Prospective Evaluation of an Australian Pertussis Toxin IgG and IgA Enzyme Immunoassay

Meryta L. May,† Suhail A. Doi,‡ David King,§ Jenny Evans,∥ and Jennifer M. Robson∥

Department of Microbiology, Sullivan Nicolaides Pathology, Brisbane, Australia,† and Clinical Epidemiology Unit, School of Population Health, University of Queensland, Brisbane, Australia‡,§,∥

Serological diagnosis of recent pertussis infection is an important part of both clinical assessment and epidemiological documentation of this disease. Standardization of serological testing and interpretation remains challenging despite international efforts to improve it. Currently, determining the anti-pertussis toxin (PT) IgG titer is recommended as the most accurate serological test in Europe and the United States, while Australia relies predominantly on measurement of Bordetella pertussis IgA antibody responses. Using B. pertussis PCR and the WHO clinical case definition as reference standards, the diagnostic utility of in-house anti-PT IgG and anti-PT IgA assays was evaluated prospectively in an Australian community-based cohort (n = 327). Patients provided up to four consecutive serum samples to document the kinetics of antibody response and decay. Previously validated cutoffs for positivity were converted to international units by using WHO-approved reference sera. At currently used cutoffs, both anti-PT IgG (>94 IU/ml) and anti-PT IgA (>20 IU/ml) assays had good specificity (80% [95% confidence interval [95% CI], 68 to 88%] and 87% [95% CI, 77 to 94%]), but anti-PT IgG assay was consistently more sensitive than anti-PT IgA assay across a range of cutoffs (60 to 79% [95% CI, 53 to 84%] versus 41 to 62% [95% CI, 34 to 69%]). The combination of anti-PT IgG and anti-PT IgA assays performed no better than anti-PT IgG assay alone. The anti-PT IgA response in children under 12 years of age was poor. The accuracy of serology was optimal between 2 and 8 weeks after symptom onset. Cutoffs of >94 IU/ml for anti-PT IgG and >20 IU/ml for anti-PT IgA correlated well with recent pertussis infection and were consistent with recent recommendations from the EU Pertstrain group. Anti-PT IgG assay was superior to anti-PT IgA assay as the test of choice for the diagnosis of pertussis from a single sample.

Bordetella pertussis infection is reemerging internationally, despite high rates of vaccination in early childhood (8). Accurate diagnosis of recent infection remains encumbered by the nonspecific nature of the clinical illness and the lack of well-standardized serological tests for diagnosis of late presentations. Culture of the organism has mostly been superseded by the use of PCR, which has become the test of choice early in the course of infection. Diagnosis of later presentations relies on serology, but approaches to serological diagnosis vary widely due to differences in assay characteristics, antigens used, and antibody isotypes detected. Currently, commercial enzyme immunoassay (EIA) antigens include pertussis toxin (PT), filamentous hemagglutinin (FHA), pertactin (PRN), and a whole-cell lysate which contains multiple antigens. Whole-cell IgA is nonspecific in comparison to PT-based assays, while FHA, PRN, and fimbrial antigens show cross-reactions among other Bordetella species and other common respiratory pathogens, including Haemophilus influenzae, Mycoplasma pneumoniae, and Chlamydia pneumoniae (14, 15).

The World Health Organization (WHO) criteria for a laboratory-confirmed case include seroconversion observed in paired serology samples, but collection of serial samples is infrequent in practice. Across Europe, there is emerging acceptance of a single high titer of anti-PT IgG (50 to 120 IU/ml) as a specific serological indicator of acute infection (9). In Australia, the emphasis in serological testing and reporting was initially on the use of whole-cell IgA and, more recently, on a combination of anti-whole-cell or -FHA IgA and anti-PT IgA (2). Australian data on the relevance of anti-PT IgG are limited (18, 20), and it is only since April 2011 that an elevated anti-PT IgG level has been part of Australia’s national notifiable disease case definition of pertussis.

Significant investment has been made by the U.S. Centers for Disease Control and Prevention (CDC) and the European Union Pertstrain group to standardize pertussis serology internationally (9, 16, 20). As of 2009, a WHO-approved international standard can be purchased by laboratories wishing to benchmark their pertussis assays (24).

Using B. pertussis PCR and the WHO clinical case definition as reference standards, we set out to examine the accuracy of in-house anti-PT IgG- and IgA-based EIAs for the diagnosis of acute B. pertussis infection in a prospective community-based study and to observe the kinetics of anti-PT antibody response and decay.

MATERIALS AND METHODS

Definitions. A positive result for B. pertussis PCR on combined throat and nasopharyngeal swabs was taken as a laboratory-confirmed case and became the reference standard. The clinical case definition was cough for >2 weeks and <9 weeks and at least one of the following symptoms: paroxysms of cough, posttussive vomiting or retching, and the presence of an inspiratory whoop (23).

