

Isolation of High Level Macrolide Resistant *Bordetella pertussis* Without Transition Mutation at Domain V in Iran

Bahman Mirzaei¹; Zakaria Bameri¹; Ryhane Babaei¹; Fereshteh Shahcheraghi^{1*}

¹Department of Bacteriology, Pasteur Institute of Iran, Tehran, IR Iran

*Corresponding author: Fereshteh Shahcheraghi, Department of Bacteriology, Pasteur Institute of Iran, Tehran, IR Iran. Tel: +98-2166953311, Fax: +98-2166953331, E-mail: shahcheraghifereshteh@yahoo.com

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Background: *Bordetella pertussis*, as a causative agent of whooping cough, due to the annual rise of infection cases, failure of prophylaxis and treatment by macrolides, is considered as the new concern in the health care system.

Objectives: The main objective of this study was the determination of single nucleotide polymorphisms (SNPs) at domain V, as the main binding site for macrolides, following the identification of high level macrolides resistant *B. pertussis*.

Materials and Methods: Following the identification of 11 recovered *B. pertussis* isolates, from a total of 1084 nasopharyngeal swabs, by using the biochemical and molecular methods, the activities of erythromycin, azithromycin and clarithromycin antibiotics against the recovered isolates were examined. Subsequently, A-G transition mutations in domain V were analyzed by molecular techniques, such as Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) and sequencing.

Results: After susceptibility testing, one strain was detected as a high level macrolide resistant *B. pertussis* (Erythromycin = 128 µg/mL, Clarithromycin > 256 µg/mL). After sequencing and PCR-RFLP methods, transition mutations in positions 2047 and 2058 of the mentioned domain were not observed.

Conclusions: Although previous studies have shown that A-G transition mutations in 23S rRNA gene (domain V) are the main reason for the occurrence of high level macrolides resistance in *B. pertussis*, however, the mentioned single nucleotide polymorphisms (SNPs) have not been detected in our resistant strain. This is the first report of high level macrolide resistant *B. pertussis*, without SNPs in domain V, in Iran.

Keywords: Drug Resistance, Minimum Inhibitory Concentration; Macrolides; *Bordetella pertussis*; Mutation

1. Background

Whooping cough (pertussis) is the acute respiratory infectious disease caused by the *Bordetella pertussis* bacterium (1). The *B. pertussis*, as a causative agent of pertussis, is transferred through droplets produced by coughing, sneezing, or talking (2). Infants are the age group with the highest risk of morbidity, hospitalization and mortality. Estimates from World Health Organization (WHO) suggest that, in 2008, about 16 million cases of pertussis occurred worldwide, 95% of which were in developing countries, and that about 195000 children died from the disease (3). Despite the vaccination, the incidence of pertussis in the vaccinated individuals is rising. Although macrolides are used as the drug of choice for prevention and treatment of pertussis, considering previous reports for the isolation of high level macrolides resistant strains, the emergence of resistant strains must be regarded as an alert signal for the health care system (1, 4-6).

Antimicrobial drug treatment substantially contributes to the control of disease transmission (6). According to the published study by Weisblum, in 1995, peptidyle transferase activity is a main characteristic of macrolide

binding site of domain V (7). Following detection of first erythromycin resistant *B. pertussis* in Arizona, four additional cases were detected in USA (8). Recently, two strains of erythromycin resistant *B. pertussis* were reported in France and China, too (5, 6). Transition mutations (A-G) in nucleotide positions 2047 and 2058 (critical erythromycin binding site in 23S rRNA gene) have been demonstrated, as a main cause of high level resistance against macrolides in *B. pertussis* (4-6).

2. Objectives

The main objective of this study is to determine the single nucleotide polymorphisms (SNPs) at domain V, as main binding site for macrolides, following the identification of high level macrolides resistant *B. pertussis*.

3. Materials and Methods

From May 2009 to December 2010, 1084 nasopharyngeal swab specimens from the patients having pertussis symptoms, with the age ranging from < 1 month to > 90

years, were delivered to the Pertussis Reference Laboratory at the Pasteur Institute of Iran, Tehran, Iran. Specimen swabs (Dacron-tipped swabs) were transported from the different provinces of Iran to the laboratory, on Regan-Lowe transport medium (Difco, Becton Dickinson, Franklin Lakes, NJ, USA).

