DIAGNOSIS OF PERTUSSIS USING NASOPHARYNGEAL IgA AND POLYMERASE CHAIN REACTION IN SPECIMENS FROM OUTPATIENTS IN AUSTRALIA[#]

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We assessed IgA antibodies and polymerase chain reaction (PCR) for diagnosis of pertussis in nasopharyngeal aspiration (NPA) samples from outpatients in Australia.

A total of 1700 patients (849 adults, 851 children) from Western Australia and the Northern Territory fulfilled the laboratory case definition for pertussis between 2004 and 2013: 732 specimens were positive by NPA IgA alone, 559 by PCR alone, and 409 by both tests. Overall, 968 cases (56.8%) were positive by PCR and 1141 cases (67.2%) by IgA [$p < 0.00025$]. Among pediatric patients, PCR was positive in 524 (61.3%) and IgA in 569 (67%). In 849 adult cases, the respective proportions were 52.3% and 67.4% [$p < 0.00025$].

The duration of cough in 507 patients was shorter in 262 pediatric cases (mean, 2.51 weeks; standard deviation [SD], 2.25) than 245 adult patients (3.27 weeks; SD, 2.79) [$p = 0.0009$]. PCR positivity showed a season-dependent variance (range, 5.6 to 85.9%) and peaked in the second week (71.7%) of illness. IgA antibodies peaked in the fifth week (89.5%) postinfection, and the positivity rate for NPA IgA was less variable (range, 38.3–97.2%).

Nasopharyngeal *Bordetella pertussis*-specific IgA antibodies are valuable in diagnosis of pertussis in Australia. Reliance on PCR alone misses a significant proportion of pertussis cases, especially those with a delayed presentation.

Keywords: pertussis, nasopharyngeal aspirate, PCR, IgA, serology

Introduction

Pertussis is vaccine-preventable, but cases of this potentially serious disease continue to occur worldwide, partly because of waning vaccine-derived immunity in adults [1–4].

The diagnosis of pertussis may be by clinical or diagnostic criteria, but diagnostic techniques have generally been evaluated during single outbreaks or as part of vaccine licensing trials. Few papers report the role of these techniques in routine diagnostic laboratories under field conditions, especially over multiple seasons. Classical bacteriological techniques such as culture are known to be insensitive in the diagnosis of pertussis infection [5] partly because of the fastidiousness of the organism and delays in

specimen transport to the laboratory. Mucosal *Bordetella pertussis*-specific IgA serology performed on samples obtained by nasopharyngeal aspiration (NPA) [6, 7] has long been demonstrated to be useful in the diagnosis of cases of pertussis but is not widely used in most jurisdictions. NPA IgA assays have been in routine use in one of our institutions for over 20 years [8], and specimens are easily collected in an outpatient setting with little patient discomfort. More recently, PCR testing using a number of protocols [9, 10] has also been shown to have a role in diagnosing acute cases, with a number of caveats. For example, *B. pertussis* nucleic acid is generally PCR-detectable only in the first 3 weeks of illness, and patients presenting later often have a negative result [11]. In addition, an over-reliance on PCR testing alone may result in false-positive diagnoses as

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highlighted by several recent reports of pseudo-outbreaks [12, 13]. Hence, the precise role that each testing modality has in diagnosis of pertussis is not fully resolved, particularly in a vaccinated community setting.

Jurisdictions monitor the incidence of infectious disease, such as pertussis, by notification systems. Wide variations in incidence may occur because of differing methodology in various jurisdictions, including diagnostic techniques [4]. The method of diagnosis is rarely available in reports of disease incidence, however, including for pertussis. Australia, despite having an active immunisation program with national coverage rates of up to 92% by 15 months of age, continues to suffer epidemics of pertussis. Western Australia had explosive epidemics in 2004 and 2011/2 [14], and the most recent major outbreak in the Northern Territory was in 2008 [15]. One of our laboratories (Western Diagnostic Pathology, WDP) services both of these jurisdictions, which cover areas of 2.5 and 1.3 million square kilometres with populations of 2.3 and 0.23 million, respectively. We used the Australian national case definition for laboratory diagnosis of pertussis [16] (i.e., positive culture or PCR test, seroconversion or significant increase in antibody level, or single-titre positive whole cell IgA serology [peripheral or NPA]) to study the relative contribution of *B. pertussis*-specific IgA and PCR testing of NPA specimens in diagnosis of laboratory-notified community cases of pertussis in Western Australia and the Northern Territory over 10 completed calendar years (2004–13).