Study population and sera. Sullivan Nicolaides Pathology (SNP) is a large private pathology company providing diagnostic services predominantly to community-based medical practices in all Australian states. All patients referred for B. pertussis PCR and serology from January 2009 to
March 2010 at Queensland or northern New South Wales SNP collection centers were invited to participate. In addition, to increase the recruitment of laboratory-confirmed cases, starting in July 2009, all *B. pertussis* PCR-positive patients aged >5 years were contacted. Subjects were excluded if they had another laboratory-proven respiratory infection, cough for more than 8 weeks at presentation, or receipt of a vaccination against pertussis in the previous 6 months. All patients had to have both PCR and serology testing for pertussis and provide information regarding vaccination status and length of symptoms. Demographic and clinical data were collected, including duration and clinical features of cough, previous pertussis infection or exposure, and history of pertussis vaccination. Telephone verification was made from the patient or physician for those reporting recent vaccination. Ethics approval was obtained from the Royal Australian College of General Practitioners. Study results were made available to the patients’ treating practitioners.

Specimens for serological analysis were collected at enrolment where possible, and then a maximum of 3 further samples were requested for each subject. Each sequential collection time was not fixed but was referenced to the date of onset of symptoms. Follow-up samples were collected for up to a year (median, 55 days; interquartile range [IQR], 33 to 83 days) from the estimated onset of symptoms. All laboratory testing was performed as part of routine processing of specimens by rostered laboratory scientists who had no participation in the study.

**Laboratory testing.** (i) Anti-PT IgG and anti-PT IgA assay overview. The in-house assay was an indirect EIA using PT (Kaketsuken, Japan)-coated Costar polystyrene strip wells (Corning Inc.). Horseradish peroxidase (HRP)-conjugated anti-human IgG and IgA antibodies (Millipore) and tetramethylbenzidine (TMB) comprised the detection system. The assay and clinically relevant cutoffs were validated extensively from 1998 to 2010. Previous validation samples included sera from children, Red Cross blood donors, and patients with other acute respiratory infections. Specificity data from 2010 are shown in Fig. S3 and S4 in the supplemental material.

(ii) Anti-PT IgG and anti-PT IgA in-house assay testing method. To correct for interrun variation, a calibration was used in each run to normalize optical density values. The absorbance of the calibrator was used to represent the cutoff absorbance. The sample absorbance was divided by the cutoff absorbance to give a serum/cutoff ratio (referred to as an index). The calibrator, controls, and samples were diluted 1:121 in sample diluent (1:11 dilution = 10 μl serum + 100 μl diluent 1; 1:121 dilution = 20 μl 1:11 dilution + 200 μl diluent 2). One-hundred-microliter aliquots of the diluted serum samples were added to designated microwells and incubated for 30 min at 37°C. This was followed by six washes with wash buffer, and then 100 μl of HRP-conjugated anti-human IgG or IgA was added for another incubation step of 30 min at 37°C. After a further six washes, 100 μl TMB was added, followed by incubation at 20 to 25°C for 30 min. The reaction was terminated by adding 100 μl 1 M orthophosphoric acid. Microrotator strips were read at a wavelength of 450/620 nm within 30 min. The calibrator represents the cutoff absorbance and was used in all runs to normalize absorbance values as described above, and the results are reported as laboratory indices. The end user performed data reduction via Multicalc software (Wallac Oy, Finland), and the assay production department used Anthos ADAP software (Anthos Labtec Instruments, Austria). The study samples were tested initially by the serology department, and results were reported as indices. Samples were distributed by a Tecan liquid-handling robot and processed daily with the routine pertussis testing. The samples were then retested by the assay production department, who distributed the samples manually. Sequential samples from study patients were retested on the same plate. Each plate tested included the WHO reference reagent (NIBSC code 06/142) and dilutions of the WHO standard (NIBSC code 06/140). For this study, WHO standards were reconstituted per WHO instructions, diluted in Sera Sub (CST Technologies, Inc., NY) to give 200, 100, 50, and 25 IU/ml, and then processed as outlined in the method. Assay processing was done manually in both departments. The WHO standard and reference reagent were tested on 5 kit lots for anti-PT IgG EIA and on 4 kit lots for anti-PT IgA EIA.

Laboratory indices were converted to international units (IU/ml) (see the supplemental material). The assay performance was constantly monitored by the end user, with any change in trends being reported to the assay production department for review.

