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Serological diagnosis of pertussis: evaluation of IgA against whole cell and specific *Bordetella pertussis* antigens as markers of recent infection

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SUMMARY

In Australia, notification of pertussis cases in older children or adults has increased significantly in recent years. In most cases, laboratory diagnosis is based only on a positive serological test for IgA antibody against whole cell *Bordetella pertussis*. During a 3-month period, 318 consecutive sera submitted for diagnosis of pertussis were tested for IgA antibody against whole cell (WC) sonicated *B. pertussis*, pertussis toxin (PT), filamentous haemagglutinin (FHA) and pertactin (PRN). Results of one or more of these tests were positive in sera from 175 subjects and clinical information was obtained by telephone interview from 90 subjects. Using a clinical case definition as the reference standard, the sensitivities of the four IgA assays were variable but quite low (24–64%), but the specificities were high (93–98%). For diagnosis of pertussis in subjects with a compatible clinical illness, these and other findings support the use of serological testing for IgA antibody.

INTRODUCTION

Australia has experienced a dramatic increase in notifications of pertussis in the past 6 years [1]. Reasons for this include greater awareness of the disease in adults and adolescents and consequent increased testing, especially by serology [2, 3]. In New South Wales, between 1990 and 1997, serological testing, usually on a single serum sample, was the basis for diagnosis of pertussis in 30%, 63% and 83% of notified cases in the 0–4, 5–9 and 10–85 age-groups respectively (Siranda Torvaldsen, personal communication; data from Notifiable Diseases Database, Health Outcomes Information and Statistical Toolkit, New South Wales Department of Health, 1999).

A commercially available enzyme immunoassay (EIA) for IgA against sonicated whole cell *B. pertussis* has been widely used in diagnostic laboratories in Australia since the early 1990s [4]. However, there has been limited clinical evaluation of this test and the validity of the high proportion of notified cases based on serology alone has been questioned. Previously, most reports of serological diagnosis of pertussis have been based on EIA for IgG and/or IgA against specific purified pertussis antigens, namely pertussis toxin (PT), filamentous haemagglutinin (FHA), pertactin (PRN), fimbriae and/or agglutinogen in single or paired sera. Their utility has been validated in culture-proven cases and/or their contacts [5–9].

Interpretation of positive serological tests can be difficult, as antibodies against pertussis may be detected in patients without a history of clinical disease. In Australia, pertussis vaccine is included in the routine childhood immunization schedule, and thus many subjects will have some level of pertussis immunity, usually detectable as IgG antibodies. Acellular pertussis vaccines are now replacing the previously used whole cell vaccine and these acellular

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vaccines generally include the specific purified pertussis antigens used in serological assays (PT, FHA and PRN).

Of the specific antigens, only PT is unique to *B. pertussis*. Both FHA and PRN are found in other *Bordetella* species. However, other studies have shown that a rise in FHA antibodies has good clinical correlation with *B. pertussis* infection [10]. There may be natural boosting of immunity due to infection with *B. pertussis* without overt clinical disease, with both IgA and IgG antibodies produced. IgA antibody levels decay more rapidly than IgG and therefore are considered more useful in detecting recent infection when the first serum specimen is not collected at the onset of illness.

The use of serology for diagnosis of pertussis is attractive, since isolation of *B. pertussis*, although highly specific, is insensitive [8, 11]. When antibiotics have been used and/or when cough has been present for more than 2 weeks, there is a very low yield from culture, even if the correct specimen is collected [12]. Nucleic acid detection methods, such as PCR, are more sensitive but do not remain positive for more than a few weeks after the onset illness. In this study, a clinical case definition was used as the reference standard for 'real life' evaluation of the validity of serological testing, since other laboratory data are rarely available.

The aims of the study were: (a) to determine whether serological test results correspond with a clinical case definition of pertussis, and (b) to compare the sensitivities and specificities of four IgA antibody assays against specific or whole cell sonicated *B. pertussis* antigens, alone or in combination, in subjects with suspected pertussis and in controls.

METHODS

Study design

The study was a diagnostic test evaluation. Study subjects were selected consecutively from people whose sera were received for pertussis antibody testing during the study period. Sera were tested for IgA antibody against whole cell (WC) sonicated *B. pertussis* antigen and three specific purified *B. pertussis* antigens, namely PT, FHA and PRN.