Materials and methods

NPA specimens from patients clinically suspected of acute pertussis infection were collected in the community by WDP staff and were assayed there or at Princess Margaret Hospital for Children (PMH) for *B. pertussis*-specific IgA and by real-time PCR. Data were respectively collated by computer searching for specimens received between January 1, 2004 and December 31, 2012 at WDP. The Australian national case definition used for incident pertussis cases in this study was detectable mucosal *B. pertussis*-specific IgA antibody and/or positive *B. pertussis* PCR [16]. Hence, according to this definition, PCR+/IgA+, PCR+/IgA-, PCR+/IgA equivocal, and PCR-/IgA+ cases fulfilled these criteria. PCR-, IgA equivocal, and PCR-/IgA- cases were excluded. Evidence of a clinically compatible illness (prolonged coughing including paroxysms, post-tussive whooping, and/or vomiting) and/or contact with a proven case was sought from information provided on the laboratory request form, but not used to classify cases.

This was not designed to be a study of clinically notified pertussis, but cases which had sufficient clinical information on their request forms to fulfil the Australian national clinical case definition for pertussis [20] were recorded for subanalysis. These criteria include close contact with a proven case, >2 weeks cough illness,

cough paroxysms, post-tussive vomiting, or post-tussive whooping.

Specimen collection and processing

NPAs were collected from patients with suspected pertussis into sterile collection tubes. Aspirated NP fluid was collected into 0.5 ml sterile saline. An equal volume of phosphate buffered saline (PBS) was added to the aspirate in the laboratory with vigorous vortexing. Aliquots of this suspension were subsequently used for detection of specific nasal IgA antibodies directed against *B. pertussis* via an enzyme immunoassay (EIA) using a whole cell sonicate of *B. pertussis* as the antigen, and detection of *B. pertussis* and *Bordetella parapertussis* DNA via PCR.

Detection of NPA IgA anti-B. pertussis antibodies

Identical protocols were used in both laboratories (WDP and PMH), and results from the former were originally validated in parallel with the latter laboratory. A 100 µl aliquot of the NPA specimen/PBS suspension was analyzed using a commercial anti-*B. pertussis* IgA detection kit with whole cell *B. pertussis* sonicate as antigen (Panbio *B. pertussis* IgA kit E-BPB01A, Panbio Ltd., Brisbane, Queensland, Australia). Resulting assay optical densities were converted to Panbio Units (specimen/control × 10) with results <9 being reported as negative, 9–19 as equivocal, and >19 as positive. This methodology for NPA specimens had been previously validated [17] against a published in-house NPA IgA anti-*B. pertussis* assay using whole cell sonicates [8].

Detection of B. pertussis/B. parapertussis DNA by PCR

Identical target sequences and protocols were used in both laboratories (WDP and PMH), and results from the former were originally validated in parallel with the latter laboratory. Following automated DNA extraction from NPA/PBS aliquots (MagNA Pure LC, Roche Applied Science), real-time amplification and detection of insertion sequences IS 481 (a multicopy gene which provides high sensitivity) of *B. pertussis* and IS1001 of *B. parapertussis* were performed using specific primers and fluorescent Taqman probes as previously described [17, 18]. Negative control for assay runs was PCR-grade water, and positive control was DNA extracted from local clinical isolates of *B. pertussis* and *B. parapertussis*. IS 481 is also present in some strains of *Bordetella holmesii* and *Bordetella bronchiseptica*, and PCR assays are reported with this disclaimer, although, in our experience during the study period, these species are rarely seen (80 clinical isolates of *B. pertussis* were obtained from specimens, and only one other specimen had another species present, *B. bronchiseptica*, which was actually negative in our IS 481 assay).

Statistical analysis

Proportions from different groups were compared by Fisher's exact test. Group data were compared by unpaired *t*-test [19].