(iii) Pertussis PCR. Combined nasopharyngeal flocked and throat swabs collected from patients were eluted and vortexed in 2 ml of viral transport medium (cell culture medium based on Ham’s F10, Sigma medium N6635, fetal calf serum, and l-glutamine, prepared in-house). A 200-μl aliquot was then extracted using a MagNA Pure LC total nucleic acid isolation kit (Roche Applied Sciences, Basel, Switzerland) on a MagNA Pure LC instrument (Roche Applied Sciences). Five microliters of DNA template was added to 15 μl of master mix. The sequences for the *B. pertussis* primers and TaqMan probe were selected from the multicity Bordetella pertussis IS481-like insertion sequence (GenBank accession no. BX640418). The probe was labeled at the 5’ end with a reporter fluorochrome (6-carboxyfluorescein [FAM]) and at the 3’ end with a dark quencher (BHQ-1). The forward and reverse primer sequences were CA ATAGGGTTGTATGCATGTTCATC and CGGGCTCTCTGTAGTGAA CTG, respectively, and the FAM-labeled probe sequence was acTgGaAT cGcCaaacc-BHQ1 (capitalized bases indicate locked nucleic acids). *Lactobacillus fermentum* was seeded and coextracted as an internal control. Denaturation at 95°C for 30 s was followed by annealing and extension at 60°C and cooling at 50°C for 50 cycles. Samples were reported as positive if an amplification curve was generated. Included in each PCR run were a no-template control (NTC), a “high positive” control (HPC), a “low positive” control (LPC) (*B. pertussis*; Vircell, Granada, Spain), and an *L. fermentum* positive control. The controls were calibrated such that positivity was observed for the high positive control in the *C*~r~ range of 20 to 29 and for the low positive control in the *C*~r~ range of 30 to 39. All molecular testing occurred at the same central Sullivan Nicolaides Pathology Laboratory, Taringa, Brisbane, Australia. The analytical sensitivity of the assay was calculated to be 1.1 genome equivalents per reaction.

**Statistical analysis.** Frequency tables were analyzed using the χ² test or Fisher’s exact test for categorical variables. Differences in the medians of continuous variables for two and multiple independent samples were analyzed using the Mann-Whitney test and the Kruskal-Wallis test, respectively. Kaplan-Meier (KM) analysis was used to determine median times to seropositivity/seronegativity, and stratum-specific outcomes were compared using log rank statistics. Kinetics of anti-PT IgG and anti-PT IgA postinfection in PCR-positive patients were analyzed on a per-sample basis, using median antibody values at different time intervals per the methods of Hallander and colleagues (10). The sensitivity and specificity of anti-PT IgG and anti-PT IgA EIAs were calculated from 2 × 2 contingency tables for a range of possible cutoff values and plotted as receiver operating characteristic (ROC) curves. The area under the ROC curve (AUC) and its significance were calculated according to the methods of Hanley and McNeil (11). Operating characteristics of the serological tests were computed at various cutoffs.

In an attempt to determine if baseline information could increase accuracy over serologic testing alone, multivariable analysis was performed to see which baseline variables predicted PCR status independent of serology, using forward stepwise logistic regression models. All analyses were run on a per-sample basis, not per-subject basis. All statistical analyses performed were two-sided and were declared significant if the P value was <0.05, and they were run using SPSS software (SPSS for Windows, version 13; SPSS, Inc., Chicago, IL).

**RESULTS**

**Patient characteristics.** Four hundred thirteen patients were recruited, and of these, 76 were excluded (Fig. 1). Of the remaining 337 patients, 193 tested *B. pertussis* PCR positive. Ten children who were PCR positive submitted follow-up PCR samples only, without serum, leaving 183 PCR-positive patients who provided...
Seventy-one of these 183 PCR-positive patients met the clinical case definition criteria. Of the 144 PCR-negative subjects, 46 met the clinical definition, and 98 did not.

From the 327 subjects who provided samples for serological analysis (183 PCR-positive and 144 PCR-negative subjects), a total of 639 serum samples were collected over the duration of the study. There were 268 samples collected at presentation and 209, 121, and 41 samples collected at the second, third, and fourth collection time points, respectively. In total, 63% of samples were from PCR-positive patients.

Demographic data for the study participants are shown in Table 1. The median age was 34.6 years (IQR, 13.8 to 55.7 years; range, 1.5 to 87.8 years); 69% of subjects were female. A large proportion had received vaccination. PCR-positive subjects were more likely to be children aged less than 12 years and less likely to have received vaccination. They were significantly more likely than PCR-negative subjects to have had paroxysms and whoop and to report case contact. There was no relationship between postcough vomiting and either PCR status or clinical status for PCR-negative subjects (data not shown).

Conversion of serological results to international units. Laboratory index cutoffs for both anti-PT IgG and anti-PT IgA ELAs...
obtained from the calibration curves were as follows: negative, <0.9; equivocal, 0.9 to 1.1; and positive, >1.1. In international units per ml, these indices equated to <65 IU/ml, 65 to 94 IU/ml, and >94 IU/ml for anti-PT IgG and <16 IU/ml, 16 to 20 IU/ml, and >20 IU/ml for anti-PT IgA. The dynamic linear ranges of the anti-PT IgG and anti-PT IgA assays were 10 to 200 IU/ml and 5 to 65 IU/ml.

Kinetics of anti-PT antibody response and decay. The median time from onset of symptoms to measured seropositivity was 33 days for anti-PT IgG (95% confidence interval [95% CI], 29 to 36 days) and 36 days for anti-PT IgA (95% CI, 29 to 42 days) in adults (Fig. 2). Children of <12 years of age had markedly less anti-PT IgA seropositivity but no difference in development of anti-PT IgG. There was no difference in either antibody response for those aged >60 years (data not shown).