3.1. Isolation and Identification of *Bordetella pertussis*

Isolation and identification of *B. pertussis* strains were performed according to the WHO recipes (9). After performing conventional biochemical tests, growth of colonies was confirmed using Taq Man real time-polymerase chain reaction (RT-PCR) assay. The RT-PCR assay was accomplished according to the previously described method (10).

3.2. Taq Man Real Time Polymerase Chain Reaction

Taq Man RT-PCR assay was applied for precise determination of *B. pertussis* in clinical samples. Insertion sequence 481 (IS 481) and BP 283 sequence (Codon gene between tyolase region) were targeted by using specific primers and probes (10, 11). Utilized primers, probes sequences and amplification protocol in Taq Man RT-PCR assay were described previously (10, 11). Taq Man RT-PCR was performed in an ABI 7500 instrument (Applied Biosystems, Waltham, MA, USA). *Bordetella pertussis* ATCC 9797 served as the control strain.

The amplification plots and standard curve for RT-PCR have been shown in Figure 1.

3.3. Macrolides Susceptibility Test

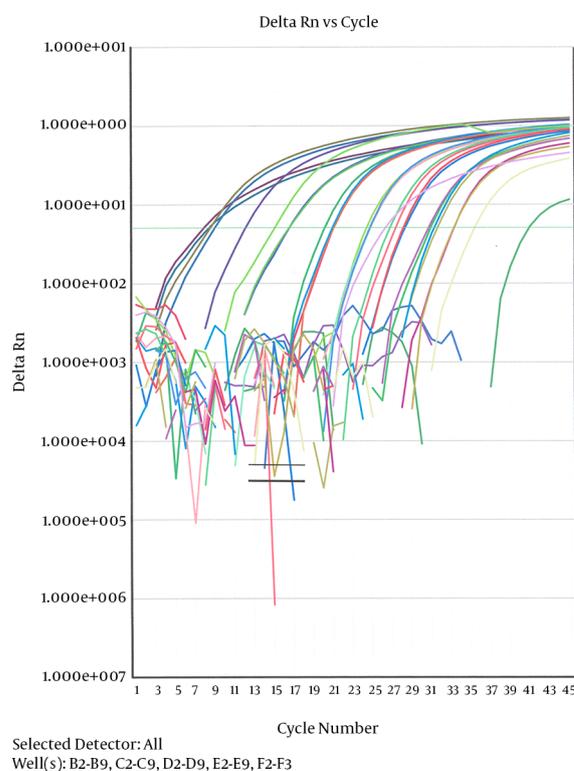
Susceptibility testing was performed according to the Clinical Laboratory Standard Institute (CLSI) 2010 guideline, applying antimicrobial agents, such as erythromycin, azithromycin and clarithromycin (Difco, Becton Dickinson, Franklin Lakes, NJ, USA) on the Muller Hinton Agar (Difco, Becton Dickinson, Franklin Lakes, NJ, USA) containing 5% sheep blood (12). Minimum inhibitory concentrations (MICs) breakpoints for the mentioned antibiotics were determined according to the recommended guideline in CLSI (12). The concentration of antibiotic agents was prepared between 0.016-512 µg/mL of antibiotics. Antibiotic free plates were inoculated to check for growth and purity. *Bordetella pertussis* ATCC 9797 and *Staphylococcus aureus* ATCC 29213 served as control strains. Strains for which the MICs of macrolides were ≤ 0.12 µg/mL were considered susceptible (8, 12).

