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Clinical Evaluation and Validation of Laboratory Methods for the Diagnosis of *Bordetella pertussis* Infection: Culture, Polymerase Chain Reaction (PCR), and Anti-Pertussis Toxin IgG Serology (IgG-PT)

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2012

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An abstract of
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Abstract

Clinical Evaluation and Validation of Laboratory Methods for the Diagnosis of *Bordetella pertussis* Infection: Culture, Polymerase Chain Reaction (PCR), and Anti-Pertussis Toxin IgG Serology (IgG-PT)

By Adria D. Lee

Introduction. The appropriate use of clinically accurate diagnostic tests is essential for the detection of pertussis, a relatively poorly controlled vaccine-preventable disease. The purpose of this study was to estimate the sensitivities and specificities of different methods of diagnosing pertussis including culture, polymerase chain reaction (PCR), serology, and the use of a clinical case definition. An additional study objective was to describe the utility of pertussis serological testing in routine clinical practice.

Methods. Clinical specimens were collected from patients with cough illness between 2007 and 2011 at seven sites across the US. Sensitivities and specificities of each diagnostic test were estimated using three alternative "gold standards"—pertussis culture, composite reference standard (CRS), and latent class analysis (LCA) results. The effect of delayed blood specimen collection on serological testing accuracy was also assessed.

Results. In the total sample with non-missing data on all diagnostic tests, PCR was the most sensitive (> 90%) diagnostic test; it was also 100% specific. The LCA and CRS approaches generated similar estimates of the sensitivity and specificity for each test. When the analysis was restricted to a sub-group of participants with optimally-timed specimen collection, LCA provided lower estimates of the sensitivity of each test, compared to using culture as the gold standard or CRS analysis. Of the participants with both acute and convalescent serology results, 94% had concordant results at both time points. However, only 12% of participants with positive acute serology and 20% of participants with positive convalescent serology were classified as pertussis cases by LCA.

Conclusions. Convalescent pertussis serology is useful as an additional confirmatory diagnostic test in the clinical setting. Additionally, estimates of sensitivity and specificity and the accuracy of the diagnostic result are affected by the timing of specimen collection.

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Introduction

Pertussis, commonly known as whooping cough, is a respiratory illness caused by the Gram-negative bacterium *Bordetella pertussis*. Infection occurs in three stages, and may last several weeks or months (1, 2). The patient is most contagious during the catarrhal stage, which usually spans the first two weeks of illness. During this stage, non-specific symptoms, such as sneezing and mild coughing, are present. "Classical" pertussis symptoms may appear during the paroxysmal stage, and include paroxysmal coughing, which is followed by a high-pitched inspiratory whoop and post-tussive vomiting. Recovery begins during the convalescent stage, when paroxysmal attacks become less frequent (2).

Pertussis is currently one of the most poorly controlled vaccine-preventable diseases in the United States (1). The annual incidence of pertussis has increased dramatically in recent decades, from a historic low of 1,010 cases reported in 1976 to 48,277 cases reported in 2012 (3). About 40%-50% of pertussis cases occur in adolescents and adults (4, 5). Adolescents and adults are more likely to have undiagnosed mild illness, and a recent nonhuman primate model suggests that currently licensed pertussis vaccines do not protect against asymptomatic infection (6, 7), both allowing for continued transmission of the pathogen.

Clinically accurate diagnostic tests are essential for the detection of pertussis. The Council of State and Territorial Epidemiologists (CSTE) defines a clinical case as a patient with cough illness lasting two or more weeks and at least one of the characteristic pertussis symptoms, in the absence of another diagnosis. In order to be considered a confirmed case, patients must either have a cough and positive culture, meet the clinical case definition and have a positive polymerase chain reaction (PCR) test, or meet the clinical case definition and have had contact with a laboratory-confirmed pertussis case (8).

Culture is considered the "gold standard" diagnostic test. However, culture has several limitations. Although it is 100% specific, it has very low sensitivity, with estimates ranging from 12%-60% (9). It typically takes a 5-10 day incubation period to obtain results, during which the patient would still be able to transmit the infection to others if not empirically treated. To obtain viable bacteria for

isolation, nasopharyngeal (NP) specimens should be collected within the first two weeks of illness, when symptoms are more likely to be non-specific and physicians may not consider pertussis as the diagnosis. Additionally, the organism can be difficult to isolate, especially if the patient has previously been vaccinated or received antibiotics against pertussis (10).

PCR has become the most commonly used pertussis diagnostic test (11). Real-time PCR can be completed in 2 to 24 hours, which allows for the rapid diagnosis of patients. Since PCR does not require viable bacteria to be positive, it is more sensitive than culture, with estimates ranging from 70% to 99%. Optimal sensitivity occurs during the first 3 to 4 weeks of cough when bacterial DNA is still present in the nasopharynx (9). Multitarget real-time PCR assays can be used to differentiate between *Bordetella* species, and are highly specific (12). However, environmental contamination of clinical specimens in clinics and cross-contamination within laboratories has been associated with falsely positive PCR results and several pseudo-outbreaks of pertussis in recent years (13, 14, 15).

Although not included in the CSTE case definition, serologic test results may also be used to confirm recent pertussis infection. Serology has recently been used to identify cases during outbreak investigations (16) and to rule-out pertussis during pseudo-outbreaks caused by false-positive PCR results (13). Optimal sensitivity of anti-pertussis toxin IgG (IgG-PT) serology testing generally occurs 2-8 weeks after cough onset (17). The lack of well validated and standardized serology diagnostic assays currently limits its use in routine clinical practice.

