube with leak-proof seal in the water bath at 95 ° C for 10 minutes until inactivate the bacteria and ensure the release of its genome. The suspension was centrifuged in 10000g for 10 minutes after cooling and the supernatant was separated and was passed through a 0.2 µl syringe filter. The clear solution contains the genetic material of bacteria and was kept in the lab freezer or refrigerator until use. The suspension is used directly in PCR reactions.

B- Finding in silico after loci VNTR:

By the end of March 2017, strains of genome 289 in Bordetella Pertussis have been fully sequenced and their information is available to the public (accessible via <https://www.ncbi.nlm.nih.gov/genome/genomes/1008>). In this study, laboratory and vaccine genome known strains of B. pertussis Tohama, B. pertussis B202, B pertussis B203, B pertussis C393 as well as B. pertussis 18323 have been selected. Tandem Repeat Finder

Software Ver 4.07 is used for simultaneous screening of the five selected genome and searching for the VNTR repeating units. Software settings were kept as default and unchanged. The results obtained collected with the help of Excel software and set respectively based on the correspondence between repeating units (Tandem Repeats = TRs) per locus VNTR, the size (length) of repeating units and the number of these units at each locus. Next, a group of TRs were selected which have a perfect match between repetitions in each locus that have different degrees of length polymorphism within 5 genomes under investigation.

C- Primer design:

Specialized software of Artemis (20) was used to locate loci in the genome of Tohama lab strain. Part of the genome of this strain of approximately 2 Kb of each locus was selected as the locus is located approximately in the central part of the BE segment. Primer 3 program was used to design primers (21). Two pairs of primers VNTR9f (5' CGAAGGTTGAAACGGCTCAG 3') and VNTR9r (5' GG TAGTTCTTGCCGCCGATA 3') and VNTR10f (5' GGGCTGATAATCCTTGCGGT 3') and VNTR10r (5' GGCCCGTAATAACCGCCATA 3') were selected for this study and made by Macrogen South Korea.

D- PCR:

Optimizing the temperature and components of PCR was performed using the existing protocol (22). Totally four reactions of PCR (each consisting of 6 reactions) have been designed and six different annealing temperatures (55, 56.7, 59.1, 61.7, 63.9, 64.9 degrees Celsius) have been applied in each series. The primer in each reaction in two series of four series of reactions was 1 pmol and in two other series was 5 pmol. The amount of magnesium chloride in two series was equal to 1mM and in other twelve reactions was set as 2.5 mM. Providing materials and components needed for PCR reactions (except primers and DNA template) was performed using Ampliquor ready to use commercial

kits (Ampliquor®, Denmark). The volume of all PCR reactions are set equal to 12 µL and Double Distilled PCR water was used as a negative control.

Amplification protocol includes an initial denaturation under the terms of 45 s / 95 °C, followed by 30 consecutive Denaturation cycles at 95 °C for 45 seconds, annealing for 45 seconds, Extension at 72 °C for 60 seconds and ended with Final extension for one minute at 72°C (table 1).

Gel electrophoresis and imaging:

Agarose gel (Invitrogen®, USA) was used at 1.5% of concentration and pre-painted with Red Safe ®. Gel electrophoresis carried out after loading PCR products (10µl) per well for 2 hours in the electric field of 2 V / cm. DNA size marker from Razi institute (23) was used as a marker.

PCR optimization and applying the settings:

The best treatment in case of changes in temperature and concentration of the components (magnesium chloride and primer) has been selected through reviewing the gel electrophoresis findings obtained from Vigesimal and quartet testing of each locus under test (Table 2) and vaccine strains of *B. pertussis* 134 and *B. pertussis* 509 have been used in the amplification of the loci using genome.

Nucleotide sequencing of PCR products:

Genetic parts of the amplification of loci 9 and 10 in the genome strains of *B. pertussis* 134 and *B. pertussis* 507 were sequenced in cooperator lab (Microgen of South Korea). Chromatograms processed using specialized software such as Chromas lite Version 2.1. (24) and Clustal (25) and the number of repeating units were determined using TRF software.

