Comparison of Multiplex Polymerase Chain Reaction, Culture, and Serology for the Diagnosis of *Bordetella pertussis* Infection

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Background: Accurate diagnosis of *Bordetella pertussis* infection is difficult. Polymerase chain reaction (PCR) tests are more sensitive than culture, but the reported sensitivity is variable. We prospectively compared the performance of culture, serology, and a multiplex PCR for the detection of *B. pertussis*.

Methods: A total of 193 paired nasopharyngeal (NP) swab specimens were examined by both culture and a multiplex PCR. Serology results were available in 103 patients. Medical charts of the patients with discrepant laboratory findings were reviewed and compared with the United States Centers for Disease Control and Prevention (CDC) clinical case definition.

Results: Of the 193 specimens, 11 were positive on both culture and PCR, and 14 were positive on PCR only. Of the 103 specimens with serology results, 3 were positive with all three methods, and 69 were negative with all methods. Eleven of the 14 PCR-positive only cases and 6 of the 19 serology-positive only cases were defined as true pertussis cases according to an expanded standard which includes either (1) culture positive or (2) PCR or serology positive with clinical features fulfilling the CDC clinical case definition and the patients having received macrolides treatment for more than 10 days. The sensitivity and specificity of the multiplex PCR were 79% and 98%, respectively, while those for serology were 47% and 85%, and for culture 39% and 100%.

Conclusions: Our data confirm the superior sensitivity of the multiplex PCR in detection of *B. pertussis*, compared with conventional culture and serology. Clinical validation indicates that the multiplex PCR offers specific detection of *B. pertussis* from NP specimens.

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Key words: ***Bordetella pertussis*, multiplex PCR, nasopharyngeal swab specimens.**

Pertussis is presently one of the ten most common causes of death from infectious disease worldwide. Fifty million pertussis cases and 350,000 deaths worldwide per year have been reported by the World Health Organization, mainly among unvaccinated infants and children.[1-3]

In Taiwan, diphtheria, pertussis and tetanus combined vaccines have been offered to children

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since 1954. The number of reported cases has decreased yearly from 691 in 1955 to less than 10 in 1970. This incidence remained low from 1971 to 1991 until a sudden increase of 226 cases occurred due to an outbreak in 1992. In 1992, the Epidemiology Meeting of the Department of Health formulated a set of standards for the reporting and classification of pertussis cases. According to a report from the Center for Disease Control, Department of Health (DOH), Taiwan in 2000,[4] there were 7, 6, 26, 15, 101, and 34 confirmed cases, and 63, 51, 86, 146, 477, and 283 reported cases each year, between 1993 and 1998. There were two peaks in the age distribution of cases; one was young children under nine years old, and the other was their caretakers in the 30-39 year-old age group. The age of patients seemed to have moved upward, and the number of patients older than 10 years has increased each year. Many infections in vaccinated persons, especially adults and older children, lack typical symptoms of pertussis and are not diagnosed or properly treated.

Accurate laboratory diagnosis of *Bordetella pertussis* infections is therefore very important but unfortunately remains problematic. Isolation of *B. pertussis* from respiratory specimens is the "gold standard" for the diagnosis of pertussis. Culture is highly specific, but time consuming. Sensitivity can be affected by many factors, including patient's age, immunization status, antibiotic treatment, and specimen culture and transport conditions.[5-7] Direct detection of *B. pertussis* by fluorescent-conjugated-antibody stain can provide a rapid result but also has the disadvantages of poor sensitivity and specificity.[8] Although serologic tests are useful for epidemiological studies, they are not suitable for routine diagnosis. Due to the increased awareness of *B. pertussis* infection and the limitations of traditional diagnostic methods, several polymerase chain reaction (PCR)-based assays have been developed to detect *B. pertussis* DNA in patients' specimens.[5-18] These assays have consistently been shown to provide a more sensitive, specific, and rapid method of detecting *B. pertussis* infections than other methods.[5-18] In the present study, we attempted to validate the performance of a multiplex PCR in comparison with culture and serology in samples from patients with clinically suspected whooping cough.