There was a biphasic pattern of antibody decay of anti-PT IgG, with a change from a rapid to a slower decline after around 4 months (Fig. 3a). A similar pattern was seen with IgA, although the change in rate of decay was seen earlier (Fig. 3b). The median time to measured seronegativity (≤94 IU/ml) for anti-PT IgG was 200 days (95% CI, 170 to 229 days), and for anti-PT IgA was 101 days (95% CI, 24 to 178 days) (Fig. 4). Patients with anti-PT IgG levels of >94 IU/ml at the third collection point (n = 62) were requested to submit a further sample more than 5 months after symptom onset. Of the 37 patients who provided a sample, 19 (48%) still had anti-PT IgG levels of ≤94 IU/ml. Only eight subjects in the <12-year-old age group submitted follow-up serum 90 days (range, 96 to 299 days) after the onset of symptoms. Three of these (37.5%) had anti-PT IgG levels of ≤94 IU/ml at this time.

Follow-up PCR results. Eighty PCR-positive patients provided a follow-up sample for PCR when providing repeat serum samples. The median duration of positivity was a maximum of 43 days (95% CI, 36 to 50 days) from the reported onset of symptoms. By 54 days, over 80% of subjects were negative.

Multivariable analysis. Forward stepwise logistic regression

### TABLE 1 Demographic data and reported clinical history of participating patients

| Characteristic                        | PCR-positive cases (n = 183) | PCR-negative and clinical case definition-positive cases (n = 46) | PCR-negative and clinical case definition-negative cases (n = 98) | P value*   |
|--------------------------------------|-----------------------------|----------------------------------------------------------------|-----------------------------------------------------------------|-----------|
| No. (%) of children of <12 yr        | 55 (30)                     | 2 (4)                                                           | 18 (18)                                                        | <0.0001   |
| No. (%) of adults of ≥60 yr          | 29 (16)                     | 11 (24)                                                         | 21 (21)                                                        | 0.214     |
| Median age (range) (yr)              | 32 (1–87)                   | 41 (7–77)                                                       | 41 (1–84)                                                      | 0.001     |
| Sex                                  |                             |                                                                 |                                                                | 0.008     |
| No. (%) of males                     | 70 (38)                     | 6 (13)                                                          | 24 (24)                                                        |           |
| No. (%) of females                   | 113 (62)                    | 40 (87)                                                         | 74 (76)                                                        |           |
| Median duration of symptoms (range) (days) | 14 (0–56)                  | 28 (17.5–49)                                                    | 10.5 (3.5–70)                                                  | 0.038     |
| No. of subjects with symptom or history/total no. of subjects (%) |                     |                                                                 |                                                                |           |
| Paroxysms                            | 149/171 (87)                | 44/45 (98)                                                      | 64/96 (67)                                                    | 0.021     |
| Whoop                                | 95/165 (58)                 | 27/41 (66)                                                      | 26/89 (29)                                                    | 0.002     |
| Posttussive vomiting                 | 106/169 (63)                | 28/45 (62)                                                      | 43/93 (46)                                                    | 0.056     |
| Contact with pertussis               | 91/169 (54)                 | 10/45 (22)                                                      | 31/94 (33)                                                    | <0.0001   |
| Past pertussis vaccination           | 139/157 (89)                | 35/38 (92)                                                      | 86/87 (99)                                                    | 0.015     |

*Comparing PCR-positive cases with all others. The Mann-Whitney U test was used for continuous variables, and Fisher’s exact test was used for categorical variables.
analysis selected the following as being independently predictive of PCR status: time point (>2 weeks compared to 0 to 2 weeks), age (<12 years), anti-PT IgG level (65 to 94 IU/ml and >94 IU/ml compared to <65 IU/ml), history of case contact, and the presence of whoop. Other symptoms, gender, and anti-PT IgA levels were not selected in the model. This suggests that those presenting more than 2 weeks after onset, with an age of <12 years, with a contact history, or with a whoop had a higher likelihood of being PCR positive. However, use of the model did not result in an appreciable improvement in diagnostic accuracy over serology alone and thus was not pursued further.

**ROC analysis.** ROC analysis using the clinical definition as the reference standard was not discriminatory for either anti-PT IgG or anti-PT IgA (Table 2). With PCR result as the reference standard, the AUC showed significant predictive value for both serological tests after 2 weeks, with peak performance 4 to 8 weeks after the onset of symptoms. Although confidence intervals overlapped, the trend favored anti-PT IgG over anti-PT IgA at all time intervals. The combination of anti-PT IgG and anti-PT IgA performed no better than anti-PT IgG alone.

**Operating characteristics of serology.** The sensitivity, specificity, and likelihood ratios for anti-PT IgG and anti-PT IgA EIAs at various cutoff points are shown in Table 3. At currently used cutoffs, the anti-PT IgA assay (>20 IU/ml) had high specificity (87% [95% CI, 77 to 94%]) but poor sensitivity (50% [95% CI, 43 to 57%]). The anti-PT IgG assay (>94 IU/ml) had a comparable specificity (80% [95% CI, 68 to 88%]) and greater sensitivity (66% [95% CI, 59 to 73%]). Simultaneous anti-PT IgG with anti-PT IgA testing produced a marginal increase in sensitivity (71% [95 CI, 64 to 77%]) but a lower specificity (75% [95% CI, 64 to 85%]) and no change in the likelihood ratio, suggesting that very few subjects might be expected to express anti-PT IgA positivity without anti-PT IgG positivity.