Results

Over the span of 10 years, 14,135 NPA samples were tested and 2559 specimens were positive for *B. pertussis* DNA by PCR and/or had detectable titres for *B. pertussis*-specific NPA WC-IgA by EIA. Twenty-five samples had *B. paraptussis* DNA detected by PCR but were not included in the cohort as paraptussis is not notifiable in Australia.

Of the 2559 cases mentioned above, 1700 unique patients fulfilled the strict laboratory case-definition for incident pertussis. An amount of 859 IgA-equivocal PCR-negative cases were excluded as they did not satisfy the national case definition, but IgA-equivocal PCR-positive cases were included as they did fulfil these criteria. Fifty-three cases were from the Northern Territory, and 1647 were from Western Australia. Overall, 926 (54.5%) patients were female, and 774 (45.5%) were male. The mean age was 23.9 years (SD, 20.9; range, 1 week–82 years). The mean age for males was lower (21.4 years; SD, 19.7; range, 1 day–82 years) than for females (26 years; SD, 21.7; range, 1 day–81 years) [$p < 0.001$ by unpaired *t*-test]. A total of 851 cases (50.1%) were 14 years or younger (mean age for children 7.22 years; SD, 4.18) and classified as pediatric cases, and 849 cases were above 14 years and categorized as adults (mean age, 40.67 years; SD, 17.32). Of the pediatric group and the adult group, 437 (51.4%) and 491 (57.8%), respectively, were female.

A total of 732 specimens were positive by NPA IgA EIA alone, 559 by PCR alone, and 409 by both tests (261 positive and 148 equivocal for NPA IgA) (Table 1).

- Agreement overall was lower for PCR (89.5) than for IgA (91.9) [proportions statistically different by Fisher's exact test; Chi square, 847.22; $p < 0.00025$].
- Positive agreement was lower for PCR (56.8) than for IgA (67.2) [proportions statistically different by Fisher's exact test; Chi square, 57.95; $p < 0.00025$].
- Negative agreement was lower for PCR (94.0) than for IgA (95.4) [proportions statistically different by Fisher's exact test; Chi square, 21.96; $p < 0.00025$].

PCR was positive in 524 (61.3%) and NPA IgA detectable in 569 (67%) of the pediatric group. In contrast, in the adult group, the relative percentages were 52.3% (444 cases) and 67.4% (572) [proportions for PCR positivity in each age group statistically different by Fisher's exact test; Chi square, 29.84; $p < 0.00025$].

The duration of cough was recorded for 507 patients (mean of 2.88 weeks; SD, 2.55). The mean duration was shorter in 262 pediatric (2.51 weeks; SD, 2.25) than 245 adult patients (3.27 weeks; SD, 2.79) [$p = 0.0009$ by unpaired *t*-test]. A total of 294 PCR-positive patients had a shorter cough duration reported (mean of 2.31 weeks; SD, 2.55) than the 213 patients with detectable NPA IgA (mean of 3.56 weeks; SD, 2.93) [$p = 0.0001$ by unpaired *t*-test]. The prevalence of a positive PCR result peaked in the second week of illness and progressively fell with each succeeding week, with the last positive result recorded in a patient with 12 weeks of symptoms (Table 3). The prevalence of a positive NPA IgA result peaked in the fifth week and slowly fell but remained high in each succeeding week, with the latest positive result recorded in a patient 26 weeks after the onset of cough. The proportion of cases with a positive PCR result (67.9%) was higher than those with a positive NPA IgA result (59%) in the first week (Table 3; Fisher's exact test; Chi square, 15.15; $p < 0.0005$), was not different in the second week, and thereafter was statistically lower than NPA IgA in each subsequent week (Table 3).