METHODS

Specimen collection, isolation and identification

From June 1997 through December 2001, a total of 193 paired nasopharyngeal (NP) swab specimens collected from patients who had clinically suspected pertussis at Chang Gung Memorial Hospital were tested by both culture and a multiplex PCR. One hundred and three serum samples requested for serology testing by physicians were collected from suspected patients. NP swab specimens for culture and the multiplex PCR were collected using calcium alginate and Dacron swabs (Copan Diagnostics, Corona, CA, USA), respectively. Calcium alginate swabs are superior to Dacron swabs for the isolation of *B. pertussis* but contain substances which inhibit PCR;[19,20] Dacron swabs do not have inhibitory substances and are therefore better for PCR.[20,21] The specimens on calcium alginate swabs were inoculated into freshly prepared Bordet-Gengou agar plates containing 20% sheep blood and 40 μg cephalexin per ml. The inoculum on the agar plates was streaked for isolation, and the plates were incubated at 35˚C for 7 days in a moist chamber. Preliminary identification was done by examining catalase/oxidase production and Gram staining. Suspected colonies were confirmed by the multiplex PCR method described below. The specimens on Dacron swabs were placed into glass tubes containing 0.5 ml saline. Aliquots of the suspension were subsequently tested by the multiplex PCR.

Evaluation of specificity and sensitivity

Laboratory strains of *B. pertussis* were used as positive controls in the multiplex PCR. The following clinical isolates obtained from the clinical microbiology laboratory were used to evaluate the specificity of the PCR assay: *Staphylococcus aureus, Haemophilus influenzae, Moraxella catarrhalis, Pseudomonas aeruginosa, Klebsiella pneumoniae,* and *Streptococcus pneumoniae*. Bacterial strains were grown on routinely used media, under standard conditions.[22] Colonies grown on solid media were suspended in water (>10^8 CFU/ml, based on turbidity), vortexed, and boiled for 10 min. After centrifugation, DNA was extracted and subjected to the multiplex PCR.

To evaluate the sensitivity of the multiplex PCR, purified *B. pertussis* DNA was extracted by a pro-

teinase K-sonication procedure. Serial dilutions were made to the purified DNA. Aliquots of each solution containing the DNA were used for the multiplex PCR.

Multiplex PCR

The method described previously by Wadowsky et al. was used with some modification.[5] DNA was extracted from NP swabs by a proteinase K-sonication procedure. Fifty microliters of extracted DNA was added to 50 μl of PCR mixtures containing 2 U Taq DNA polymerase (ABgene, Epsom, Surrey, UK), 2.5 mM $MgCl_2$, 100 μM (each) deoxynucleoside triphosphates (GeneTeks BioScience, Inc., Taipei, Taiwan), and 0.25 μM of each oligonucleotide primer in 1x PCR buffer. Ten nanograms of human DNA (included as an optimal amount of template for the HAC3 and HAC5 primers since swab specimens do not provide a reliable target) was added into each PCR reaction. PCR tubes were placed in a model 9600 thermal cycler (Perkin-Elmer Biosystems, Foster City, CA, USA), denatured at 94˚C for 2 min, then subjected to 32 cycles of 94˚C for 1 min, 64˚C for 30 s, and 72˚C for 1 min. A final extension step at 72˚C for 10 min was performed and the products were stored at 4˚C until used. Each run included a positive and a negative control, and a reagent control for every five specimens. After agarose gel electrophoresis, the ethidium bromide-stained PCR products were visualized under UV light. Specimens yielding two bands corresponding to the 153 and 203-bp products were considered positive. Specimens that yielded no band corresponding to the β-actin gene product were initially interpreted as nonevaluable and were further treated with phenol chloroform to remove inhibitory substances, and the samples were tested by the multiplex PCR.