Bioinformatics analysis:

Location of six loci proposed by Scholes and two loci identified by Kurniawan and loci found in this study in the genome have been identified in an international collection of 20 laboratory and clinical strains of

Bordetella pertussis with the greatest diversity reported in terms of time and geographic location of initial isolation and history of use in the production of vaccines by Artemis program and the size of equivalent TRs is determined in each strain. Genetic diversity indices for each independent locus, also known as Nei's diversity index (NDI), is calculated on the basis of $1 - \sum (\text{Allele frequency})^2$ (26). In each MLVA locus, the size of equivalent genetic part is determined and Total Information of Decuple Locus have been transfer to Excel Software BioNumerics version 6.7 software (Applied Maths, Kortrijk Belgium) and was used to draw Minimum spanning tree based on the genetic relationship among 20 strains of *Bordetella pertussis* under test.

Results

A large number of repeating units were identified in 5-strain genome screening during the search for VNTR among which two TR were selected containing a repeat unit with 6 nucleotides (AAGCCC) and a repeat unit with 9 nucleotides (GGCTGGCCG) which were named as VNTR9 and VNTR10, respectively. The initial in silico assessment showed the existence of polymorphism at these loci in the 5-solate genomes. According to the results of electrophoresis and PCR amplicon sequencing, loci VNTR9 in the genome strains of *B. pertussis* 134 and *B. pertussis* 509 of Razi was 666 (includes 5 copies of the repeat unit) and 660 (4 copies) base pairs and loci VNTR10 was about 593 base pairs (equivalent to 3.83 copies) in both strains.

Ability to perform PCR using a universal protocol in terms of temperature and components of the reaction using both loci were shown (Figure 1 and Table 2) and PCR products in both strains of *B. pertussis* 134 and *B. pertussis* 509 have been produced and their nucleotide sequencing are identified.

Table 1: components and protocol structure of time-temperature cycles of the PCR reaction used in this study.

PCR protocol	PCR master mix (μl)	Primer forward1 (μl)	Primer reverse1 (μl)	MgCl22 (μl*)	DNA template (μl)	PCR water (μl)	Total volume (μl)
1	6	1	1	0	2.5	1.5	12
2	6	1	1	0.36	2.5	1.14	12
3	6	0.2	0.2	0	2.5	3.15	12
4	6	0.2	0.2	0.36	2.5	2.74	12
Universal	6	0.2	0.2	0	1.5	4.1	12

* Magnesium chloride solution 50 mM was used

Table 2: PCR products of the decuple locus VNTR1-10 in the 20-strain genomes of international Bordetella pertussis, the number of variants (alleles) at each locus and Nei's diversity index for each locus is known.

Strain (aliens)	Specifications	Source	Collection date	VNTR1	VNTR2	VNTR3	VNTR4	VNTR5	VNTR6	VNTR7	VNTR8	VNTR9	VNTR10
H788	Laboratory	USA (VT)	2011	621	425	774	678	778	198	718	720	666	593
I344	Laboratory	USA (MN)	2012	651	406	774	642	778	208	718	720	666	593
J445 (134)	Vaccine	India	ND	651	425	702	690	778	216	718	720	666	593
B202	Laboratory	USA (PA)	1946	651	425	774	690	778	216	718	720	666	593
H622	Laboratory	USA (CA)	2010	666	406	774	651	778	198	718	720	666	593
18323	Laboratory	USA	1946	666	413	730	618	778	153	727	677	660	584
B137	Vaccine	Brazil	ND	666	425	738	678	814	198	718	714	660	593
D420	Laboratory	ND	ND	666	425	748	678	772	198	718	714	660	593
B203	Laboratory	USA (MI)	1939	666	425	764	678	814	198	718	714	660	593
B1838	Laboratory	Netherlands	1999	666	425	774	666	778	198	718	720	666	593
VA-10	Laboratory	USA (Vir)	2001	666	425	774	678	778	198	718	702	666	593
VA-150	Laboratory	USA (Vir)	2010	666	425	774	678	778	198	718	720	666	593
B3405	Laboratory	Netherlands	2010	666	425	774	678	778	198	718	720	666	593
H321	Laboratory	France	2007	666	425	774	678	778	198	718	720	666	593
I979	Laboratory	USA (NY)	2013	666	425	774	678	778	198	718	720	666	593
B3582	Laboratory	Sweden	2009	666	425	779	678	778	198	718	720	666	593
J448 (25525)	Laboratory	India	ND	666	425	779	678	784	189	718	720	666	593
C393 (CS)	Vaccine	China	1951	681	425	774	678	784	189	718	720	666	593
E476 (Tohama1)	Vaccine	Japan	1954	681	449	774	702	784	234	718	720	666	593
J446 (509)	Laboratory	India	ND	696	425	764	678	814	208	718	714	660	593
No of variants				5	4	7	7	4	6	2	4	2	2
Nei's diversity index				0.54	0.35	0.61	0.56	0.53	0.61	0.1	0.47	0.38	0.1

