Searching the *B. pertussis* (Real Time PCR) in Cases with Prolonged Cough (>2 weeks) in Population with Unvaccinated Adult: A Cross Sectional Study. Tehran, Iran

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Abstract

**Aims:** searching the *B. pertussis*-DNAs by Real time PCR in nasopharyngeal samples of cases with prolonged cough (>2 weeks) in population with unvaccinated adult.

**Study design:** A cross sectional/analytic study done in Rasoul Akram hospital (May 2007-March 2009); Tehran, Iran.

**Methodology:** According to WHO criteria; 170 cases with cough (>2 weeks) with either paroxysm of cough, inspiratory whoop, or post-tussive vomiting and other cases with cough (in contact with the index case) selected for study. *B. pertussis* searched in nasopharyngeal samples by conventional and Real time-PCR.

**Results:** Out of 170 studied cases (mean age 27.3 years). 34.3% had previous contact with other cases with prolonged cough. 50% was febrile. 67.6% presented with WHO criteria and 32.4% had not any other symptom except prolonged cough. Abnormal chest x-ray reported in 61.6%. Positive *B. pertussis* -DNAs detected in samples by conventional-PCR and Real time-PCR was 20% and 2.4% respectively. 2 cases with positive Real time-PCR were >6 month and 2 adults in contact with chronic cough children.

**Conclusion:** Whooping cough caused by the bacteria *B. pertussis* and *B. parapertussis*. High false positive (20% and 2.4%) results by conventional PCR usually indicated to nasopharyngeal carriage state not *B. pertussis* disease so it is preferred to use Real time-PCR for definite diagnosis of *B. pertussis* in suspected cases. For disease prevention in adolescents and control the circulation of the organism, booster vaccination (with acellular pertussis vaccines) in older children, adolescents, and adults are needed.

**Keywords:** *B. pertussis*; Chronic cough; Polymerase chain reaction; Real time-PCR

Introduction

Whooping cough is a worldwide infectious disease caused by the bacteria *B. pertussis* and *B. parapertussis* [1,2]. Incidence is highest in children under five except where infant vaccination programs have been very effective and a shift has occurred to adolescents. Whooping cough is not only a childhood disease. Routine mass vaccination of infants and children has been effective in decreasing the mortality and morbidity of the disease in children but has not eliminated the circulation of *B. pertussis* [3,4]. The older children and adolescents can then become a source of infection for neonates and young infants who have not yet completed their vaccination schedules. It is dramatic for neonates and infants but can be very severe for children and adults [3,4]. Two weeks after the beginning of the cough it becomes very difficult to isolate the bacteria, suggesting that most of the symptoms are due to toxins released by the bacteria. When treated with erythromycin, clarithromycin or azithromycin, patients are no longer contagious after five days of treatment [4]. PCR has been found to be more sensitive and more specific than other methods [5].

The WHO case definitions are based on clinical presentation alone, but do include recommendations on laboratory diagnostics [6]. Until pertussis can be accurately diagnosed, its burden will remain underestimated, making the introduction of epidemiologically appropriate preventive strategies difficult [7]. Common laboratory diagnostic methods used for pertussis diagnosis include culture, direct- fluorescent-antibody testing (DFA), serology and polymerase chain reaction (PCR). Culture of *B. pertussis* is highly specific but fastidious and has limited sensitivity [8,9]. DFA provides a much more rapid result, but has the disadvantage of poor sensitivity and specificity. Serology is not useful in infants. In older persons, it is hampered by the limitations of paired sera and it provides mainly a retrospective diagnosis [10]. ELISA tests for *B. pertussis* and pertussis toxin antibodies allow a diagnosis to be made even if the patient does not seek treatment during early stages [10]. In addition, antibody testing can also verify immunity after vaccination such limitations of conventional diagnosis testing have led to the development of PCR [11]. In previous serological studies performed in different countries, pertussis protective antibodies were reported in a wide range of 30% to 97% [12].