Serology

Sera were available in 103 of the 193 patients and were sent to the Center for Disease Control, DOH, Taiwan for further processing. Serology testing consisted of the measurement of IgA and IgM antibodies against whole cells of *B. pertussis* in enzyme-linked immunosorbent assay (ELISA), according to methods described previously.[23,24]

Analysis of discrepant results and expanded standard

A positive result obtained by culture was considered to be true positive. Specimens positive by multiplex PCR or serology alone were considered to be potentially false positive, and the true status was decided by reviewing the patient's medical record. In the United States, the Centers for Disease Control and Prevention (CDC) clinical case definition is a cough illness lasting ≥2 weeks with one of the following: paroxysms of coughing, inspiratory "whoop," or post-tussive vomiting, without other apparent cause.[25] An expanded standard was therefore established: a true positive pertussis case was either (1) culture positive; or (2) multiplex PCR or serology positive with clinical features fulfilling the CDC clinical case definition and the patient having received macrolides treatment for more than 10 days.

RESULTS

Analysis of PCR analytical sensitivity and specificity

The multiplex PCR simultaneously amplified two separate DNA targets (153 and 203 bp) within a *B. pertussis* repetitive element IS481 and a 438-bp target within the β-actin gene of human DNA (PCR amplification control). Testing of *B. pertussis* DNA with serial dilutions demonstrated a sensitivity of approximately 50 fg (equivalent to 10 genomic copies) per PCR. DNA from a wide variety of bacteria from the upper respiratory tracts of children do not yield PCR products.

Detection of *B. pertussis* in clinical specimens

The results from all 3 methods are summarized in Table 1. Twenty-one (11%) of the 193 samples had to be treated with phenol chloroform, which in all cases removed inhibitory substances for amplification. Two of the 21 samples yielded positive results. Of the 193 paired NP swab specimens tested by both culture and the multiplex PCR, positive results were obtained with both assays in 11 pairs, and negative results were obtained with both assays in 168 pairs. The yield of multiplex PCR for the detection of *B. pertussis* was 2.3-fold (25 positive samples) higher than that of culture (11 positive samples). All culture-positive specimens were also positive with the multiplex PCR. Culture-negative, multiplex PCR-positive results were obtained in 14 pairs. Nine of the 14 pairs had serology results that were

negative. Of the 103 specimens tested by culture, multiplex PCR and serology methods, 3 were positive by all three methods, and 69 were negative by all methods. Three of the 6 culture-positive specimens were negative with serology. Nineteen specimens were positive with serology alone. Compared to that of culture, the sensitivities for the multiplex PCR and serology were 100% and 50%, respectively, and the specificities were 92% and 80%, respectively (Table 1).

Review of medical records and resolution of discrepant data

Medical records of the patients examined were reviewed to resolve discrepant results. Eleven of the 14 patients who tested positive on the multiplex PCR only were defined as true pertussis cases because they met the expanded standard. Of the three remaining patients who did not meet the pertussis case definition, one, a 1-month-old boy with paroxysms and a whooping cough that lasted for 6 days, was considered to be a likely case. The other two with unresolved positive PCR results were not considered to be true cases because they were shown to have mycoplasma and chlamydia infections on serology examination.

Medical records were available for 18 of the 19 patients who tested positive on serology only. Six patients were defined as positive cases because they met the expanded standard. Three patients who met the pertussis case definition did not receive a full treatment course for a pertussis and thus were defined as negative cases. Three of the remaining nine patients who did not meet the pertussis case definition had a whooping cough that lasted for only about 10 days and were therefore categorized as neg-

Table 1. Numbers of Positive and Negative Results Tested by the Multiplex PCR and Serology Methods, and the Comparison Between the Two Methods and the Standard Culture Method, Respectively.

Test	Culture		Sensitivity	Specificity	PPV	NPV
	Positive	Negative	(%)	(%)	(%)	(%)
Multiplex PCR						
Positive	11	14	100	92	44	100
Negative	0	168				
Serology						
Positive	3	19	50	80	14	96
Negative	3	78				

Abbreviations: PCR: polymerase chain reaction; PPV: positive predictive value; NPV: negative predictive value.

Table 2. Numbers of Positive and Negative Results Tested by Culture, Multiplex PCR, and Serology Methods, and Their Comparison with Expanded Standard

Test	Expanded standard*		Sensitivity	Specificity	PPV	NPV
	Positive	Negative	(%)	(%)	(%)	(%)
Culture						
Positive	11	0	39	100	100	91
Negative	17	165				
Multiplex PCR						
Positive	22	3	79	98	88	96
Negative	6	162				
Serology						
Positive	9	13	47	85	41	88
Negative	10	71				

Abbreviations: PCR: polymerase chain reaction; PPV: positive predictive value; NPV: negative predictive value.