3.4. Pulsed Field Gel Electrophoresis

Pulsed field gel electrophoresis (PFGE) analysis was done according to the standardized recommendations for epidemiological typing of *B. pertussis*, in a previously published study (13). Chromosomal DNA samples from 11 strains were digested with *Xba*I (Fermentas, Vilnius, Lith-

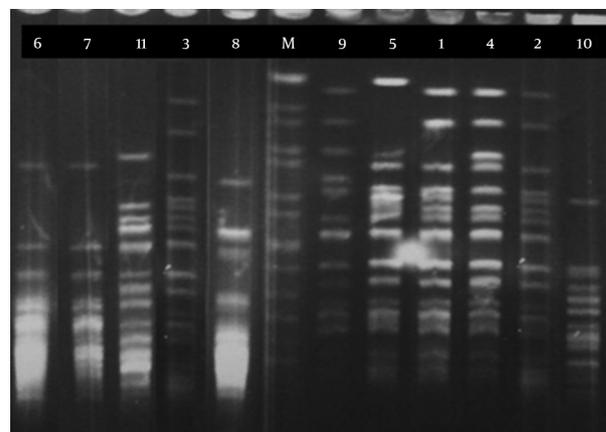
uania) and examined by PFGE. Subsequently, the profiles of PFGE were analyzed by using BioNumerics software, version 4.61 (Applied Maths NV, Sint-Martens-Latem, Belgium) (Figure 2). *Salmonella Braenderup* H9812 was used as molecular weight marker (13).

Figure 1. Taq Man Real Time-Polymerase Chain Reaction Amplification Plots



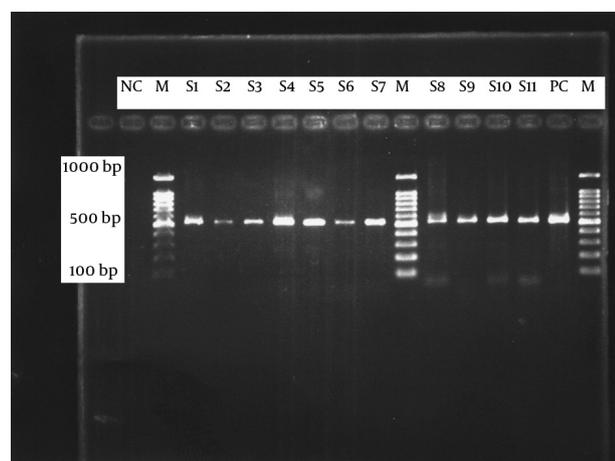
All samples that were positive in culture test were also positive in RT-PCR. They are shown as sharp curve. (Several *B. pertussis* samples double tested in RT-PCR). Abbreviations: RT-PCR, real time-polymerase chain reaction.

Figure 2. Pulsed Field Gel Electrophoresis Pattern of the *Bordetella pertussis* Strains Digested With *Xba*I



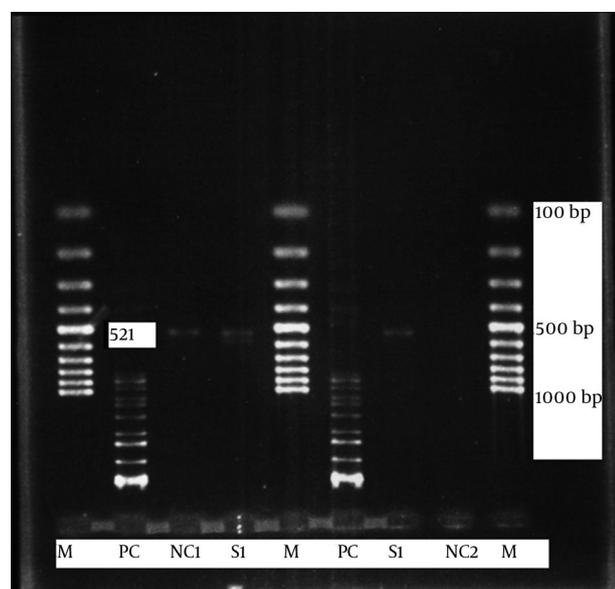
Lane M, genomic pattern of *Salmonella Braenderup* H9812 as a marker.

Figure 3. Amplified 521bp Domain V in *Bordetella pertussis* Strains by Polymerase Chain Reaction



Abbreviations: MP, 100bp marker plus; NC, negative control; PC, positive control; S, samples.

Figure 4. Screening for the A2047G and A2058G Mutations by Polymerase Chain Reaction - Restriction Fragment Length Polymorphism Analysis



The 521bp fragment of the 23SrRNA gene amplified by PCR from the *B. pertussis* clinical isolates and controls; Abbreviations: *B. pertussis*, *Bordetella pertussis*; PCR, polymerase chain reaction.