Accurate and reliable diagnostic tests are essential for the development and evaluation of pertussis prevention and control strategies. Imperfect diagnostics contribute to an underestimation of disease burden across the age spectrum; compromise prevention programs, surveillance activities, vaccine effectiveness studies, and outbreak management; and enable continuous transmission of the bacterium. Additionally, pseudo-outbreaks and false-positive PCR results lead to wasted resources during unnecessary outbreak responses, and the exclusion of serology from the CSTE pertussis case definition limits the diagnosis of patients who seek medical attention after the first few weeks of illness. For all of the above reasons, it is important to understand the relative diagnostic utility of various available pertussis

tests. The current project seeks to address these issues by evaluating the sensitivities and specificities of the clinical case definition, culture, multi-target PCR, and an IgG-PT serology test for the diagnosis of *Bordetella pertussis* infection using various "gold standard" measures and latent class analysis (LCA). Additionally, we will assess the effect of the timing of the blood specimen collection for serologic testing (acute vs. convalescent) on the accuracy of the diagnostic result.

Methods

Study Enrollment

Data were collected from July 2007 to February 2011 at seven different study sites across the United States. The study sites included state and local public health departments, academic centers, and Emerging Infections Programs (EIP) located in California, Colorado, Georgia, Minnesota, New Mexico, and New York. Investigators at each site identified persons with cough illness through physician office visits, pertussis case investigations, or outbreak responses. In addition, case ascertainment involved a prospective review of hospital admissions, diagnostic test orders, and emergency department visits.

Individuals at least three years of age and meeting the inclusion criteria as listed in Box 1 were invited to participate in the study. Patients were not eligible for the study if they had a chronic cough illness (cough lasting ≥ 30 days) or were prisoners. Infants and children under the age of 3 were excluded from the study due to their recent completion of the childhood diphtheria, tetanus, and acellular pertussis vaccine (DTaP) priming series (8).

Each enrolled participant provided written informed consent. Written parental permission was obtained for participants less than 18 years of age in addition to adolescent assent for participants aged 11-18 years. Each participant was assigned a unique identifier, which was used to link the study data to the clinical specimens. Demographic and clinical information, including vaccination history, presence of pertussis symptoms, duration of cough illness, and recent use of antibiotics, was collected from each participant during the enrollment visit.

Specimen Collection

An NP aspirate or swab specimen, and a blood specimen were collected from each participant at the enrollment visit. NP aspirates were collected using N-pak kits (N-Pak, Baxter, MN). NP swab samples were obtained from the posterior nasopharynx using a polyester- or rayon-tipped probe with an aluminum shaft. After collection, the aspirate and swab specimens were placed into a tube containing 2 mL of 1% casamino acids broth, stored at 4-8°C using cold packs, transported to the site laboratory, and

divided into four 300 µL aliquots within 24 hours of collection. Gloves were changed between each specimen, and the aliquots were prepared in a clean, *Bordetella* species DNA-free biological safety cabinet located in a clean room where no *B. pertussis* culture tests were performed. Aliquots not used for testing at the site laboratory were stored at -40 to -80°C and shipped to CDC.

Blood was collected using the Vacutainer™-type needle system and Vacutainer™ serum separator tube (Becton, Dickinson and Company, Franklin Lakes, NJ). Participants under the age of 9 provided 5 mL of blood. For all other participants, 10-mL blood samples were collected. No more than three blood draw attempts per participant were allowed. Whole blood was allowed to clot for 30-45 minutes at room temperature and centrifuged within two hours of collection at 1100-1300 x g for 10 minutes for swing-head units or at 15 minutes for fixed angle rotors. The serum was divided into 300 µL aliquots within 24 hours of blood collection. Aliquots were frozen at -40 to -80°C until shipped to CDC. Patients who had been coughing for less than 2 weeks at enrollment were asked to return in 2-4 weeks for collection of a convalescent serum specimen.

Participants were reimbursed for their time and travel expenses in the form of a retail gift card. Each participant received a $40.00 gift card at the enrollment visit and a second $30.00 gift card for the second blood draw visit. All study sites and CDC obtained human subjects approval prior to study initiation by their respective Institutional Review Boards.

Laboratory Testing

Culture was performed at the site laboratories for all specimens not collected during a CDC-led outbreak investigation. Specimens collected as part of CDC-led outbreak investigations were only tested at CDC. NP specimens were plated onto Regan-Lowe agar plates with and without cephalexin within 24 hours of collection. Plates were incubated at 37°C with high humidity under ambient air, and were examined daily for 7-10 days. Colonies were stained to check for Gram-negative coccobacilli, and *Bordetella* species were identified using confirmation tests, as available at each laboratory site. The

confirmation tests used were the direct fluorescent antibody (DFA) test, slide agglutination using specific antisera, and biochemical tests.

PCR was performed as previously described (9, 12). Briefly, NP aliquots were shipped from the collection sites to CDC, where DNA was extracted. The published interpretation algorithm for the multitarget real-time PCR (RT-PCR) assay allows for distinguishing between *B. pertussis*, *B. parapertussis*, *B. holmesii*, and *B. bronchiseptica*. The four RT-PCR targets used were insertion sequence 481 (IS*481*), ptxS1, hIS*1001*, and pIS*1001*.