Individuals whose sera contained antibody against one or more antigens were defined as subjects and were eligible to be contacted by telephone to ascertain clinical case status. Interviews were restricted to those in whom one or more test was positive, to reduce the number of interviews in a potentially large sample, whilst providing information about relative sensitivity and specificity of the tests [13].

Subjects were divided into 'cases' and 'non-cases'. 'Cases' were those who fulfilled a clinical case definition of pertussis, modified from one agreed upon in Geneva, Switzerland in 1991 at a World Health Organisation consensus conference on pertussis [14]. A case of pertussis was defined as a patient with cough lasting more than 3 weeks with one or more of paroxysms of coughing, inspiratory whoop or post-tussive vomiting, without other apparent cause. 'Non-cases' were those who did not meet these criteria or who had a history of cough for more than one year.

Sensitivity of the diagnostic tests were assessed from subjects who were clinical cases. Specificity was assessed from three different samples of individuals who were assumed not to have pertussis, namely (a) subjects who were 'non-cases' based on the clinical definition; (b) individuals for whom pertussis antibody testing was requested, but whose sera were negative in all four tests, and (c) a separate group of age-matched controls who had had blood submitted to the laboratory for routine pre-operative tests or diagnosis of other conditions (see Controls, below). Clinical data were not available for the latter two groups.

Clinical data

Consent for inclusion in the study was obtained from subjects or their parents, via their doctor or directly by telephone. Members of the New South Wales Health Department Survey Team, who were unaware of serology results, administered a questionnaire during a 5-min telephone interview. The questionnaire was based on one developed by the South Australian Health Department [15].

Study population

Sera received for pertussis antibody testing between 1 February 1998 and 5 May 1998 were tested routinely for WC IgA and, in addition, for IgA antibody against the three specific *B. pertussis* antigens. 23 patients had sufficient sera only for WC IgA, PT and FHA testing. Sera were received from subjects aged 2 months to 95 years from a wide geographic area including metropolitan Sydney and Canberra and

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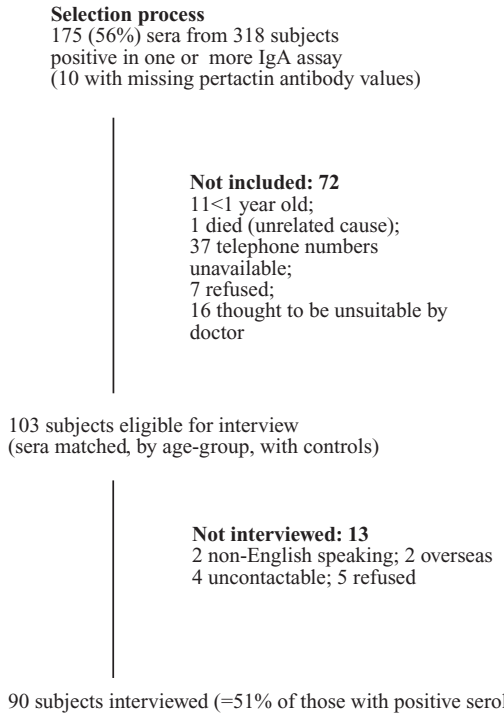

Fig 1. Selection process.

rural areas of New South Wales. Children less than 1 year of age were excluded because of their unreliable IgA antibody response to *B. pertussis* [16–18]. Only a single specimen of serum from each subject was included. Subjects were divided into those who fulfilled the clinical case definition for pertussis (cases), and those who did not (non-cases). However, as a strict clinical case definition was used, some 'non-cases' may have actually represented mild or atypical *B. pertussis* infection.

Controls

Sera from subjects were grouped by age (1–5 years, then in 10-year age groups) as age was considered a potential modifier of test performance characteristics. Subjects' sera within each age-group were matched with an equivalent number of consecutive sera received for diagnosis of another illness or for routine pre-operative testing. Control sera were tested by the same four serological assays as those of subjects. Clinical data were not available for controls.

Serological testing

B. pertussis IgA EIA, (PanBio, Windsor, Queensland Australia Cat BPA-300) was performed, according to the manufacturer's instructions.