ND = Not Defined

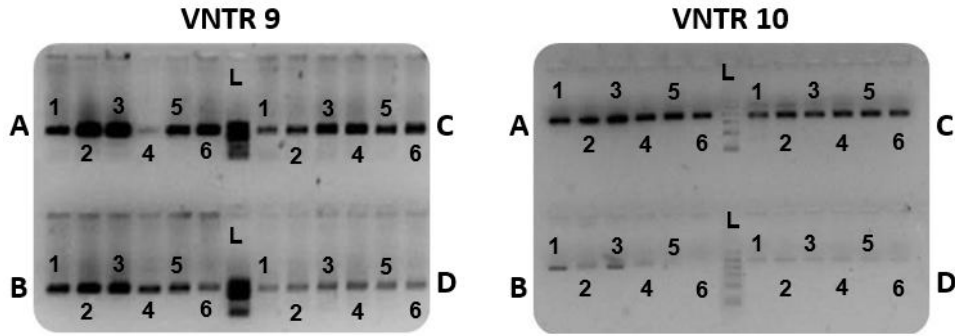


Figure 1: applying the optimization process in the PCR amplification of VNTR9 and VNTR10 loci and the electrophoretic results of the implementation of the protocols A, B, C, D. Temperature gradients of annealing are marked with the numbers 1 to 6, respectively as 56.1, 56.7, 60.1, 62.7, 63.9 and 65.9 degrees Celsius. There is a pmol of each propulsion and boys primer in series A and B in each PCR reaction, while this primer is five pmol in series C and D. The concentration of MgCl₂ in B and D series is 1mM and in A and C is 2.5 mM. Intermediate columns (L) relate to DNA size marker and each has 5 bands (100, 300, 500 700 and 900 bp).