IgG-PT enzyme-linked immunosorbent assays (ELISA) were performed at CDC as previously described (18). Blood specimens collected during the first two weeks of illness were considered acute, and all other specimens were considered convalescent. Acute and convalescent blood antibody concentrations ≥ 94 EU/mL were considered positive for pertussis, concentrations 49-93 EU/mL were considered indeterminate, and concentrations < 49 EU/mL were considered negative (19).

Data Analysis

Statistical analyses were conducted using SAS 9.3 (SAS Institute, Inc., Cary, NC, USA) and Latent Gold® 4.0 (Statistical Innovations, Inc., Belmont, MA, USA). Sensitivities and specificities of PCR, acute and convalescent serology, and the clinical case definition were calculated using culture as the "gold standard" test. However, due to its low sensitivity, culture is an imperfect gold standard and analyses may produce biased, underestimated values of the specificities (20). Therefore, sensitivities and specificities were also estimated using a composite reference standard (CRS) and latent class analyses (LCA).

In LCA, gold standard bias is reduced by considering all diagnostic tests as imperfect. The statistical model combines the results of each test to define an unmeasured latent variable that indicates true disease status. The model calculates the probability of each participant being classified as a case or a non-case based on the results of at least three diagnostic tests and an assumption of conditional independence between the tests. This assumption states that the result of one test depends on the

participant's true disease status, not the outcome of the other tests (20). Bivariate residuals (BVR) are local measures of model fit that are used to determine whether or not the independence assumption is met, and are calculated by dividing the Pearson Chi-square value by the model's degrees of freedom (21). $BVR \geq 3.84$ indicates a violation of this assumption, suggests that the results of two diagnostic tests are conditionally dependent, and results in an overestimation of the sensitivity and specificity of each test (22, 23). To address this issue, a conditional dependence model is fit that includes a direct effect between the two tests. Good model fit is indicated by low, non-significant likelihood ratio statistics (L^2) and a low proportion of classification errors (21).

The CRS was created by combining culture and PCR results, and the resulting CRS-based gold standard was then used to estimate the sensitivity and specificity of serology and the clinical case definition. This method has also been shown to reduce the imperfect gold standard bias (20).

Results

Description of the Study Population

A total of 868 patients were eligible for participation in the study. The majority of the participants were enrolled in 2008 and 2009 (Figure 1). Study sites in Georgia, New York, and Minnesota had the highest levels of enrollment (Figure 2). The mean age at enrollment was 32 years (range: 3-83 years), and 88.36% of participants were ≥ 9 years of age. As shown in Table 1, the majority of the participants were female, Caucasian, and non-Hispanic.

Clinical Symptoms

About 57% of the participants enrolled during the first two weeks of their cough illness (Table 1). Of the participants with non-missing clinical symptom data, 60.48% (508/840) reported that they experienced at least one "classical" pertussis symptom, and 57.38% reported experiencing paroxysmal coughing. Only 8.86% (45/508) reported experiencing all three symptoms. Of the 296 participants who reported having contact with a confirmed pertussis case patient, 68.58% (203/296) reported that their illness was related to an outbreak of pertussis. The clinical case definition was met by 42.59% of the participants.

Laboratory Diagnostic Test Results

Culture results were available for 824 participants (Table 2). Culture was positive for *B. pertussis* in 2.67% of the participants, and 68.18% of these positive specimens were collected during the first two weeks of cough illness. No other *Bordetella* species were identified by culture. PCR results were available for 806 participants. Only 3.85% were positive for *B. pertussis*, and 77.42% of these positive specimens were collected within the first three weeks of cough illness. The remaining specimens were identified as negative, indeterminate for *B. pertussis*, or positive for *B. parapertussis* or B. *holmesii*.

Acute serology results were available for 451 participants, and 5.32% were pertussis positive. On average, these specimens were collected 8.00 days after cough onset (range: 1-14 days). Convalescent

serology results were available for 650 participants, and 12.77% were pertussis positive. All of the positive convalescent specimens were collected during weeks 2-8 of illness (Table 2). Both an acute and convalescent blood specimen was collected from 305 participants. These specimens were collected 20.53 days apart, on average (range: 10- 62 days), and 94.10% of these participants had concordant results at both time points. Of those with concordant results, 91.99% were negative, 4.53% were indeterminate, and 3.48% were positive.

In total, 97 specimens were considered pertussis positive by serology (Table 3). Paired specimens were available for 21.65% (21/97) of these participants. Of these, 47.62% were positive at both time points, 33.33% were positive at the convalescent but not the acute time point, and 23.81% were also confirmed by both culture and PCR. The remaining 76 participants only had a specimen collected at one of the two time points. Of the 11 participants with a positive acute result, one was also positive by culture and PCR. Sixty-five participants with positive convalescent serology enrolled in the study after their second week of illness and therefore only provided a convalescent blood specimen. Of these, 9 participants were also positive by either culture or PCR, and 3 were positive by both.