**Recent vaccination.** As documented in Fig. 1, 10 patients, aged 4 months to 49 years, had confirmed pertussis vaccination in the past 6 months and were excluded from prior analysis; 5 of these patients...
were also PCR positive for *B. pertussis*. These five patients all had anti-PT IgG levels of >94 IU/ml, and three had anti-PT IgA levels of >16 IU/ml. Of the remaining five PCR-negative subjects with recent vaccination (4 to 178 days postvaccination), none had anti-PT IgG levels of >94 IU/ml or anti-PT IgA levels of >20 IU/ml. One adult aged 34 years had an anti-PT IgG level of 87 IU/ml and an anti-PT IgA level of 12 IU/ml 4 days after receiving a pertussis vaccination. A follow-up sample taken after 2 months recorded an anti-PT IgG level of 61 IU/ml and an anti-PT IgA level of 8 IU/ml. In all 24 PCR-negative subjects aged less than 12 years who were appropriately vaccinated for their age, only 2 (8%) had an anti-PT IgG level of >94 IU/ml, and none had an IgA level of >16 IU/ml.

**DISCUSSION**

Significant work has been undertaken internationally over the last 10 years to achieve standardization and clarify the utility of serology for the purposes of pertussis diagnosis (9, 16, 20). While PCR is currently the gold standard for most routine laboratory diagnoses of pertussis, serology is used for diagnosis in patients with late presentations, in those who have received prior antibiotics, and in outbreak investigations and epidemiologic surveys. The majority of pertussis notifications to public health authorities in Australia are based on a single sample, tested for either whole-cell *B. pertussis* IgA or anti-FHA, -PT, and/or -PRN IgA (2). This no longer reflects the most appropriate serological approach to diagnosis.

For interpreting single samples, anti-PT IgG has repeatedly been shown to be the most robust serological marker for acute pertussis infection (1, 5, 9). The dilemma has been defining a clinically relevant diagnostic cutoff likely to represent acute infection rather than immunization or past exposure. While numerous epidemiological studies have been undertaken to determine this cutoff (3, 6, 7, 17), this is the first prospective community-based assessment of the diagnostic capability of a single-sample anti-PT IgG assay in an Australian population. We have demonstrated a sensitivity of 66 to 72% and a specificity of 75 to 80% for anti-PT IgG levels of

### TABLE 2 ROC analysis of anti-PT IgG, anti-PT IgA, and combined anti-PT IgA and anti-PT IgG

| Variable and time interval | Area  | SE  | Asymptotic significance | Lower bound | Upper bound |
|---------------------------|-------|-----|-------------------------|-------------|-------------|
| **Anti-PT IgG**            |       |     |                         |             |             |
| PCR standard              |       |     |                         |             |             |
| 0 to 2 weeks (n = 149)    | 0.685 | 0.047 | <0.001                  | 0.592       | 0.777       |
| 2 to 4 weeks (n = 124)    | 0.780 | 0.045 | <0.001                  | 0.692       | 0.868       |
| 4 to 8 weeks (n = 145)    | 0.793 | 0.045 | <0.001                  | 0.705       | 0.881       |
| 8 to 18 weeks (n = 113)   | 0.728 | 0.063 | 0.001                   | 0.605       | 0.851       |
| Clinical standard         |       |     |                         |             |             |
| 2 to 4 weeks (n = 126)    | 0.428 | 0.052 | 0.166                   | 0.326       | 0.530       |
| 4 to 8 weeks (n = 148)    | 0.505 | 0.048 | 0.924                   | 0.411       | 0.599       |
| 8 to 18 weeks (n = 116)   | 0.487 | 0.054 | 0.816                   | 0.382       | 0.593       |
| **Anti-PT IgA**            |       |     |                         |             |             |
| PCR standard              |       |     |                         |             |             |
| 0 to 2 weeks (n = 149)    | 0.685 | 0.044 | <0.001                  | 0.598       | 0.772       |
| 2 to 4 weeks (n = 124)    | 0.710 | 0.048 | <0.001                  | 0.616       | 0.804       |
| 4 to 8 weeks (n = 145)    | 0.725 | 0.049 | <0.001                  | 0.629       | 0.822       |
| 8 to 18 weeks (n = 113)   | 0.628 | 0.062 | 0.052                   | 0.506       | 0.749       |
| Clinical standard         |       |     |                         |             |             |
| 2 to 4 weeks (n = 126)    | 0.413 | 0.051 | 0.092                   | 0.313       | 0.513       |
| 4 to 8 weeks (n = 148)    | 0.486 | 0.048 | 0.779                   | 0.392       | 0.581       |
| 8 to 18 weeks (n = 116)   | 0.483 | 0.054 | 0.754                   | 0.377       | 0.589       |
| **Combined anti-PT IgA and IgG (whichever had the higher result)** |       |     |                         |             |             |
| PCR standard              |       |     |                         |             |             |
| 0 to 2 weeks (n = 149)    | 0.694 | 0.044 | <0.001                  | 0.607       | 0.780       |
| 2 to 4 weeks (n = 124)    | 0.762 | 0.046 | <0.001                  | 0.672       | 0.852       |
| 4 to 8 weeks (n = 145)    | 0.797 | 0.047 | <0.001                  | 0.705       | 0.889       |
| 8 to 18 weeks (n = 113)   | 0.729 | 0.063 | 0.001                   | 0.606       | 0.852       |
| Clinical standard         |       |     |                         |             |             |
| 2 to 4 weeks (n = 126)    | 0.410 | 0.051 | 0.081                   | 0.310       | 0.509       |
| 4 to 8 weeks (n = 148)    | 0.479 | 0.048 | 0.661                   | 0.385       | 0.572       |
| 8 to 18 weeks (n = 116)   | 0.491 | 0.054 | 0.874                   | 0.386       | 0.597       |

