Comparison of nasopharyngeal culture, polymerase chain reaction (PCR) and serological test for diagnosis of pertussis

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This prospective study, which was designed to compare nasopharyngeal culture, polymerase chain reaction (PCR) and serology in the diagnosis of pertussis, covered 35 children aged between 0 and 16 who were admitted to Hacettepe University İhsan Doğramacı Children's Hospital between 1 March 2005 and 31 August 2006 with coughing for 7 days or longer, paroxysmal cough of any duration, or cough with inspiratory whoop and/or vomiting (or apnea) after coughs. The demographic data and vaccination history of the patients were recorded. During the initial examination, samples were taken from the posterior nasopharynx for *Bordetella pertussis* (*B. pertussis*) culture and PCR analysis. In order to determine antibody positivity and antibody levels against *B. pertussis* antigens, serum samples were taken during the initial examination (acute phase) and two weeks later (convalescent phase). In the first serum sample, immunoglobulin M (IgM) was determined against pertussis toxin. In the first and second samples, IgA and IgG antibodies were evaluated against pertussis toxin and filamentous hemagglutinin. Culture yielded negative results in all of the patients. PCR was positive in two cases (5.7%). In the PCR-positive patients, IgM, IgA and IgG type anti-pertussis antibodies were found to be positive in the first serum samples, and IgA and IgG antibodies were found to be positive in the second serum samples. Therefore, it was considered that serology could be as sensitive as PCR when type IgM, IgA and IgG antibodies were found to be positive against a minimum of two antigens of *B. pertussis*. In conclusion, both PCR and serologic tests -if evaluating all types of antibodies to a minimum of two antigens of *B. pertussis* obtained in both acute and convalescent sera- could be more sensitive than culture in the diagnosis of pertussis.

Key words: pertussis, culture, polymerase chain reaction (PCR), serology.

Pertussis is one of the most common causes of death from infectious diseases worldwide, and pertussis epidemics still prevail in developing countries due to the low vaccination coverage of children[1]. According to the World Health Organization (WHO) reports, pertussis causes 20-40 million cases and 350,000-400,000 fatalities per annum worldwide, primarily including the unvaccinated children in developing countries[1,2]. However, it is believed that the actual figures are much higher[1]. In order to avoid the complications and mortality of the disease, laboratory techniques that enable accurate diagnosis in a short period of time are required.

Isolation of *Bordetella pertussis* (*B. pertussis*) through nasopharyngeal culture is regarded as the gold standard for the diagnosis of pertussis due to its very high specificity (100%)[1,3,4]. However, it takes 7-12 days to obtain the results of pertussis culture[4,5], and the sensitivity of the culture has been shown to be low and

may vary depending on the immunity from past infection or from vaccination, the stage of disease, how the specimen is handled, the age of the patient, and the effective antibiotic pretreatment prior to culture[1,3,6-8].

For these reasons, studies are being conducted on methods providing faster results for diagnosis with higher sensitivity. In recent years, the detection of *B. pertussis* DNA through polymerase chain reaction (PCR) in nasopharyngeal secretions obtained through aspiration or swab has gained further importance as a highly sensitive (80-100%) and specific method for the diagnosis of pertussis and as a method faster than culture[1,3,4]. However, with non-standardized PCR, false-positive and false-negative PCR test results may occur[3,9,10] and PCR cannot be applied everywhere due to technical reasons. In addition, a Food and Drug Administration (FDA)-licensed PCR test is not yet available.

Various serological tests have been used to detect the presence of antibodies to *B. pertussis* antigens or to measure the level of antibodies in serum[1,3,4,7,8,11-18]. Because the enzyme-linked immunosorbent assay (ELISA) method is specific, easier to perform, cheaper and requires less serum, it has become the preferred serological method of detecting *B. pertussis* antibodies in the last 20 years[3,11]. The generally accepted serological criterion in the diagnosis of pertussis is the detection of, or significant increase in the concentration of, one or more immunoglobulin type (IgM, IgG and IgA) antibodies to one, multiple or all antigens of *B. pertussis* in acute and convalescent serum samples[1,19]. It has been reported that it would be appropriate to take the first serum sample within two weeks after the cough starts, and to take the second sample 2-4 weeks later[12].