EIA for IgA against FHA, PT and PRN were performed using standardized methods. Antigens used were PT, FHA (List Biologicals, CA, USA, Cat 181 and 170, respectively) and PRN (kindly provided by Dr Rino Rappuoli, IRIS, Siena, Italy). Briefly, 100 μl of antigen (1·0 μg/ml in carbonate-bicarbonate buffer pH 9·6) was adhered to the wells of polystyrene microtitre plates (Greiner, Cat 655101) overnight at 4 °C. Samples were diluted 1:100 for the anti-FHA and anti-pertactin EIA and 1:50 for the anti-PT EIA, in phosphate buffered saline with 1% bovine serum albumin (PBS-BSA), Sigma, Cat A7638).

The plates were incubated at 37 °C for 1 h prior to the first wash, and washed five times with saline/Tween20. 100 μl of diluted samples and controls were added to antigen-coated and uncoated wells, in triplicate, and incubated at 37 °C for 90 min. The plates were washed five times with PBS with 0·05% Tween20 (PBST), and 100 μl of horseradish peroxidase conjugated anti-human IgA (DAKO, Denmark, Cat P-214), diluted 1:1000 in PBST-BSA, was added to every well and incubated at 37 °C for 90 min. After incubation with test serum and anti-human IgA conjugated with horseradish peroxidase, specific antibody was detected by addition of a colourigenic enzyme.

After incubation at 37 °C for 90 min, the plates were washed five times with PBST, and 100 μl of TMB/E solution (Chemicon, CA, Cat ES001) was added to the wells. The plates were then incubated at 37 °C for 20 min, after which 50 μl of 2N H_2SO_4 was added to each well, and the absorbance of the wells read in a microplate reader (Biotek EL312), at 450 nm and 630 nm. Using the technique, the intensity of colour change is proportional to the antibody level, except at very low or high titres or when other isotypes of specific antibody are present in excess.

The raw optical density (OD) was corrected by subtracting the mean OD of the uncoated wells for each sample from the mean OD of the coated wells for the corresponding sample. Results were rejected if the OD of uncoated wells was greater than 0·1. As there is no international reference standard serum for IgA antibodies to pertussis antigens, a positive result was defined as a sample having a corrected OD greater than the mean plus 3SD of reference negative sera (pooled pre-immunization sera from participants in a pertussis vaccine trial). The laboratory personnel performing the serological assays were unaware of the clinical case status of the subject or of the results of the other IgA assays.

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Table 1. *Demographic characteristics of subjects*

Characteristic	Clinical cases† ($n = 66$)	Non-cases† ($n = 24$)
Age: years – mean (range)	29 (1–70)	25 (1–71)
Male : female – ratio	1·4 : 1	1 : 1
Urban : rural residents – ratio	0·9 : 1	0·7 : 1
Vaccination status – *n* (%)*		
Full ($\geqslant 3$ vaccinations)	36 (54)	13 (54)
Partial	13 (20)	4 (17)
None	6 (9)	4 (17)
Unknown	11 (17)	3 (12)
Returned consent forms – *n* (%)	46 (70)	16 (67)

* Obtained by history.

† No statistically significant differences between cases and non-cases.

Data analysis

Sensitivity and specificity for each test were calculated, with 95% confidence intervals, using clinical case status of subjects as the reference standard. McNemar's χ^2 test was used to test whether sensitivity and specificity of the alternative tests differed significantly, using the method described by Chock *et al.* [13, 19].

RESULTS

During a 3-month period (1 February to 5 May 1998), sera were received for pertussis IgA testing from 318 people. One hundred & seventy-five (56%) were positive in one or more test, including 10 of 23 of which there were insufficient sera to test for pertactin antibody. Seventy-two of the 175 seropositive subjects were unsuitable or unavailable for interview, leaving 103 eligible subjects whose sera were matched, by age-group, with control sera. Thirteen subjects could not be interviewed for various reasons and thus data were analysed for 90 subjects and 103 controls (Fig. 1). The 90 subjects had had all four serological tests performed on their sera. Sixty-six subjects (73%) fulfilled the case definition for pertussis (clinical cases) and 24 did not (non-cases). There were no significant differences in demographic characteristics of the two groups (Table 1).

There were seven participants with family members in the study, including two siblings, a set of twins and a mother, father and son. All were clinical cases, except the father in the latter family who had a 2-week history of cough with vomiting and was WC IgA positive.

Twenty-eight (31%) subjects were 10 years old or less and eight (9%) were over 65. Neither the proportion of cases nor the proportion of subjects with positive WC IgA tests differed significantly between those subjects 10 years old or younger and those older than 10 years of age, so data were analysed without stratification by age.