*Either (1) culture positive, or (2) multiplex PCR or serology positive with clinical features fulfilling the CDC clinical case definition and having received macrolide treatment for more than 10 days.

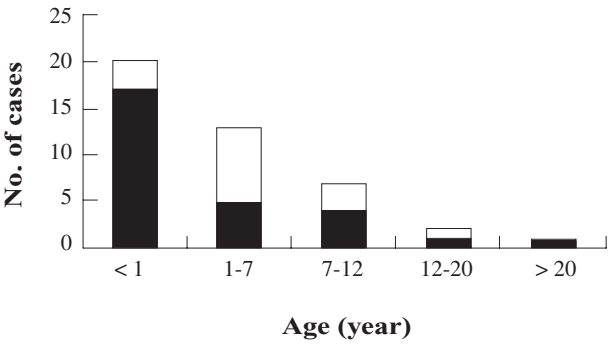

Fig. 1 Number of cases in different age groups. ■, number of cases that met the expanded standard for diagnosis; □, number of cases that did not meet the expanded standard but had a positive result in either serology or multiplex PCR.

ative. One patient with a whooping cough lasting for 7 days, also tested positive for respiratory syncytial virus on culture and therefore was not considered to represent a true case. The remaining five patients were also defined as negative cases because they all had nonspecific coughs and three of them tested positive for mycoplasma, chlamydia, and adenovirus.

The performance of culture, serology and multiplex PCR were calculated according to the expanded case definition for a pertussis infection. The sensitivities for culture, multiplex PCR, and serology methods were 39%, 79%, and 47%, respectively, and the specificities were 100%, 98%, and 85%, respectively (Table 2).

The age distribution of the patients enrolled in this study is shown in the Fig. 1. Seventeen (61%) of the 28 patients who met the expanded standard were less than 1 year of age.

DISCUSSION

In this study, the accuracy of the multiplex PCR in detecting *B. pertussis* was evaluated in comparison with that of culture and serology. The assay has a high degree of analytical sensitivity and specificity. It was able to detect as few as 10 genome copies of *B. pertussis* DNA and did not cross-react with other bacteria commonly isolated from the upper respiratory tracts of children. The findings are comparable with those of previous studies.[16] Inhibition of PCR due to various substances present in clinical samples occurs at frequencies ranging from 6% to 26% in the detection of *B. pertussis*.[5] In our study, inhibition of the multiplex PCR occurred in 11% of the specimens. The inclusion of an internal control detects inhibition and thus prevents false-negative results.

The multiplex PCR-based assay had a 2.3-fold higher yield of positive results than the traditional culture method, which was in agreement with previous findings.[6,9-13,18,26] In spite of the growing acceptance of PCR as a diagnostic test during the past decade, concerns remain over false-positive results due to contamination or cross-reactivity.[6,13,18,26] In addition, there has been no evaluation using local materials in Taiwan. In the current study, 11 (44%) of 25 multiplex PCR-positive results were confirmed by culture. Furthermore, 14 discrepant test results (multiplex PCR-positive only) were resolved by review of the patient's medical records. After comparison of the performance of culture, serology, and the multiplex PCR against an expanded standard, it became apparent that the difference in the positive rates between the multiplex PCR and culture was more likely due to false-negative culture results rather than to false-positive PCR results. Indeed, when compared with the expanded standard, culture and the multiplex PCR showed relatively different sensitivities of 39% and 79%, respectively.

Though specificity of the culture method was high, its sensitivity varied depending on the culture conditions. Isolation of *B. pertussis* was more likely to be successful at the end of the incubation period, during the catarrhal stage, and at the early stage of paroxysms.[7] However, pertussis infection was rarely suspected at these stages. Most specimens were collected from suspected patients at the mid-paroxysmal stage in our hospital. Thus only 11 of 193 (6%) specimens were positive by culture in this study.