There are studies in the literature that compare the efficacy of culture, serology and PCR in the diagnosis of pertussis[4]. As compared with culture, it has been reported that the number of cases diagnosed through PCR is at least three times higher, that the results of serology and PCR are highly consistent diagnostically, and that the number of positive cases detected through serological test is much higher than the number of positive cases detected through culture[4]. The literature particularly emphasizes that *B. pertussis* infection may be diagnosed more rapidly through PCR and serological tests.

This study was intended to: compare the efficacy of culture, PCR and serology in the diagnosis of pertussis, since a consensus has not yet been reached as to which laboratory method is more reliable; identify an appropriate and reliable laboratory method under the circumstances in Turkey, where the whole-cell pertussis vaccine is routinely used; determine whether PCR or serology presents any advantages as compared with culture; and determine whether it would be appropriate to use one of these methods in place of or in addition to culture.

Material and Methods

The study group consisted of patients aged 0 to 16 who were admitted to Hacettepe University İhsan Doğramacı Children's Hospital between 1 March 2005 and 31 August 2006 with suspicion of pertussis due to a coughing history of seven days or longer, paroxysmal cough episodes of any duration, and cough with inspiratory whoop and/or vomiting (or apnea) after coughing without any other apparent cause. These selection criteria were based on the ''suspected case'' definition of the Centers for Disease Control and Prevention[20-22]. Patients who had taken an effective antibiotic (macrolides or trimethoprim-sulfamethoxazole) in the last two weeks were excluded from the study. Written informed consent to participate in the study was obtained from the parents and from the children themselves if they were old enough. During the first visit (when the patients were enrolled in the study), a standardized questionnaire was filled in by the patients, including the demographic data of patients, the symptoms and findings of disease (cough, whether the cough is of paroxysmal nature, presence of inspiratory whoop, post-tussive vomiting or post-tussive apnea), their duration, immunization histories, presence of fever, city of residence, and presence of relatives with similar symptoms.

The protocol for this study was approved by the Hacettepe University Medical Faculty Ethics Committee for Medical, Surgical and Pharmaceutical Researches. This study was supported by Hacettepe University Scientific Researches Unit.

Microbiology

During the first examination of patients, specimens (secretions) were taken from the posterior nasopharynx for *B. pertussis* culture and PCR assay.

Culture: Nasopharyngeal specimens were obtained using a Dacron swab inserted slowly through the nostril to the posterior pharynx. The swab was left in the region for 10-15 seconds. All specimens (swabs) were inoculated onto freshly made Bordet-Gengou agar. The media were incubated at 35-36°C in a moist atmosphere and examined every day for 14 days.

PCR: The nasopharyngeal samples taken for PCR assays were placed in 1 ml phosphate-buffered saline and were stored at -80°C before genomic DNA extraction. DNA isolation was done through MagNa Pure LC Total Nucleic Acid Isolation Kit (Roche Diagnostics; Mannheim, Germany) according to the manufacturer's protocol. The DNA presence of *B. pertussis* was detected through the pre-defined PCR method[23,24]. The primers were selected from the repeated gene element of *B. pertussis* (IS481)[23,25]. The final PCR product was analyzed by gel-electrophoresis by using 1.4% agarose and the amplicon was 181 bp in size. PCR products were visualized and photographed under UV light with wave length of 254 nm.

Serology

Blood samples were taken and serum was separated during first examination (acute period) and two weeks later (convalescent period). Acute and convalescent serum samples were stored at -20°C until they were tested. In the first serum samples, the presence of type IgM antibody against pertussis toxin (PT), and in the first and second samples the presence of type IgG and IgA antibodies against PT and filamentous hemagglutinin (FHA) were tested qualitatively and semi-quantitatively through the ELISA method, in line with the instructions of the kit (Genzyme Virotech®; Rüsselsheim, Germany). Test results were read at the wavelength of 420/620 nm, using spectrophotometric reader (ELX808, BioTek®; Vermont, USA) on 96-well microplates. Because seven cases were not brought back by their parents, their second serum samples could not be obtained and therefore their convalescent antibody levels could not be studied. During the phone interview with the parents of these seven children, the parents declined having blood samples taken from their children for a second time, since their child's cough complaints had resolved and their general situation was good.