a When anti-PT IgG, anti-PT IgA, or the combination of both cannot distinguish between positive and negative results for each standard, i.e., when there is no difference between the two distributions, the area equals 0.5. When there is a perfect separation of the values for the two groups, i.e., there is no overlap of the distributions, the area under the ROC curve equals 1.

b Under the nonparametric assumption.

c Null hypothesis: true area = 0.5.

d Samples taken >18 weeks from the onset of symptoms were excluded.

---

February 2012 Volume 19 Number 2

cvi.asm.org 195
IgG EIA in diagnosing recent pertussis, using a dual cutoff of 65 to 94 IU/ml on a single sample. Previous epidemiological studies in Australia have concurred with the general recommendation that the most specific cutoff for anti-PT IgG is probably around 100 IU/ml (4), as first described in the Netherlands by de Melker et al. (5). Others have suggested that a cutoff of 100 IU/ml may be relatively insensitive in comparison to lower values, such as 50 to 65 IU/ml (9, 10), particularly in an outbreak situation with a high pretest probability (12). In recently published guidelines from the EU Pertstrain Group Reference Laboratories, it was recommended that a dual cutoff between 50 IU/ml and 120 IU/ml is advisable for diagnosis from a single serum sample (9). This is in keeping with the cutoffs used in our assay, with results reported as “equivocal” for values of 65 to 94 IU/ml and “positive” for values of >94 IU/ml.

In contrast to the substantial progress made in defining clinically relevant cutoffs for anti-PT IgG in the international literature, there is little established guidance for anti-PT IgA. Our results concur with those of Guiso and colleagues (9), who suggest that the likely cutoff is between 10 and 20 IU/ml, close to the minimum level of quantitation. In Australia, the use of IgA titers has traditionally been favored for serological diagnosis of pertussis, partly due to the belief that the more rapid decay of IgA titers produces significantly greater specificity. Our analysis, despite confirming a higher rate of decay, showed that anti-PT IgA has a secondary role in the serological diagnosis of pertussis. Sensitivity was consistently inferior to that for anti-PT IgG (Table 3), and the combination of anti-PT IgG and anti-PT IgA showed no significant advantage over the use of anti-PT IgG alone. The sensitivity of anti-PT IgA was particularly problematic in children. It is well recognized that IgA responses in children younger than 2 years are not robust, but this was demonstrated for older children as well. Similar results were found by Wirsing von Konig and colleagues, who found elevated convalescent-phase levels of anti-PT IgA in only 20% of children aged 5 to 10 years, while 74% had elevated anti-PT IgG levels (22). Our results concur with others who suggest that in the presence of a well-validated anti-PT IgG test, the role of anti-PT IgA testing is limited (19).

The optimum time for performance of serology appears to be 2 to 8 weeks after the estimated onset of symptoms, which correlates with the decline in sensitivity of PCR testing. In contrast to the case for anti-PT IgG, we found no significant age-related differences in seroconversion of anti-PT IgG. Since repeat serum collections were not performed at fixed time intervals, the time to seropositivity represents the maximum time interval to seroconversion, but the data are consistent with previous literature describing the longitudinal dynamics of anti-PT IgG measurement following infection (5, 10, 21).

The median time for anti-PT IgG and anti-PT IgA to decay below laboratory cutoffs was approximately 6 months for anti-PT IgG and 3 months for anti-PT IgA, with 80% of subjects reaching anti-PT IgG levels of <94 IU/ml by 310 days (11 months). In clinical practice, these dynamics mean that even with serial samples, a diagnostic rise may be missed, and convalescent-phase samples may show only a minor decline in antibody levels, underscoring the importance of defining the interpretation of a single serum result.