Serology has been used as a reference method against which other laboratory tests have been compared. In some studies,[6,15] the specificity for PCR was estimated by comparing with serology. In two of the largest studies, with 2,421 and 833 samples, the specificities were estimated as 99% and 98%, respectively, which were similar to that observed in our study. After comparison with the expanded standard, the specificity for the multiplex PCR-based assay was 98% in this study.

Our data showed that the serology method had poor sensitivity and lacked specificity; only 3 (50%)

of the 6 culture-confirmed cases were positive on serology. The data revealed that the sensitivity and specificity for serology were 47% and 85%, respectively, when compared with the expanded standard. It is well known that the sensitivity and specificity of serology can be affected by multiple factors, including age, vaccination, and the availability of paired serum samples.[7] For these reasons, CDC guidelines for laboratory confirmation of pertussis cases do not include serologic testings.[25] However, the serology method remains a requisition for the diagnosis of *B. pertussis* infection in symptomatic adolescents and adults because pertussis in these patients is usually diagnosed late in the course of the disease when negative results for culture are frequently encountered.

In accord with earlier reports,[1,2,4,27] this study showed that the majority of the cases of pertussis in Taiwan occurred in infants less than 1 year of age, who had not completed the vaccination course at the onset of the disease. These young infants usually presented with atypical symptoms such as cyanosis or apnea and were more likely to develop complications following the infection.[1,2,27]

In summary, the present study confirms the superior sensitivity of the multiplex PCR-based assay compared with that of culture. If careful internal and external controls are applied, PCR offers specific and rapid detection of *B. pertussis* from NP swab specimens. The result can be obtained within 1 day with relatively high sensitivity and specificity.

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多引子聚合酶鏈鎖反應，細菌培養及血清學檢查三種診斷百日咳感染方法的比較

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背 景： 正確診斷百日咳鮑特氏菌（*Bordetella pertussis*）所引起的百日咳感染並不容易。一般使用細菌培養，而以聚合酶鏈鎖反應（PCR）作爲診斷方法，則具有較高敏感性，但相關文獻對於敏感性的報告並不一致。因此，本篇文章以回溯研究的方式，將臨床上用以鑑定百日咳感染的細菌培養、血清學檢查以及一多引子聚合酶鏈鎖反應（Multiplex PCR）三種診斷方法加以比較。

方 法： 將193位病患取得的成對鼻咽拭子，同時進行細菌培養及多引子聚合酶鏈鎖反應，其中的103位病患有血清學檢查的結果。當培養結果與血清學檢查或是多引子聚合酶鏈鎖反應的結果不一致時，則進一步分析病患的臨床資料，看是否符合美國疾病管制中心（CDC）對於百日咳臨床病例的認定標準。

結 果： 在193對鼻咽拭子中，有25個檢體爲多引子聚合酶鏈鎖反應陽性，而其中的11個可培養出百日咳鮑特氏菌。在103個有血清學檢查結果的檢體，有3個檢體的三種診斷方法皆爲陽性，另有69個則皆爲陰性。以細菌培養爲標準，則多引子聚合酶鏈鎖反應及血清學檢查的靈敏度分別爲100%與50%，專一性分別爲92%與80%。當標準延展爲 (1) 細菌培養陽性或是 (2) 多引子聚合酶鏈鎖反應或是血清學檢查陽性，而其臨床症狀符合美國疾病管制中心對於百日咳臨床病例的認定標準，且已接受抗生素治療10天以上，在14個單獨多引子聚合酶鏈鎖反應陽性及19個單獨血清學檢查陽性的病患中，分別有11個及6個患者被認定爲百日咳病例。則多引子聚合酶鏈鎖反應的靈敏度與專一性分別爲79%及98%，血清學檢查爲47%及85%，而細菌培養的專一性爲100%，但靈敏度降爲39%。

結 論： 本實驗證實了此多引子聚合酶鏈鎖反應的高靈敏度與專一性，可提供臨床上快速且正確地診斷百日咳感染。

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關鍵字： 百日咳鮑特氏菌，多引子聚合酶鏈鎖反應，鼻咽拭子。

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