Results

Of the 35 cases enrolled in our study, 23 were male (65.7%) and 12 were female (34.3%). Their ages varied between 2 months and 13 years (mean: 6.5 ± 3.9 years) (Table I). Three of the cases were aged ≤3 months. Four cases were aged <1 year. There were 4 cases in the age group 1-4, 11 cases in the age group 5-8, 11 cases in the age group 9-12, and 5 cases in the age group 13-16 (Table I). Thirty-four of the cases (97.1%) were fully vaccinated according to their ages. One case aged 2 months was not vaccinated.

The cough duration of cases ranged from 3 to 90 days (mean cough duration: 34.6 ± 26.6 days). Cough duration was <7 days in 2 cases, between 7-13 days in 4 cases, between 14-20 days in 8 cases, between 21-27 days in 5 cases, and between 28-90 days in 16 cases (Table I). Paroxysmal cough was detected in 22 cases (62.9%), inspiratory whoop was detected in 14 cases (40%), and vomiting following cough was detected in 9 cases (25.7%). None of the cases had apnea.

Twenty-one of the cases were enrolled in the study in spring, 12 in winter and 2 in autumn.

Hospitalization was not required for any of the patients.

B. pertussis could not be isolated in any of the patients' nasopharynx culture. PCR was found to be positive in 2 cases (5.7%) (Table II). These patients were aged 5 and 8. The cough duration of both cases was ≥14 days; the number of their pertussis vaccines conformed to that recommended for their ages.

Because antibody levels were tested qualitatively and semi-quantitatively in our study and 34 of the cases (97.1%) were fully vaccinated according to their ages, a cut-off value to determine whether the positivity in their antibody level was due to vaccination or infection could not be specified.

In the PCR-positive cases, type IgM, IgA and IgG antibodies were found to be positive in the first serum samples, and type IgA and IgG antibodies were found to be positive in the second serum samples (Table II).

Discussion

The gold standard in the diagnosis of pertussis is isolation of *B. pertussis* from the posterior nasopharynx through culture[1,3]. When the

Table I. Demographic Data and Symptoms of Patients

Case No.	Age Yr (mo)	Sex	Vaccination status	Cough duration (day)	Paroxysmal cough	Inspiratory whoop	Vomiting	Month of application
1	6 (74)	M	FV	30	N	N	N	March
2	12 (155)	M	FV	60	Y	N	N	March
3	5 (70)	M	FV	20	Y	N	Y	April
4	2 (32)	M	FV	45	Y	N	N	April
5	5 (69)	M	FV	90	Y	Y	N	April
6	10 (130)	M	FV	90	Y	N	N	April
7	8 (107)	F	FV	14	Y	N	Y	April
8	0 (7)	F	FV	10	Y	N	N	April
9	9 (111)	M	FV	30	N	N	N	April
10	4 (57)	F	FV	45	Y	Y	N	April
11	8 (100)	M	FV	24	Y	Y	N	May
12	9 (118)	M	FV	60	Y	N	N	May
13	8 (97)	M	FV	75	N	N	N	May
14	4 (49)	M	FV	90	N	N	N	May
15	7 (104)	M	FV	18	Y	Y	Y	May
16	4 (51)	F	FV	21	Y	N	N	November
17	9 (113)	F	FV	90	N	Y	Y	November
18	4 (49)	M	FV	3	Y	Y	N	December
19	5 (67)	F	FV	45	N	N	N	December
20	12 (154)	M	FV	20	N	N	N	January
21	11 (134)	F	FV	21	N	Y	N	January
22	13 (156)	F	FV	60	N	Y	N	January
23	10 (125)	F	FV	21	N	N	N	January
24	4 (55)	M	FV	30	Y	Y	Y	January
25	2 (25)	M	FV	20	N	N	N	January
26	2 (32)	M	FV	7	Y	Y	N	February
27	13 (163)	F	FV	14	N	Y	N	February
28	10 (128)	M	FV	14	N	N	N	February
29	13 (157)	M	FV	40	Y	Y	N	February
30	0 (2.5)	M	FV	7	Y	Y	Y	March
31	0 (2)	M	Un-vaccinated	4	Y	Y	Y	March
32	0 (3)	M	FV	20	Y	N	N	April
33	7 (88)	M	FV	25	Y	N	N	May
34	7 (95)	F	FV	40	Y	N	Y	May
35	3 (46)	F	FV	10	Y	N	Y	May