The pertussis vaccination program in Iran includes three doses of a whole-cell pertussis vaccine together with diphtheria and tetanus toxoid (DT w P) at months 2, 4, and 6 of life and then two additional

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booster doses at 1.5 and 4–6 years [13]. In spite of a more than 95% coverage rate for the third dose of pertussis vaccine, a resurgence of pertussis is suspected in Iran. The incidence of pertussis in Iran has decreased from 40% in 100,000 populations in 1978 to 0.5% in 100,000 populations in 2007 [13]. The Iranian Centers for Disease Control reported that the incidence of B. pertussis in Iran was 0.5 cases per 100,000 population in 2008 – higher than the previous year, 0.19 cases per 100,000 population [14]. Zahraei and Doosti [15] reported the vaccine response and seroconversion for pertussis in 70.3% of the in Iranian preschool children [13]. In last decade at least 3 studies have been carried out about pertussis seroprevalence in Iran [16-18]. Sherkat et al. [16] reported 48% positive serology for IgG-PT in adolescents with prolonged cough in Isfahan [16]. Eslamifar et al. [18] study showed that B. pertussis infection is on the rise in Iranian adolescents and young adults [18]. The elevation of pertussis antibody rates and levels in Iranian subjects reflects the acquisition of natural infection with B. pertussis following the joining cases to crowded community with a high probability of communicable disease. They recommended for using a more immunogenically effective vaccine to ensure sufficient immunity. These conflicting data may be related to various factors such as the size and age of the study groups, and the demographic and epidemiologic factors [16-18].

Acellular pertussis (aP) vaccines have now been used in adolescents and adults and they are effective and safe for pertussis protection but not in Iran [19-23].

**Main Goal of Study**

Searching the B. pertussis-DNAs by Real time PCR in nasopharyngeal samples of cases with prolonged cough (>2 weeks) in Iranian population with unvaccinated adult.

**Material and Methods**

A cross sectional/analytic study was carried out in Rasoul Akram hospital (May 2007-March 2009) Tehran, Iran. The study was approved by the Ethical Committee in Research Center of Pediatric Infectious Diseases in Tehran University of Medical Sciences.

**Data collection**

After obtaining parental consent, all children with a cough of 2-week duration or more and other cases with cough in contact with the index cases selected for study. Initially, a questionnaire was completed by an authorized physician for each case (e.g., age, gender, other relevant demographic variables), clinical manifestations of pertussis viz. duration of cough, presence of coughing paroxysms, whooping and vomiting after cough, cyanosis.

**Case definition**

According to WHO criteria cases with cough more than 14 days with either paroxysm of cough, or inspiratory whoop, or post-tussive vomiting were taken in this study.

**Exclusion criteria**

All cases with bacterial or other known causes for cough; proven bacteria or viral causes; children with an immune deficiency or congenital heart diseases, lung anomalies or chronic lung disease (asthma, cystic fibrosis, gastro esophageal reflux) abnormal cardiac or lung finding in examination, excluded from study.

Two dacron swabs from the posterior nasopharynx obtained from the cases, 1 swabs inoculated onto the Regan–Lowe medium tube and send to other research center for specific culture.

Other Swabs was washed with buffer solution. The washed off solution was transmitted to micro-tubes for PCR tests.

Extraction of DNA from specimens was performed using the DNA extraction kits (Roche, Germany).

Detection of B. pertussis was based on the amplification of a section of the IS481. The amplification of the sequences IS1001 was used for detection of B. parapertussis (8.1 µl of primer for preparation of 50 µl work stock). The proper primers were supplied by MWG (Germany), and synthesized on an automated DNA synthesizer.