Prior vaccination history may theoretically confound interpretation. However, previous work by Pebody and colleagues in Europe (17) suggested that high titers of anti-PT IgG in vaccinated children of ≥2 years of age are highly probable to indicate recent infection. The Australian immunization schedule includes an acellular pertussis vaccine at 2, 4, and 6 months of age, with a booster at 4 years and during adolescence. Estimated compliance is 91.4% at 12 months and 94.9% at 24 months of age (13). The five patients in this study who were vaccinated in the previous 6 months all showed anti-PT IgG levels of <94 IU/ml. There was little background anti-PT IgG detected in noncase subjects, including vaccinated children, and this was supported by previous in-house validation data. Since 1999, validation data have included multiple evaluations of background anti-PT IgG and anti-PT IgA levels, particularly after any changes in vaccination type and schedule. Most recently, in 2008, only 3 of 210 (1.4%) children aged 0 to 8 years with no acute respiratory illness had anti-PT IgG levels of >94 IU/ml. Only one child had an anti-PT IgA level of >16 IU/ml. This is not conclusive but suggests that in the majority of cases, a significant anti-PT IgA or IgG response to vaccination is relatively short-lived, and single high-titer (>94 IU/ml) anti-PT IgG results can still have diagnostic value for children of <10 years of age. Conversely, if an individual of any age

### Table 3: Sensitivity, specificity, and likelihood ratios at various cutoffs for individual and combined serology 2 to 8 weeks after onset of symptoms.

| Variable and cutoff (IU/ml) (laboratory index) | Sensitivity (95% CI) | Specificity (95% CI) | Likelihood ratio (95% CI) |
|-----------------------------------------------|----------------------|----------------------|--------------------------|
| **Anti-PT IgG**                               |                      |                      |                          |
| >50 (0.75)                                    | 79 (72–84)           | 73 (60–83)           | 2.9 (2.4–3.4)            | 0.3 (0.2–0.5) |
| >65 (0.9)                                     | 72 (65–78)           | 75 (64–85)           | 2.9 (2.5–3.4)            | 0.4 (0.2–0.6) |
| >94 (1.1)                                     | 66 (59–73)           | 80 (68–88)           | 3.3 (2.8–3.8)            | 0.4 (0.3–0.7) |
| >125 (1.28)                                   | 60 (53–67)           | 84 (73–92)           | 3.8 (3.2–4.4)            | 0.5 (0.3–0.8) |
| **Anti-PT IgA**                               |                      |                      |                          |
| >10 (0.6)                                     | 62 (55–69)           | 75 (64–85)           | 2.5 (2.1–3)              | 0.5 (0.3–0.8) |
| >16 (0.9)                                     | 55 (47–62)           | 81 (70–90)           | 2.9 (2.4–3.4)            | 0.6 (0.3–0.9) |
| >20 (1.1)                                     | 50 (43–57)           | 87 (77–94)           | 3.8 (3.2–4.5)            | 0.6 (0.3–1.1) |
| >25 (1.35)                                    | 41 (34–48)           | 90 (80–96)           | 4 (3.4–4.9)              | 0.7 (0.3–1.3) |
| **Anti-PT IgA or IgG**                        |                      |                      |                          |
| >16 or > 65 (>0.9)                            | 80 (73–85)           | 71 (59–81)           | 2.7 (2.3–3.2)            | 0.3 (0.2–0.5) |
| >20 or > 94 (>1.1)                            | 71 (64–77)           | 75 (64–85)           | 2.9 (2.4–3.4)            | 0.4 (0.2–0.6) |
has had a pertussis-containing vaccine within the last 3 to 6 months, intermediate levels of anti-PT IgG (e.g., 65 to 94 IU/ml) should be interpreted with caution. Anti-PT IgA values typically peak and decline more quickly than anti-PT IgG levels, so it is unlikely that vaccination will give a prolonged anti-PT IgA response; however, there are insufficient postvaccination data from this study to confirm this expectation.

Due to the lack of a clear gold standard, we chose *B. pertussis* PCR positivity and the WHO-recognized clinical definition as reference points. ROC analysis clearly showed that the clinical definition in isolation has no discriminatory power. The poor performance of the clinical case definition has significance not only for clinicians attempting to diagnose pertussis but also for research in which the clinical case definition alone is used as the determination of true infection. It is our view that the majority of PCR-negative but clinical definition-positive cases in this study were probably not true infections. Of these 46, only 6 had a significant change in serial anti-PT IgG and/or IgA levels. Despite the rise in paired serology, these six cases were treated as noncases in ROC sensitivity and specificity analysis due to being PCR negative. This may have produced some bias against the performance of our assay and highlights the difficulties inherent in analysis of a diagnostic test in the absence of a clear gold standard.

In conclusion, this study has demonstrated the clinical utility of an in-house PT-based EIA and the value of a single high-titer anti-PT IgG result for diagnosing pertussis in an Australian population. Anti-PT IgA contributed little further diagnostic information, particularly in children. In clinical practice, levels of anti-PT IgG of >65 IU/ml are likely to be consistent with pertussis infection within the last year, and levels of >94 IU/ml are indicative of pertussis infection within the last 6 months. It is notable that irrespective of the various vaccination strategies, cutoffs for single-sample serology are comparable throughout the countries in which they have been evaluated. An increased availability of reliable commercial PT-based EIAs is needed to improve the accuracy of serological diagnosis of pertussis in Australia.

**ACKNOWLEDGMENTS**

M.L.M., J.E., and J.M.R. designed the trial and led the laboratory analyses. M.L.M. and S.A.D. executed the analyses, and M.L.M., S.A.D., D.K., and J.M.R. interpreted the results. All authors contributed to critical reviews and revisions of the report and have seen and approved the final version.