3.5. DNA Extraction

Genomic DNA was extracted from isolated bacteria by a commercial high pure extraction kit (Roche Diagnostics GmbH, Mannheim, Germany). Quantity of extracted DNA was measured by NanoDrop ND-1000[®] Spectrophotometer (NanoDrop, Wilmington, DE, USA) and adjusted to 500 ng μL^{-1} .

3.6. Polymerase Chain Reaction

The A-G transition mutations at 23SrRNA (domain V) were analyzed by using the PCR method. The amplification protocol was originally described in another study (4). Briefly, PCR was performed by 5 μL genomic DNA, as template, in a total volume of 25 μL , containing 10 μL master amplicon (Promega, Fitchburg, WI, USA), 1907U primer 5'-TTCCTTGTCGGGTAAGTCC-3'/1pmol/ μL , 2408L primer (Bioneer, Daejeon, Korea) 5'-GCGGTATCAGCCTGTTATCC-3'/1pmol/ μL and 8 μL distilled water. The PCR process was optimized with purified DNA of *B. pertussis* (ATCC 9797), as positive control. Amplification of 521bp products was performed on Gene Amp PCR system (Applied Biosystems, Waltham, MA, USA) utilizing a program as follows: an initial cycle of denaturation 95°C for 5 minutes, followed by 30 cycles of 95°C for 1 minute, 60°C for 1 minute and 72°C 1 minute. Amplified products were visualized on 1.5% agarose gel (KBC, Maw Pure agarose, Spain) stained by ethidium bromide, by a Gel Logic 212 Pro imaging system (University of Alaska, AK, USA) (Figure 3). Utilized primers were synthesized by Bioneer Corporation (Bioneer, Daejeon, Korea).

3.7. Sequencing

The PCR products were purified using a commercial gel purification kit (QIAquick PCR Purification Kit, Qiagen, Venlo, The Netherlands). The sequencing process was accomplished by applying the dye terminal method in an ABI 377 sequencer (Applied Biosystems, Waltham, MA, USA). Sequencing results were interpreted through a query to the Gen Bank local alignment search tool (BLAST) (blast.ncbi.nlm.nih.gov/Blast.cgi?).

3.8. Restriction Fragment Length Polymorphism

Transition mutation in domain V (23SrRNA) at position 2047 or 2058 is predicted to generate a new restriction site for BbsI enzyme (Fermentase, Vilnius, Lithuania). Amplified products were targeted for the activities of digestive enzymes BbsI, according to the manufacturer recommendations. Finally, products were resolved by Tris Borate EDTA gel by electrophoresis on the 2% agarose. Restriction fragments length polymorphism (RFLP) pattern was stained and observed in 2% agarose gel (KBC, Maw Pure agarose, Spain) by a Gel Logic 212 Pro (University of Alaska, AK, USA) instrument (Figure 4).

4. Results

Out of 1084 collected nasopharyngeal swabs from suspected patients, 11 specimens (1.01%) were positive by conventional test, such as bacterial culture. Using the Taq Man RT-PCR, all *B. pertussis* strains were approved and then, by the PFGE method, genetic relationships between the isolates were determined. To find genetic relationships between the isolates, we obtained genomic patterns of the isolates by PFGE analysis, which revealed six PFGE profiles

(A-F), among which three and two isolates have the same patterns in profiles A and B, respectively. Hopefully, macrolide resistant *B. pertussis* strain was not among the isolates in profile A, which was considered as a predominant profile (with five isolates) in this research.

Susceptibility tests for mentioned antimicrobial agents showed that: all *B. pertussis* isolates were susceptible to azithromycin; one isolate of *B. pertussis* with MIC break point consisting: erythromycin = 128 µg/mL, clarithromycin >256 µg/mL, was screened as high level macrolide resistant. Excluding one isolate with MIC = 0.12 µg/mL to azithromycin, all the isolates showed MIC ≤ 0.06 µg/ml to this antibiotic. From 11 strains, which were examined for MIC, strains five and six showed MIC ≥ 2 µg/mL to erythromycin. Data demonstrated that azithromycin can be a good choice of drug to treat patients infected by *B. pertussis*, in our country. The detailed data are listed in the Tables 1 and 2.