Latent Class Model Results

Complete data for culture, PCR, convalescent serology, and the pertussis clinical case definition were available for 62.79% (545/868) of the participants (Table 4). Of these participants, 54.31% were negative and 1.28% was positive for all tests. Bivariate residuals of the initial LCA model indicated that the conditional independence assumption was violated and that there was an association between serology and the clinical case definition (BVR= 18.8). Therefore, a second model was fit that included a direct effect between these two variables. This model had an L^2 value of 8.4 (p-value= 0.14), indicating good model fit. The rate of expected classification error was 0.18%, and the estimated prevalence of pertussis was 3.6% (95% CI 1.9%- 5.4%). Nineteen participants had a probability of having pertussis ≥ 0.50, and were classified as cases (Table 4). This probability was highest when culture or PCR was positive. Of the 19 cases, 57.89% were enrolled after the second week of illness, 78.95% were over the age of 9 years,

and 94. 74% reported that they experienced paroxysmal coughing. Fourteen of the cases had positive convalescent serology. Overall, the LCA model classified 80.28% of the participants with positive convalescent serology as non-cases.

A third model was fit that included acute serology in addition to culture, PCR, and the clinical case definition (N= 349). This model also had good fit (L^2=4.4; p-value= 0.62), and the rate of expected classification error was 0.32%. Fifteen participants were classified as cases (Table 5), and the prevalence of pertussis was 4.56% (95% CI 2.03%- 7.09%). Overall, 87.50% of the participants with positive acute serology were classified as non-cases.

Sensitivity and Specificity Estimates

The sensitivity and specificity of each test was estimated among the 545 participants with non-missing data on culture, PCR, convalescent serology, and the clinical case definition (Table 6). When all other tests were compared to culture, PCR had the highest sensitivity (92.3%; 95% CI 66.7%- 98.6%) and specificity (98.9%; 95% CI 97.6%- 99.5%). Convalescent serology was the least sensitive test (61.5%; 95% CI 35.5%- 82.3%), and the clinical case definition was the least specific (58.5%; 95% CI 54.6%- 62.9%). Use of LCA increased the sensitivity estimates for convalescent serology and the clinical case definition. LCA also increased the specificity estimates of PCR, convalescent serology, and the clinical case definition. Culture was the least sensitive diagnostic test, but was 99.9% specific. When culture and PCR results were combined to create a CRS, the sensitivity for both convalescent serology and the clinical case definition increased compared to the corresponding estimates obtained using culture as the gold standard. There was also a slight increase in the specificity estimates for each test. The CRS-based sensitivity and specificity estimates for convalescent serology and the clinical case definition were similar to the LCA-based estimates.

Sensitivities and specificities were also estimated among 349 participants with non-missing data on culture, PCR, acute serology, and the clinical case definition (Table 6). When compared to culture, acute serology was 16.7% sensitive (95% CI 4.70%- 44.8%) and 95.9% specific (95% CI 93.2%- 97.5%).

PCR was the most sensitive (83.3%; 95% CI 52.2%- 95.3%) and specific (99.1%; 95% CI 97.4%- 99.7%) diagnostic test, and the clinical case definition was the least specific test (77.2%; 95% CI 72.4%- 81.3%). When LCA and CRS were used, the sensitivity of acute serology decreased to 13.3%. The LCA- and CRS-based estimates of the sensitivity and specificity of PCR and the clinical case definition were similar to the culture-based estimates.

The sensitivity and specificity of each test was also estimated in a sub-group of 262 participants that had optimally timed NP specimens in addition to a convalescent serology (Table 6). NP specimens collected during the first two weeks of cough illness were considered optimally timed. Again, PCR had the highest sensitivity (87.5%; 95% CI 52.9%- 97.8%) and specificity (100%; 95% CI 98.5%-100%) when culture was considered the gold standard. When LCA was used as the gold standard, there was a decrease in the sensitivity of PCR, convalescent serology, and the clinical case definition. The sensitivity and specificity of convalescent serology and the clinical case definition from the CRS analysis were identical to the estimates using culture as the gold standard.

Discussion

The goals of this project were to estimate the sensitivities and specificities of pertussis diagnostic tests, and to evaluate the utility of pertussis serological testing in routine clinical practice. In assessing the diagnostic performance of a given test, the main challenge is selection of the appropriate gold standard. Traditionally the role of a gold standard test was assigned to pertussis culture; however, LCA and CRS may be used as alternatives to provide less biased estimates of sensitivity and specificity (20). In the current study, PCR was the most sensitive and specific diagnostic test by all three methods, and acute serology was the least sensitive. The LCA- and CRS-based estimates for convalescent serology and the clinical case definition were similar in the total sample and the sub-group with optimally timed specimen collection.

While it is essential that diagnostic tests be accurate and reliable, they must also be used at the appropriate time during the cough illness. Culture requires the collection of viable bacteria and PCR requires the presence of *Bordetella* DNA in the nasopharynx. Therefore, delayed NP specimen collection may result in falsely-negative results (17). While previous studies have estimated that culture is less than 60% sensitive (9), our results demonstrate that culture can be over 90% sensitive when performed during the first two weeks of cough illness. Our findings also suggest that convalescent serology may be useful as an additional confirmatory diagnostic test in a clinical setting. Convalescent serology was more sensitive than acute serology, and both were highly specific. IgG-PT titers peak later in the illness, when infection is less likely to be confirmed by culture or PCR (17) and the "classical" pertussis symptoms typically begin to appear. Convalescent serology could be used to identify additional pertussis cases that would be missed by culture or PCR. The LCA model only classified a participant as a case if culture or PCR was positive, and classified the majority of those with positive acute or convalescent serology as non-cases. Therefore, LCA may have resulted in misclassification of patients with positive serology results and an underestimation of the prevalence of pertussis in this sample.