We declare that we have no conflicts of interest. No external funding was received.

**REFERENCES**

1. Andre P, et al. 2008. Comparison of serological and real-time PCR assays to diagnose Bordetella pertussis infection in 2007. J. Clin. Microbiol. 46: 1672–1677.
2. Australian Government Department of Health and Ageing. March 2004, posting date. Australian national notifiable diseases case definitions: pertussis case definition. http://www.health.gov.au/internet/main/publishing.nsf/content/cda-surveil-nndss-casedefs-cd_pertus.htm.
3. Baughman AL, et al. 2004. Establishment of diagnostic cutoff points for levels of serum antibodies to pertussis toxin, filamentous hemagglutinin, and fimbriae in adolescents and adults in the United States. Clin. Diagn. Lab. Immunol. 11:1045–1053.
4. Cagney M, McInalty CR, McIntyre P, Puech M, Giammanco A. 2006. The seroepidemiology of pertussis in Australia during an epidemiologic period. Epidemiol. Infect. 134:1208–1216.
5. de Melker HE, et al. 2000. Specificity and sensitivity of high levels of immunoglobulin G antibodies against pertussis toxin in a single serum sample for diagnosis of infection with Bordetella pertussis. J. Clin. Microbiol. 38:800–806.
6. Giammanco A, et al. 2003. European Sero-Epidemiology Network: standardisation of the assay results for pertussis. Vaccine 22:112–120.
7. Giammanco A, et al. 2008. European Sero-Epidemiology Network 2: standardisation of immunoassay results for pertussis requires homogeneity in the antigenic preparations. Vaccine 26:4486–4493.
8. Quinto-Ocampo H, Bennett JE, Attila MW. 2008. Predicting pertussis in infants. Pediatr. Emerg. Care 24:16–20.
9. Guiso N, et al. 2011. What to do and what not to do in serological diagnosis of pertussis: recommendations from EU reference laboratories. Eur. J. Clin. Microbiol. Infect. Dis. 30:307–312.
10. Hallander HO, Ljungman M, Storsaeter J, Gustafsson L. 2009. Kinetics and sensitivity of ELISA IgG pertussis antitoxin after infection and vaccination with Bordetella pertussis in young children. APMIS 117:797–807.
11. Hanley JA, McNeil BJ. 1982. The meaning and use of the area under a receiver operating characteristic (ROC) curve. Radiology 143:29–36.
12. Horby P, et al. 2005. A boarding school outbreak of pertussis in adolescents: value of laboratory diagnostic methods. Epidemiol. Infect. 133:229–236.
13. Hull BP, Mahajan D, Dey A, Menzies RI, McIntyre PB. 2010. Immunisation coverage annual report, 2008. Commun. Dis. Intell. 34:241–258.
14. Jackson LA, Cherry JD, Wang SP, Grayston JT. 2000. Frequency of serological evidence of Bordetella infections and mixed infections with other respiratory pathogens in university students with cough illnesses. Clin. Infect. Dis. 31:3–6.
15. Matteo S, Cherry JD. 2005. Molecular pathogenesis, epidemiology, and clinical manifestations of respiratory infections due to Bordetella pertussis and other Bordetella subspecies. Clin. Microbiol. Rev. 18:326–382.
16. Menzies SL, et al. 2009. Development and analytical validation of an immunoassay for quantifying serum anti-pertussis toxin antibodies resulting from Bordetella pertussis infection. Clin. Vaccine Immunol. 16:1781–1788.
17. Pembley RG, et al. 2005. The seroepidemiology of Bordetella pertussis infection in Western Europe. Epidemiol. Infect. 133:159–171.
18. Quinn HE, McIntyre PB. 2007. Pertussis epidemiology in Australia over the decade 1995–2005—trends by region and age group. Commun. Dis. Intell. 31:205–215.
19. Riffelmann M, Thiels K, Schmetz J, Wirsing von Konig CH. 2010. Performance of commercial enzyme-linked immunosorbent assays for detection of antibodies to Bordetella pertussis. J. Clin. Microbiol. 48:4459–4463.
20. Tondella ML, et al. 2009. International Bordetella pertussis assay standardization and harmonization meeting report. Centers for Disease Control and Prevention, Atlanta, Georgia, United States, 19–20 July 2007. Vaccine 27:803–814.
21. Versteegh FG, et al. 2005. Age-specific long-term course of IgG antibodies to pertussis toxin after asymptomatic infection with Bordetella pertussis. Epidemiol. Infect. 133:737–748.
22. Wirsing von Konig CH, Gounis D, Laukamp S, Bogaerts H, Schmitt HJ. 1999. Evaluation of a single-sample serological technique for diagnosing pertussis in unvaccinated children. Eur. J. Clin. Microbiol. Infect. Dis. 18:341–345.
23. World Health Organization. 2001. Pertussis surveillance. World Health Organization, Geneva, Switzerland.
24. Xing D, et al. 2009. Characterization of reference materials for human antisera to pertussis antigens by an international collaborative study. Clin. Vaccine Immunol. 16:303–311.