Following the PCR process, 23SrRNA PCR products were observed in all *B. pertussis* strains (Figure 4). After performing RFLP, results demonstrated that there was no cutting site for BbsI enzyme in the 23SrRNA PCR product of macrolide resistant *B. pertussis* (Figure 4). The result of sequencing showed that there was no cutting site for BbsI enzyme or transition mutation (A-G) at nucleotide positions 2047 or 2058, in a critical region for erythromycin binding of the 23SrRNA gene.

5. Discussion

Due to poor lifelong immunity, whooping cough continues, despite national immunization programs. Widespread antibiotics were administered for the treatment and control of transition *B. pertussis* bacterium, leading to emergence of resistant strains. Then improvement of vaccines and vaccine schedules for pertussis is a necessity. Laboratory diagnosis of pertussis is traditionally based on culture, which is highly specific, even though it is maximally sensitive only in the initial phases of the disease (14).

There is a need for more rapid and sensitive diagnostic methods that have high positive predictive value, especially in the early stages of disease. As is the case for other fastidious organisms, PCR offers an attractive alternative for detecting *B. pertussis* and *B. parapertussis* in clinical specimens (15, 16). All positive *B. pertussis* colonies were confirmed by Taq Man RT-PCR. Erythromycin has been used in the USA for over 45 years, for the treatment of patients with pertussis and is currently recommended for prophylaxis, as well (16). The high level erythromycin resistant *B. pertussis* isolates in the USA carry an A-G transition at nucleotide position 2047 of the 23SrRNA gene, in a region critical for erythromycin binding (4).

We detected just one strain, as high level macrolide resistant *B. pertussis*. After performing RFLP-PCR and

Table 1. Detailed Data From Eleven Isolated Bacteria out of 1084 Specimens^a

Isolates No.	Strains	Age of Patients	Province of Iran	Year	PFGE Pattern	MIC (Eryth)	MIC (Clarith)	MIC (Azith)
1	BpT1	2, mon	Tehran	2009	A1	< 0.06	< 0.06	< 0.06
2	BpM1	3, mon	Mazandaran	2009	A1	< 0.06	< 0.06	0.125
3	BpT2	3, mon	Tehran	2009	A1	< 0.06	< 0.06	< 0.06
4	BpE1	2, y	Isfahan	2009	A2	< 0.06	< 0.06	< 0.06
5	BpQ1	18, y	Qom	2009	A3	< 0.06	2	< 0.06
6	BpM2	3, y	Mazandaran	2009	B	4	8	< 0.06
7	BpK1	5, mon	Khuzestan	2010	B	4	32	< 0.06
8	BpKO1	4, y	Khorasan	2009	C	4	8	< 0.06
9	BpM3	1, mon	Mazandaran	2009	D	< 0.06	0.5	< 0.06
10	BpK2	1.5, mon	Khorasan	2009	E	2	< 0.06	< 0.06
11	BpK3	26, d	Khorasan	2009	F	128	> 256	< 0.06

^a Abbreviations: Azith, azithromycin; Clarith, clarithromycin; d, days; Erythr, erythromycin; MIC, minimum inhibitory concentration; mon, months; y, years.

Table 2. Detailed Data From All Samples, Such as Age and Vaccinated Suspected Patients to Pertussis

Variables	Values				
Specimens (Vaccinated)	152	586 (486)	157 (143)	111 (63)	78 (68)
Isolated Bacteria (Vaccinated)	3 (0)	4 (3)	2 (2)	1 (0)	1 (0)

sequencing of 23S_rRNA gene, in this strain, we realized that the high level resistance was not dependent on the A–G transition at nucleotide position 2047 or 2058 of the 23S_rRNA gene. We suggest that other mechanisms, responsible for the resistance of this bacterium, should be considered. Given the low frequency of macrolides resistant *B. pertussis*, and to prevent the spread of these bacteria in high risk groups, a program for the identification of resistant bacteria in the laboratories and improvement of vaccines, vaccine schedules regarding pertussis, is necessary (4-6, 8).

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