A major limitation of this study is the amount of missing data. Many participants were missing data on one or more diagnostic test, and were excluded from the sensitivity and specificity estimations.

Reasons for missing data include participant refusal of specimen collection, inability to collect the specimen, insufficient specimen volume for diagnostic testing, and loss to follow-up before a convalescent blood specimen could be collected. These exclusions may have decreased the estimated prevalence of pertussis in this sample. Participants with indeterminate PCR or serology results were also excluded from sensitivity and specificity calculations. In the multi-target PCR assay, an indeterminate result indicates the presence of very little bacterial DNA, and specimens falling within this range are considered uninterpretable by PCR (12). In IgG-PT serology, the indeterminate range is used to indicate elevated antibody levels that are below the positivity cutoff, but may indicate a pertussis infection (18).

Despite the aforementioned limitations, a distinguishing feature of this study is that it is supposed to inform routine clinical practice. Data were collected from a large sample of patients across the country using standardized procedures, and all enrolled participants were tested for pertussis. In true clinical practice, patients would only be tested for pertussis if the physician suspected pertussis. Physician recognition of pertussis can be affected by a variety of factors, including age, vaccination status, previous antibiotic usage, and stage of illness at the time of the visit (10). Although the prevalence of pertussis was low in this sample of patients, the number diagnosed with pertussis may be higher than the number that would have been diagnosed during routine practice.

Overall, these data support the hypothesis that convalescent IgG-PT testing can be used in routine clinical practice as an additional confirmatory test for *Bordetella pertussis* infection. Each diagnostic test should be used during its optimal time period to ensure that all true pertussis cases are identified in the clinical setting.

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Tables and Figures

Box 1: Enrollment criteria for participation in the Clinical Validation Study, 2007-2011

- Cough 5-29 days duration, or
- Cough < 30 days duration with at least one of the following "classical" pertussis symptoms:
  - ~ Paroxysms of coughing
  - ~ Inspiratory "whoop"
  - ~ Post-tussive vomiting, or
- Close contact[1] of a CSTE- or PCR-confirmed case, plus cough < 30 days duration

[1] Close contacts are persons who have shared a confined space of ≤ 3 feet for at least 1 cumulative hour per day with a confirmed case or have direct contact with respiratory secretions from a confirmed case.

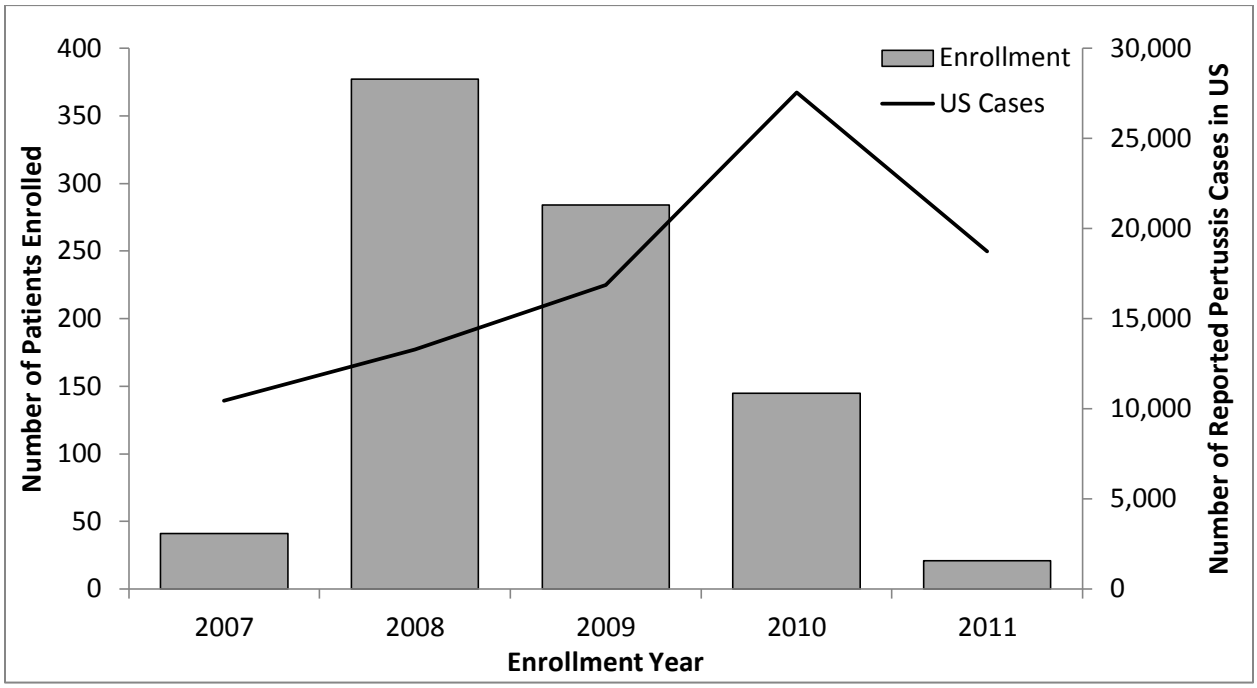

Figure 1: Comparison of the number of participants enrolled in the Clinical Validation Study and the number of reported pertussis cases in the US between 2007 and 2011.