M: Male. F: Female. N: No. Y: Yes. FV: Fully vaccinated for her/his age.

Table II. Culture, PCR and Serology Results of Patients

			Serology				
			First serum sample			Second serum sample	
Case No.	Culture	PCR	Anti-PT IgM	Anti-PT and anti-FHA IgA	Anti-PT and anti- FHA IgG	Anti-PT and anti-FHA IgA	Anti-PT and anti-FHA IgG
1	N	N	N	N	N	N	N
2	N	N	N	N	N	N/A	N/A
3	N	P	P	P	P	P	P
4	N	N	N	N	N	N	N
5	N	N	N	N	P	N	P
6	N	N	N	N	P	N	P
7	N	P	P	P	P	P	P
8	N	N	N	N	P	N	P
9	N	N	N	N	N	N	N
10	N	N	N	N	N	N/A	N/A
11	N	N	N	N	P	N	P
12	N	N	N	N	P	N	P
13	N	N	N	N	N	N/A	N/A
14	N	N	N	N	N	N	N
15	N	N	P	N	P	N	P
16	N	N	P	N	N	N	N
17	N	N	P	P	N	P	N
18	N	N	N	N	N	N	N
19	N	N	P	N	P	N	P
20	N	N	N	N	P	N	P
21	N	N	P	N	N	N	N
22	N	N	N	N	P	N/A	N/A
23	N	N	N	N	P	N	P
24	N	N	P	P	P	P	P
25	N	N	N	N	P	N/A	N/A
26	N	N	P	N	P	N	P
27	N	N	P	N	P	N	P
28	N	N	P	N	P	N	P
29	N	N	N	N	P	N	P
30	N	N	N	N	P	N	P
31	N	N	N	N	P	N	P
32	N	N	N	N	P	N/A	N/A
33	N	N	N	N	P	N/A	N/A
34	N	N	N	N	P	N	P
35	N	N	N	N	N	N	N

FHA: Filamentous hemagglutinin. PT: Pertussis toxin. N: Negative. P: Positive. NA: Not available.

specimen (secretion) is obtained from the posterior nasopharyngeal wall in the form of nasal swab or nasopharyngeal aspirate, the sample must be immediately and directly (without placement on transport medium) placed on the culture medium (Bordet-Gengou), thereby increasing the possibility of isolating *B. pertussis*[1,3]. It has been reported that the rate of *B. pertussis* isolation from the nasopharynx is lower in fully vaccinated patients as compared with unvaccinated patients[3,7]. Although our patients had not used macrolides before the culture was taken and the specimens obtained for culture were directly inoculated into the freshly prepared Bordet-Gengou agar, the fact that *B. pertussis* could not be isolated in any of the cases is consistent with the literature, which shows that the sensitivity of culture is low[1,3]. The fact that our cases did not include any patient with culture-confirmed pertussis may be attributed to the fact that 97.1% of the cases were fully vaccinated according to their ages.

It has been determined that the possibility of detecting *B. pertussis* in the later stages of the disease is higher with PCR than with culture[3]. In addition, it was demonstrated that PCR-positive and culture-negative results are more common among the vaccinated patients and patients receiving antibiotic therapy[3].

Because the number of patients enrolled in our study was insufficient, the efficacy of culture, serology and PCR in the diagnosis of pertussis could not be statistically compared.