The sequences of the oligonucleotide primers were as followed:

- B. pertussis-sense primer (PPert)
- ATCAAGCACCGCTTTACCC
- B. pertussis-antisense primer (APpert)
- TTTGGGAGTTCTGGTAGGTGTG
- B. parapertussis-sense primer (PPParaP)
- GATATCAACGGGTAGCCGATC
- B. parapertussis-antisense primer (APParaP)
- GTATGCCAACCAGTTGCAA

Real time PCR (International AG Metabion Kïagen Company; Iran, Tehran) used as Kosters et al. methods [5]. B. pertussis ATCC 9797 and B. parapertussis ATCC 9305 (Pasteur Institute of Iran) prepared for positive and negative controls. 40 cycle, Fam method

**Statistical analysis**

The student’s t test was used to determine significant in means for continuous variables. The mann-whitney test and the chi-square test were used to compare groups. p-values less than 0.05 were considered statistically significant.

**Results**

Out of 170 studied cases, 45.9% male and 541% female; mean age 27.3 years were enrolled in this study. 34.3% of cases had previous contact with other cases with prolonged cough. 50% of cases were febrile. 67.6% of cases presented had full WHO criteria (e.g. paroxysm of cough, inspiratory whoop, post-tussive vomiting, convulsion, conjunctional haemorrhage). 32.4% of cases had not any other symptom except prolonged cough.

Abnormal chest X-ray reported in 61.6%. Positive B. pertussis - DNAs detected in samples by conventional-PCR and Real time-PCR was 20% and 2.4% respectively. Two cases with positive Real time-PCR were <6 month and 2 adults in contact with chronic cough children.

**Results and Discussion**

Here, 170 subjects with prolonged cough (>2 weeks) without other apparent causes studied. 67.6% of cases presented with at least one WHO criteria (e.g. paroxysm of cough, inspiratory whoop, post-tussive vomiting, convulsion, conjunctional haemorrhage). 32.4% of cases had not any other symptom except prolonged cough. Abnormal chest X-ray detected in 61.6%. Positive B. pertussis - DNAs obtained by conventional and Real time-PCR was 20% and 2.4% respectively.

Unfortunately all culture results for B. pertussis were negative, near 95% of all studied cases had received antibiotics before study. Although the Bordetella infection confirmed by Positive conventional - PCR in 20% of studied cases but just 2.4% of those had positive Real time-PCR.
Real time PCR has greater specificity than the conventional PCR since the conventional appears to have greater sensitivity for all Bordetella species. Negative cultures support this theory.

Two confirmed cases with a positive Real time-PCR were <6 month and 2 adults in contact with chronic cough children. This study confirms that in children, pertussis is characterized by paroxysmal cough, whooping cough and post-tussive vomiting; but in adults the disease is often atypical and sometimes only manifested by a protracted cough, which are close to Cherry et al. [2] and Ghanaei et al. study [6]. Our study confirmed the B. pertussis in 2 adult cases which were in contact with chronic cough children. Like us the frequency of diagnosed cases by a positive PCR in Ghanaei et al. [6] study increased significantly with age, but this relationship did not persist for para pertussis [4].

Bamberger et al. [23] showed that clinical criteria had no significant association with infection in recently vaccinated children. 76% of unvaccinated, 39% of recently vaccinated, and 40% of post-vaccinated children with a positive PCR did not meet the CDC diagnostic criteria for B. pertussis [21].

Positive B. pertussis -PCR by gold standard test was 2.4% which is lower than Ghanaei et al. [6] study in School children (with prolonged cough). They showed; positive PCR for B. pertussis and B. parapertussis, positive culture for B. pertussis 6.4%; 1.8%; and 2% respectively. None colony of B. parapertussis was found on culture. The specificity of PCR was 15.0%. 94.9% agreement between PCR and culture reported. Although the different age group between 2 studies could explain those differences, different case definition or methods is another reason. The false positive results (6.4%) in Ghanaei et al. study [6] (vs. 2.5% of present study and 1.8% of positive culture) might be due to nasopharyngeal carriage state in school aged children not B. pertussis disease. Differentiation of B. parapertussis from B. pertussis just by conventional PCR is difficult and misleading in Ghanaei et al. [6] study, overestimated the B. pertussis in school children. B. pertussis and B. parapertussis are similar species but B. parapertussis lacks the expression of the gene coding for pertussis toxin. Differentiation between B. parapertussis and B. pertussis is based on culture, biochemical and immunologic differences [2].