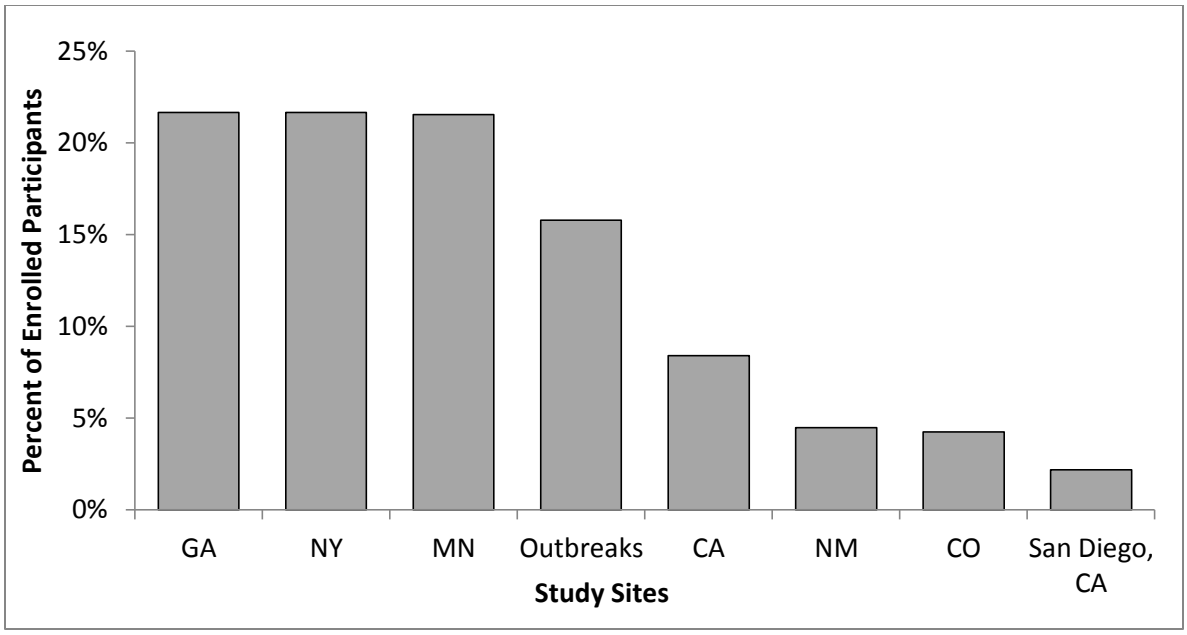

Figure 2: Percent of participants enrolled in the Clinical Validation Study at each study site between 2007 and 2011.

Table 1: Demographic, clinical, and epidemiological characteristics of all participants enrolled in the Clinical Validation Study (N=868)

	N (%)
Demographics	
Age ≤ 9 years	101 (11.64)
Age > 9 years	767 (88.36)
Female	585 (67.55)
Race: Caucasian	544 (66.18)
Ethnicity: non-Hispanic	619 (78.06)
Clinical Symptoms	
Cough duration < 2 weeks at enrollment	491 (56.57)
Cough duration ≥ 2 weeks at enrollment	377 (43.43)
Paroxysmal coughing	479 (57.30)
Post-tussive vomiting	139 (16.55)
Inspiratory whoop	126 (15.57)
Apnea	118 (14.41)
Epidemiology	
Related to a pertussis outbreak	203 (27.43)
Epidemiologically linked to lab-confirmed case	162 (22.78)
Close contact of CSTE confirmed case	128 (17.83)
Household contact of CSTE confirmed case	99 (13.73)
CSTE Case Definition	
Meets clinical case definition	348 (42.59)
Probable case	185 (22.64)
Confirmed case	123 (14.17)

*Denominators may vary because of missing data

Table 2: Diagnostic Test Results and Timing of Specimen Collection for All Participants Enrolled in the Clinical Validation Study (N= 868)

	Test Results, N (%)	**Collected at Optimal Time[1], N(%)**
Culture	**824 (94.93)**	**470 (57.04)**
Negative	802 (97.33)	455 (56.73)
B. pertussis	22 (2.67)	15 (68.18)
PCR	**806 (92.86)**	**686 (85.11)**
Negative	755 (93.67)	647 (85.70)
B. pertussis	31 (3.85)	24 (77.42)
Indeterminate *B. pertussis*	13 (1.61)	10 (76.92)
B. parapertussis	4 (0.50)	2 (50.00)
B. holmesii	3 (0.37)	3 (100.0)
Convalescent Serology	**650 (74.88)**	**647 (99.54)**
Negative	521 (80.15)	518 (99.42)
Positive	83 (12.77)	83 (100.0)
Indeterminate	46 (7.08)	46 (100.0)

[1]Optimal Timing of NP collection for culture is the first two weeks of cough illness. Optimal timing of NP collection for PCR is the first three weeks of cough illness. Optimal timing of blood collection for convalescent serology is weeks 2-8 of cough illness.