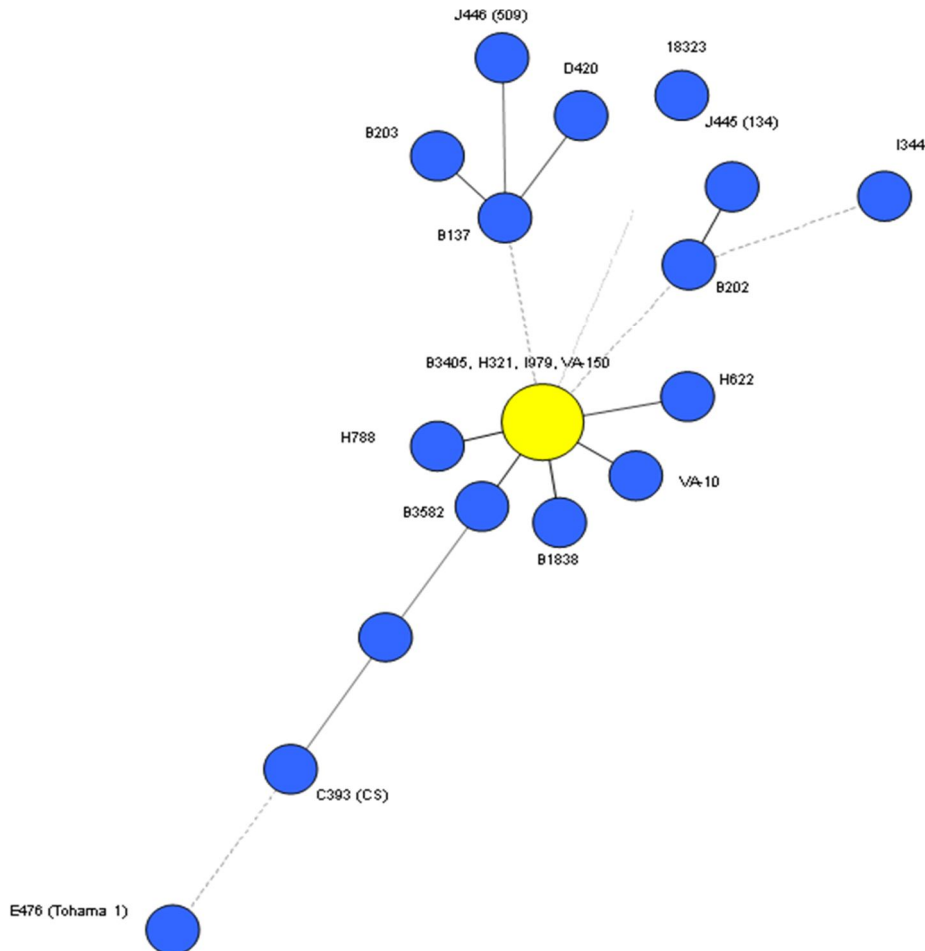


Figure 2: Minimum spanning tree image representing the genetic relationship among 20 vaccine strain and laboratory internationally recognized *Bordetella Pertussis*, based on the results of MLVA typing using 10 loci VNTR1-10. Each blue circle represents an independent genetic type (one-way) and each yellow circle represents a common genetic type among the 4 strains.

For details, see the text.

Discussion

The digital nature of the genotyping method MLVA results which provides the ability to create genetically international banks from pathogenic bacteria strains (18) have caused this method of genotyping to be applied as a standard procedure in many epidemiological studies of infectious diseases caused by bacteria with narrow genetic diversity such as *Bacillus anthracis* (27), *Mycobacterium tuberculosis* (28), *Mycobacterium bovis* (29), *Burkholderia Malei* (30) and *Yersinia pestis* (31). Currently the activates have been done on establishing international MLVA information banks of bacterial pathogens accessible through the Internet and a number of banks already have been established, for example MLVA-NET supported by the Pasteur Institute of Paris <http://www.mlva.eu/>, <http://mlva.u-psud.fr/>, and <http://www.miru-vntrplus.org>(32).

VNTR6 has the highest genetic diversity of 6 loci presented in Scholes study (1.15) defined based on the Shannon-Weiner index (SWI) among Dutch isolates of *Bordetella pertussis* and the lowest value was provided by VNTR2 (0.20) (18). Similarly, Kurniawan study on 316 isolates from 12 countries in four continents showed similar results and VNTR6 locus produced the highest level of genetic diversity in Simpson's diversity index (SDI) (0.72) and VNTR2 was identified as a locus with the lowest levels of genetic diversity (0.02) (33). Similarly, in the present study, VNTR6 still has the highest rate of 0.61 on a scale of NDI and VNTR7 has the lowest level of diversity (0.01) (Table 2). VNTR9 and VNTR10 each with 2 alleles detected among the strains among the two loci found in this study have been created the values 0.38 and 0.1 of the NDI. Currently measures are in progress in Iran on the use of VNTR9 and VNTR10 loci in genotyping clinical isolates of *Bordetella pertussis*. Due to the presence of probably native population of this bacteria in Iran that its activities

has been proved for at least the last 500 years in this country (1),

it is expected that the results of ongoing research to be helpful in demonstrating the discriminatory power of the locus in the locus introduced in Scholes and Kurniawan in Iran.

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