In our study, PCR confirmed pertussis diagnosis in 2 cases (5.7%), which is consistent with the results of studies demonstrating that the sensitivity of PCR is higher than that of culture[3,4,9,25-27]. As compared with culture, it has been reported that the number of cases diagnosed through PCR is at least three-fold[4,9]. For this reason, in the national laboratories of some European countries like Finland and Switzerland and in the United States, PCR has been used routinely for pertussis diagnosis at the national level since the 1990s[28-30].

There are studies indicating that serologic tests increase the possibility of diagnosing pertussis, as compared with nasopharynx culture[4,7,8,17,18,26].

B. pertussis has virulence factors such as PT, FHA, pertactin (PRN), fimbriae (FIM), lipopolysaccharide and agglutinogens[1,11,31]. Among people who have not received pertussis vaccine, after primary infection with *B. pertussis*, immune response develops against various antigens of *B. pertussis* and antibodies can be detected between weeks 1 and 2 following the start of symptoms[3,7]. After infection, antibodies of all isotypes (IgM, IgA, IgG) are produced[3,8]. The antibody that emerges first is IgM, followed by IgA and IgG antibodies[8,11]. The most apparent rise in antibody (especially type IgG) is against PT and FHA[11]. IgG responses to PT and FHA are observed in more than 90% of cases[3]. PT is the single antigen that is specific for *B. pertussis* and it is considered that IgG antibodies developed against PT may be responsible for long-term immunity[3,11,15]. Although it has been reported that the detection of high levels of IgG antibodies against PT in a single serum sample is diagnostic of recent or acute infection with *B. pertussis*, when antibody levels according to age groups are known in the society[19], the cut-off value indicating prevention has not yet been determined[32].

In our country, the pertussis vaccine in the childhood routine vaccination schedule is a whole cell vaccine that is administered to 2-, 3- and 4-month-old children with a booster dose at 16-24 months. In children who have received whole cell vaccine, antibodies that can be detected through ELISA develop against all known antigens of *B. pertussis* (FHA, PT, agglutinogens, lipopolysaccharide and outer membrane proteins)[3,11]. Type IgM and IgG antibodies are produced after vaccination; however, the type IgA antibodies that develop after vaccination are in very low concentrations[8,11]. Although pertussis vaccines have been in use for a long time, reliable antibody levels that determine immunity have not yet been identified[11].

In a study measuring the IgG, IgM and IgA levels against PT and FHA in acute and convalescent serum samples, Granström et al.[16] reported that the measurement of antibodies against a single *B. pertussis* antigen could not suffice for diagnosis, and that the antibody levels measured against two antigens (PT and FHA) increased the possibility of diagnosis. Halperin et al.[8] reported that serological tests (ELISA methods) are more sensitive than culture when the IgG, IgA and IgM antibodies are measured against two *B. pertussis* antigens (PT and FHA) in acute and convalescent serum

samples. Because antibody levels were tested qualitatively and semi-quantitatively in our study and 34 of the 35 cases (97.1%) were fully vaccinated according to their ages, a cut-off value to determine whether the positivity in their antibody level was due to vaccination or infection could not be specified. Among the PCR-positive cases (5.7%), all type antibodies (IgM, IgA and IgG) were found to be positive in the first serum samples, and type IgA and IgG antibodies were found to be positive in the second serum samples. For this reason, it has been determined that serological tests may be as sensitive as PCR when type IgM, IgG and IgA antibodies are found to be positive against at least two antigens of *B. pertussis*. These results obtained in our study support the results of the studies conducted by Granström et al.[16] and Halperin et al.[8]. Fry et al.[4] reported that serological test and PCR results are quite consistent diagnostically. Our study has also revealed consistency between PCR and serological test results.

In our study, it was determined that in the diagnosis of pertussis, PCR and serological tests evaluating paired serum samples (acute-convalescent) -if all antibody types are evaluated- may be more sensitive than culture. Although our study does not enable a statistical evaluation of culture, serology and PCR test results in the diagnosis of pertussis, it implied that PCR and serologic tests may be superior to culture even in the countries where whole-cell pertussis vaccination is applied. However, future studies with more cases are needed to precisely determine whether PCR or serology is more advantageous than culture in the diagnosis of pertussis given the circumstances in Turkey and whether it would be appropriate to use one of these methods alone or in combination with culture.

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