Table 3: Comparison of the culture and PCR results of all participants who were positive for pertussis by acute and/or convalescent serology (N=97)

			N (%)	
Acute	**Convalescent**	**N (%)**	**Culture +**	**PCR +**
Missing	+	65 (67.01)	3 (4.92)	9 (14.75)
+	Missing	11 (11.34)	1 (10.00)	1 (9.09)
+	+	10 (10.31)	2 (20.00)	2 (20.00)
−	+	7 (7.22)	3 (42.86)	3 (42.86)
+	Ind	3 (3.09)	0 (0.00)	0 (0.00)
Ind	+	1 (1.03)	0 (0.00)	0 (0.00)

Abbreviations: Ind, Indeterminate

*Denominators may vary due to missing data

[1]Ind, Indeterminate

Table 4: Diagnostic test result patterns and the probability of being classified as a pertussis case by LCA in all participants with non-missing data on culture, PCR, convalescent serology, and the clinical case definition (N=545)

Culture	PCR	Convalescent Serology	CCD	N	Probability of Having Pertussis
−	−	−	−	296	0.0001
−	−	−	+	173	0.0008
−	−	+	−	17	0.0021
−	−	+	+	40	0.0113
−	+	+	+	6	0.9997
+	−	−	+	1	0.7034
+	+	−	−	1	1.0000
+	+	−	+	3	1.0000
+	+	+	−	1	1.0000
+	+	+	+	7	1.0000

Abbreviations: CCD, Clinical Case Definition

Table 5: Diagnostic test result patterns and the probability of being classified as a pertussis case by LCA in all participants with non-missing data on culture, PCR, acute serology, and the clinical case definition (N=349)

Culture	PCR	Acute Serology	CCD	N	Probability of Having Pertussis
−	−	−	−	248	0.0011
−	−	−	+	72	0.0057
−	−	+	−	11	0.0039
−	−	+	+	3	0.0198
+	−	−	−	1	0.7963
+	−	−	+	1	0.9525
−	+	−	−	1	0.9378
−	+	−	+	2	0.9872
+	+	−	−	4	1.0000
+	+	−	+	4	1.0000
+	+	+	+	2	1.0000

Abbreviations: CCD, Clinical Case Definition

Table 6: Sensitivity and specificity estimates of pertussis diagnostic tests in all participants with non-missing data on culture, PCR, convalescent serology, and the clinical case definition (N=545); all participants with non-missing data on culture, PCR, acute serology, and the clinical case definition (N= 349); and all participants with specimens collected at the optimal time[1] for each test (N= 262)

		"Gold Standard" Culture		"Gold Standard" LCA		CRS[2]	
		Sensitivity (95% CI)	**Specificity (95% CI)**	**Sensitivity (95% CI)**	**Specificity (95% CI)**	**Sensitivity (95% CI)**	**Specificity (95% CI)**
Convalescent Serology, n=545	**Culture**	N/A	N/A	64.0 (41.8- 86.2)	99.9 (99.4- 100)	N/A	N/A
	PCR	92.3 (66.7- 98.6)	98.9 (97.6- 99.5)	90.7 (65.6- 100)	100 (99.4- 100)	N/A	N/A
	Con. Serology	61.5 (35.5- 82.3)	88.2 (85.1- 90.6)	73.2 (50.9- 95.6)	89.3 (86.6- 91.9)	73.7 (51.2- 88.2)	89.2 (86.2- 91.5)
	CCD	84.6 (57.8- 95.7)	58.8 (54.6- 62.9)	88.1 (73.5- 100)	59.5 (55.3- 63.7)	89.5 (68.6- 97.1)	59.5 (55.3- 63.6)
Acute Serology, n=349	**Culture**	N/A	N/A	73.8 (48.6- 98.9)	99.9 (99.1- 100)	N/A	N/A
	PCR	83.3 (52.2- 95.3)	99.1 (97.4- 99.7)	81.1 (52.6- 100)	100 (99.5- 100)	N/A	N/A
	Acute Serology	16.7 (4.70- 44.8)	95.9 (93.2- 97.5)	13.3 (0.0- 30.6)	95.8 (93.7- 98.0)	13.3 (3.7- 37.9)	95.8 (93.1- 97.5)
	CCD	58.3 (32.0- 80.7)	77.2 (72.4- 81.3)	59.7 (33.8- 85.5)	77.6 (73.1- 82.1)	60.0 (35.8- 80.2)	77.5 (72.8- 81.7)
Optimally Timed Specimen Collection, n= 262	**Culture**	N/A	N/A	93.2 (74.6- 100)	100 (99.8- 100)	N/A	N/A
	PCR	87.5 (52.9- 97.8)	100 (98.5- 100)	81.7 (54.7- 100)	100 (99.9- 100)	N/A	N/A
	Con. Serology	62.5 (30.6- 86.3)	96.1 (92.9- 97.9)	59.2 (26.1- 92.4)	96.1 (93.7- 98.5)	62.5 (30.6- 86.3)	96.1 (92.9- 97.9)
	CCD	75.0 (40.9- 92.9)	77.2 (71.6- 81.9)	71.9 (41.7- 100)	77.2 (72.0- 82.3)	75.0 (40.9- 92.9)	77.2 (71.6- 81.9)

Abbreviations: 95% CI, 95% Confidence Interval; N/A, not applicable; Con. Serology, Convalescent Serology; CCD, Clinical Case Definition

[1] Optimal Timing of NP collection for culture is the first two weeks of cough illness. Optimal timing of NP collection for PCR is the first three weeks of cough illness. Optimal timing of blood collection for convalescent serology is weeks 2-8 of cough illness

[2] CRS was defined as positive if culture or PCR were positive; otherwise, CRS was defined as negative

Appendix 1: Pertussis and Environmental Health

Pertussis has been a nationally notifiable disease in the US since 1922. From 1934-1943, 200,752 pertussis cases and 4,034 pertussis-related deaths were reported each year, on average (1). Vaccination against pertussis began in the 1940s and resulted in a dramatic decrease in the number of reported pertussis cases and deaths in the U.S. However, the annual incidence of pertussis has been on a gradual increase since the 1980's, and over 48,000 cases were reported in 2012 (2). Reasons for this increase include increased diagnosis and reporting of pertussis, low vaccine effectiveness, and immunity following vaccination and natural infection that only lasts 5-10 years after vaccination (1, 3). Understanding the clinical accuracy and reliability of pertussis diagnostic tests will help prevent severe disease in infants, and will assist in the development and evaluation of pertussis prevention and control strategies.