Serological test results are summarized in Tables 2–4. The sensitivity of individual tests for identification of clinical cases varied from 24–64%. Individual tests were positive in 13–58% of non-cases. FHA IgA was the most sensitive test and second only to the relatively insensitive WC IgA in specificity (Table 2). However, because of selection criteria, some non-cases were almost certainly infected with *B. pertussis* and would have been classified as cases had a less strict case definition been used (e.g. the family which included several clinical cases). Therefore the test specificity is very likely to be underestimated in this group. The control sera may overestimate the specificity of the tests, since the control group was comprised of subjects who were unlikely to have pertussis-like illness. However, based on the results in all three groups used to assess specificity (see Study Design, above) all four serological tests had high specificity (93–98%). WC IgA alone was insensitive for diagnosis of clinical cases but the most specific and least likely to be positive in non-cases.

When each individual antibody test (PT, FHA and PRN) was combined separately with WC IgA, the combinations did not differ significantly in the number of extra cased or non-cases identified ($\chi^2 = 3{\cdot}05$ with 2 df; $P > 0{\cdot}1$). However, the study lacked sufficient power to detect a small difference between these combinations. Examining the results for a trend, it is apparent that the combination of WC IgA and FHA IgA detected the greatest number of true positive

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Table 2. *Positive results for individual IgA antibody tests using various* B. pertussis *antigens in subjects and controls*

Antigen	Total +ve no.	Cases no. = 66 +ve (sensitivity*%; 95% CI)	Non-case no. = 24 +ve (specificity%; 95% CI)	Controls no. = 103 +ve (specificity%; 95% CI)	All non-pertussis groups† no. = 257 +ve (specificity%; 95% CI)
WC	19	16 (24·2; 14·5–36·4)	3 (87·5; 67·6–97·3)	2 (98·1; 93·2–99·8)	5 (98·1; 95·5–99·4)
PRN	39	35 (53·0; 40·3–65·4)	14 (41·7; 22·1–63·4)	4 (96·1; 90·3–98·9)	18 (93·0; 89·2–95·8)
PT	47	37 (56·1; 43·3–68·3)	10 (58·3; 33·6–77·9)	4 (96·1; 90·3–98·9)	14 (94·6; 91·0–97·0)
FHA	50	42 (63·6; 50·9–75·1)	8 (66·7; 44·7–84·4)	4 (96·1; 90·3–98·9)	12 (95·3; 91·9–97·6)

Abbreviations: WC, whole cell; PRN, pertactin; PT, pertussis toxin; FHA, filamentous haemagglutinin.
* Sensitivity is the proportion of clinical cases with positive results.
† All non-pertussis groups are (a) subjects who are not clinical cases (24); (b) those tested for pertussis antibody who were negative on all four tests (130) and (c) controls (103). The 13 subjects who were negative for WC, PT and FHA but had insufficient sera for PRN testing were excluded.

Table 3. *Accuracy of various combinations of positive test results for diagnosis of pertussis in clinical cases (compared with subjects who do not fulfil the case definition – 'non-cases')*

Test combination	All subjects (no. = 90) +ve	Cases (n = 66)				Non-cases (n = 24)			
		Both +ve	WC +ve only	Other test +ve only	Total (%)	Both +ve	WC +ve only	Other test +ve only	Total (%)
WC and/or FHA*	54	14	2	28	44 (67)	1	2	7	10 (42)
WC and/or PT*	55	9	7	28	44 (67)	2	1	8	11 (46)
WC and/or PRN*	54	13	3	22	38 (58)	1	2	13	16 (67)

Abbreviations: WC, whole cell; PRN, pertactin, PT, pertussis toxin; FHA, filamentous haemagglutinin.
* With or without other positive tests, in addition.

Table 4. *Comparing combinations of positive test results for diagnosis of pertussis in clinical cases (compared with subjects who do not fulfil the case definition – 'non-cases')*

Subjects (no. = 90) with antibody against	Total +ve	Cases (no. = 66) +ve (sensitivity)	Non-cases (no. = 24) +ve (%)
1 antigen only	41	26 (39%)	15 (63%)
Any 2 antigens only	32	24 (36%)	8 (33%)
Any 3 or 4 antigens	17	16 (24%)	1 (4%)

cases with the fewest false negatives. However the difference between PT IgA/WC IgA and FHA IgA/WC IgA was due only to one non-case (Table 3).