We agree with Ghanaei et al. [6] that the low reported incidence by CDC in Iran [15] may be due to some factors; insufficient surveillance, cyclic pattern of disease distribution, or misconception of Iranian health workers that pertussis has been eradicated by the high vaccination coverage in our country. The estimated incidence of B. pertussis and B. parapertussis and culture in school aged was 318; 2; 4 cases per 100 000 population [6].

In the pre-vaccination period, natural immunity was achieved early in the life and maintained through frequent contact with the organism, which acted as a natural booster [1-3]. B. pertussis protective antibodies were reported in a wide range of 30% to 97% [17-23] it has also been shown that the pertussis sero prevalence increases with age. As vaccine antibodies begin to wane 4 years after the last dose and immunity to pertussis vaccine decreases to 0% to 20% over a 10-year interval [17-23].

At least 4 other Iranian studies (serologically) confirmed our results. The seroprevalence of IgG antibodies against B. pertussis in Iranian population is not protective [15-18]. Two recent studies showed that B. pertussis infection is on the rise in Iranian adolescents and young adults [6,16]. Sherkat et al. [16] study is close to us, in adolescents with prolonged cough (Isfahan, center part of Iran) 48% positive serology for IgG-PT observed. Hashemi et al. [17] showed the overall prevalence of pertussis IgG -PT in asymptomatic; 48% (mean= 44 ± 47.7 U/ml); 47.6% (mean=71.7 U/ml) respectively. All studies are in contrast to Zahraei and Doosti [15]. Zarei et al. [13] reported the vaccine response and sero conversion for pertussis just in 70.3% of the in Iranian preschool children [13]. The Immunity following the vaccination or natural disease is not life-long and re infection can occur. This causes the transmission of the disease to infants prior to the completion of their routine immunization, unless passively acquired antibodies are present in sufficient titers. So, as we found, adolescents, and adults in our country are vulnerable to the infection due to waning of the vaccine-acquired immunity. Hashemi et al. [17] study describe the seroprevalence of Immunoglobulin G antibodies against pertussis toxin among asymptomatic medical students in the west of Iran (Hamedan). 47.6% seropositivity of IgG-PT, with a mean level of 71.7 U/ml [17]. Esfandiari et al. [18] survey, 39.1% of children in the 6-10 year age group showed pertussis antibodies, over half (53.1%) of the children aged 8 months to 6 years were negative for pertussis antibodies It may be due to low immunogenicity of the licensed DTaP vaccines which related to the bacterial strain used or the formulation protocol adopted for the vaccine [18]. The efficacy and antibody responses of a whole-cell vaccine were unexpectedly low in other countries [19-22]. Probably, the elevation of pertussis antibody rates and levels in Iranian subjects reflects the acquisition of natural infection with B. pertussis following the joining cases to crowded community with a high probability of communicable disease [14-16]. Some Iranian authors recommended for using a more immunogenically effective vaccine to ensure sufficient immunity [6,16].

Conclusion

It should be considered that Whooping cough is an infectious disease caused by the bacteria B. pertussis and B. parapertussis. Real time PCR has greater specificity than the conventional PCR since the conventional appears to have greater sensitivity for all Bordetella species. High false positive (20% and 2.4%) results by conventional PCR usually indicated to nasopharyngeal carriage state not B. pertussis disease, so it is preferred to use Real time-PCR for definite diagnosis of B. pertussis in suspected cases. This study confirms that in children, pertussis is characterized by paroxysmal cough, whooping cough and post - tussive vomiting; but in adults the disease is often atypical and sometimes only manifested by a protracted cough. For disease prevention in adolescents and control the circulation of the organism, booster vaccination (withacellular pertussis vaccines) in older children, adolescents, and adults are needed.

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Ethical approval: This research was approved by the ethics committee of pediatric Infectious Diseases Research Center under the Helsinki rules.

Conflict of interest: No conflict of interest to declare.

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