Infants have the most severe cases of pertussis, and often require hospitalization. Infants are often infected by adolescent and adult household contacts (4), and infants <1 year of age consistently have the highest annual incidence of pertussis in the US. In 2011, the pertussis incidence rate in infants < 1 year of age was 66.85 per 100,000 population, while the incidence rate in all other age groups ranged from 1.38 – 17.62 per 100,000 population (5). The pertussis death rate is also highest in this age group. In 2012, 83% of all reported pertussis-related deaths occurred in infants <12 months of age (6). Infantile pertussis may result in hospitalization and severe complications, including pneumonia, bacterial and viral co-infections, and apnea (7).

Many pertussis outbreaks have occurred in occupational settings, such as schools and hospitals. In 2006, an outbreak was reported in a Cook County, Illinois high school (8). During the investigation, 3.8% of the student body reported cough illness and were excluded from school for at least one week. A total of 36 cases were identified. Additionally, 26.1% of students and 63.9% of staff members received Tdap as part of a school-wide vaccination campaign. Outbreaks that begin in schools may quickly spread and infect others in the community. In 2002, an outbreak that began in an Arizona middle school led to the infection of 485 people in six different communities, including nine infants, over a six month period (9).

Few studies have estimated the monetary costs associated with response to a pertussis outbreak. In 2008, the Douglas County Health Department in Omaha, Nebraska responded to a pertussis outbreak in a local school. Over $52,000 was spent on the outbreak response, excluding the costs of prophylactic treatment for close contacts of pertussis cases and the costs of laboratory testing (10). Furthermore, several pseudo-outbreaks have been caused by the environmental contamination of NP specimens and falsely-positive PCR tests, and have resulted in the wasting of resources. CDC has recommended "best practices" for the collection and transportation of clinical specimens to prevent contamination and pseudo-outbreaks (11).

Health care workers are at high risk for contracting pertussis and transmitting the bacteria to their patients. In 2003, pertussis outbreaks occurred in hospitals in Kentucky, Pennsylvania, and Oregon (12). Pertussis was transmitted to or from a hospitalized infant ≤ 1 year of age, and infected physicians continued to see patients during the days and weeks between their symptom onset and diagnosis. In each hospital, over 100 contacts of the pertussis cases, including ICU patients, family members, and hospital employees, were identified and prescribed prophylactic treatment. To reduce the transmission of *B. pertussis* between health care workers and their patients, ACIP currently recommends that all health care personnel receive a single dose of Tdap if they have not previously received Tdap (13).

Environment is a major contributor to the spread of disease in a community. Recent pertussis outbreaks that have begun in schools and hospitals have quickly spread to other occupational settings and to households. Despite high childhood pertussis vaccination rates (14) and increasing Tdap vaccination rates in adolescents and adults (15), pertussis continues to cause disease across the age spectrum. Infants too young to be fully vaccinated are at the highest risk of developing severe pertussis infection, which may result in hospitalization and death (7). The rapid and accurate diagnosis of true pertussis cases is essential to control the spread of disease. Therefore, the availability and appropriate use of clinically accurate pertussis diagnostic tests is needed to adequately control pertussis in the United States.

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Appendix 2

Table 1: Comparison of full study population (N=868) and sample with non-missing data on culture, PCR, convalescent serology, and the clinical case definition (N=545)

	Full Study Population, N (%)	Complete Test Data, N (%)	p-value[1]
Demographics			
Age ≤ 9 years	101 (11.64)	51 (9.36)	0.18
Female	585 (67.55)	373 (68.57)	0.69
Race: Caucasian	544 (66.18)	335 (63.93)	0.65
Ethnicity: Non-Hispanic	619 (78.06)	391 (79.47)	0.55
Clinical Symptoms			
Cough duration < 2 weeks	491 (56.57)	264 (48.44)	0.00
Paroxysmal Cough	479 (57.30)	262 (50.00)	0.01
Post-tussive Vomiting	139 (16.55)	73 (13.85)	0.18
Inspiratory Whoop	126 (15.57)	64 (12.70)	0.15
Apnea	118 (14.41)	71 (13.76)	0.74
Epidemiology			
Related to a Pertussis Outbreak	203 (27.43)	112 (23.14)	0.09
Epidemiologically Linked to Lab-Confirmed Case	162 (22.78)	93 (19.91)	0.24
Close Contact of CSTE Confirmed Case	128 (17.83)	61 (13.12)	0.03
Household Contact of CSTE Confirmed Case	99 (13.73)	42 (9.05)	0.02
CSTE Case Definition			
Meets Clinical Case Definition	348 (42.59)	230 (42.20)	0.89
Probable Case	185 (21.31)	125 (22.94)	0.47
Confirmed Case	123 (14.17)	70 (8.06)	0.48

*Denominators may vary because of missing data

[1] p-values based on X^2 tests of independent proportions