DISCUSSION

We used a clinical case definition of pertussis as the reference standard to simulate a "real life" clinical situation. There is potential for non-differential misclassification of cases and non-cases, but this should not affect the relative sensitivities and specificities of the tests being compared. The clinical reference standard has limited sensitivity and specificity [20] but in practice it is often the basis for diagnosis and notification of cases since tests for detection of *B. pertussis* (culture or nucleic acid amplification) often are not attempted or are negative. The modified version of the WHO definition we used [14] requires a history of 3 weeks cough. It is stricter than an alternative definition that requires only 2 weeks of coughing, which has been recommended in Australia by the National Health and Medical Research Council [2] and elsewhere [21] for notifi-

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cation of pertussis cases. We chose this definition so as not to overestimate the specificity of the laboratory tests. Most cases would have fulfilled either definition.

Ideally, serological diagnosis should be based on demonstration of seroconversion or a significant rise in antibody level. However the diagnosis of pertussis is often not considered until after antibody levels have reached their peak and paired sera are rarely submitted. In this study, only 5 of 175 people had more than one serum sample sent. Thus, analysis of data on single sera is analogous to 'real-life'.

This study has shown that the presence of whole cell (WC) pertussis IgA, as measured by a commercially available ELISA, is a specific indicator of recent illness consistent with pertussis. However WC IgA alone is quite insensitive. Tests for antibody against specific pertussis antigens, especially FHA and PT, are more sensitive and only slightly less specific than IgA against WC. These results suggest that pertussis notifications, based on positive WC IgA tests, do not overestimate, and if anything underestimate, the true incidence of the disease.

The greater the number of tests that were positive, the more likely it was that the subject would fulfil the case definition (Table 4). It would be impractical and expensive to perform all four assays routinely. The combinations of WC IgA with either FHA IgA or PT IgA were the most sensitive in identifying clinical cases (67%), without loss of specificity (96%) (Table 2). The combination of WC IgA with FHA IgA was positive in slightly fewer non-cases and thus the specificity of this combination for the identification of cases was correspondingly better (Table 3). FHA IgA has been found to be an important diagnostic tool in other studies [10] and our results supports this.

Comparing subjects less than 10 years of age to those who were 10 years and older, there was no difference in the proportion of clinical cases who were WC IgA positive. Although we may not have detected a significant difference because of the small sample size, this finding allowed us to analyse our data without age-stratification. This increased the power of the study.

This study has a number of significant limitations, including a low response rate (55% at best), potential selection and recall bias. Selection bias was possible if WC IgA positive subjects were more likely to respond because they had been told that they had had pertussis. Recall bias may have occurred if people who had been told they had pertussis were more likely to remember symptoms.

Because of the method of selection of subjects and the case definition used, it is almost certain some subjects classified as non-cases had actually been infected with *B. pertussis*. Therefore, two additional groups were used to evaluate specificity, namely people with suspected pertussis (for whom requests for pertussis serology were received) who were seronegative on all four tests, and controls whom we assumed had no respiratory symptoms. Although no clinical data were available for either of these groups, we believed them to be representative of a population of people having sera submitted for testing. The specificity of all four tests in these groups combined with non-cases was high and confirms our assumption that they had not had recent pertussis.

Despite these limitations, this small study has helped to validate the use of serological testing for diagnosis of pertussis. Tests for antibody against individual *B. pertussis* antigens have been validated in other studies [5–9]. Post-vaccination IgA and IgG antibody levels against pertussis toxin, pertactin and agglutinogens decrease rapidly, and IgA disappears, after the initial antibody response. Subsequent increases in antibody levels occur after exposure to infected persons or symptomatic illness. Thus an increase in IgG or the presence of IgA antibody, unless closely following vaccination, indicates exposure to *B. pertussis*. Prospective serological studies support the view that unrecognized pertussis is common despite high immunization rates [22].

The test that is currently widely used in Australia, WC IgA, is highly specific and a positive result corresponds well with a clinical diagnosis of pertussis. It is possible that decreasing the cut-off for a positive result in the commercial WC IgA assay would increase the sensitivity without loss of specificity. Further evaluation would be required to confirm this. The use of FHA IgA or PT IgA assays, in combination with WC IgA would correctly identify additional cases of pertussis among patients with prolonged cough. These tests provide a useful guide to the clinician in confirming the diagnosis of pertussis and the results of this study support notification based on a positive result, in a patient with compatible clinical symptoms.

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