Table 1. PCR and NPA IgA results in laboratory-defined cases

	PCR positive	PCR negative	Total
IgA positive	261	732	993
IgA equivocal	148	(859)	148
IgA negative	559	0	559
Total	968	736	1700

() Cases excluded

Table 2. PCR and NPA IgA results in clinically defined cases

	PCR positive	PCR negative	Total
IgA positive	134	300	434
IgA equivocal	75	0	75
IgA negative	270	0	270
Total	479	300	779

Table 3. Effect of duration of cough on PCR and NPA IgA positivity

Week of cough	<i>n</i>	PCR detected (%)	NPA IgA detected (%)	<i>p</i> value
1	112	76 (67.9)	59 (52.7)	<0.0005
2	138	99 (71.7)	83 (60.1)	0.1–0.2
3	98	54 (55.1)	68 (69.4)	<0.0001
4	58	25 (43.1)	47 (81)	<0.0005
5	19	4 (21.1)	17 (89.5)	<0.0005
6	15	5 (33.3)	12 (80)	<0.0005
7–26	29	9 (31)	23 (79.3)	<0.005

Table 4. Percentage positivity of NPA IgA and PCR in different years

Year	<i>N</i>	% PCR positive (<i>N</i>)	% IgA detected (<i>N</i>)	Cough duration, weeks (mean, SD)
2004	357	68.9 (246)	70.3 (251)	3.29 (2.96)
2005	133	30.8 (41)	88.7 (118)	4.67 (3.11)
2006	107	5.6 (6)	97.2 (104)	4.07 (3.06)
2007	19	26.3 (5)	84.2 (16)	10.67 (13.32)
2008	51	68.6 (35)	58.8 (30)	2.66 (1.98)
2009	79	68.4 (54)	39.2 (31)	2.77 (1.99)
2010	128	85.9 (110)	38.3 (49)	2.88 (2.43)
2011	430	57.2 (246)	66.3 (284)	2.50 (1.78)
2012	314	52.5 (165)	63.4 (197)	2.38 (1.42)
2013	96	62.5 (60)	62.5 (59)	2.63(1.48)

The positivity rate for different NPA tests fluctuated markedly in different seasons (*Table 4*). For PCR in particular, the rate ranged from a low of 5.6% in 2006 to a high of 85.9% in 2010. The rate for NPA IgA was more stable (range 38.3–97.4%) and was only lower than the PCR rate in 3 years (2008, 2009, and 2010). The PCR positive rate was highest in epidemic years (2004 and 2011) at 62.5% and was statistically higher than in interepidemic years (51.3%; Chi square, 18.51; $p < 0.0005$). The positivity rate for NPA IgA did not alter significantly between epidemic and interepidemic years (68.0% vs. 65.2%, respectively; Chi square, 1.69; $p < 0.2 > 0.1$).

Although this was not designed as a study of clinically notified pertussis, 779 cases had sufficient clinical information on their request forms to fulfil the Australian national clinical case definition for pertussis [20], including close contact with a proven case (371 cases, 47.6%), >2 weeks cough illness (352 cases, 45.2%), cough paroxysms (121 cases, 15.5%), post-tussive vomiting (78 cases, 10.0%), or post-tussive whooping (15 cases, 1.9%). The remaining cases had either no clinical details submitted (517 cases) or insufficiently precise information (404 cases) to determine their clinical status: including “whooping cough,” “pertussis,” “bronchitis,” “tracheitis,” “bronchiolitis,” “cough,” “dry cough,” “chronic cough,” or “unrelenting cough” which were not inconsistent with the diagnosis.

For the cases fulfilling the clinical case definition, 300 specimens were positive by NPA IgA assay alone, 272 by

PCR alone, and 211 by both tests (134 positive and 75 equivocal for NPA IgA) (*Table 2*). Hence, of the clinically defined cases, 509 (65.3%) had detectable NPA IgA (positive or equivocal) and 479 (61.5%) were PCR positive [proportions not statistically different by Fisher’s exact test; Chi square, 2.49; $p > 0.10$].

Discussion

Our data demonstrates that both NPA PCR and IgA testing contribute significantly to laboratory notification of pertussis in our jurisdictions in the western half of Australia. The clinical data suggest that the described cohort had a high pretest clinical likelihood of pertussis.

Several studies have confirmed the increased sensitivity of PCR testing over culture for *Bordetella* infection [9, 10, 21]. It has been previously reported that pertussis cases are most likely to be PCR positive within the first 3 weeks of onset of symptoms, but may remain positive for significantly longer [11]. Results from our cohort are consistent with those published observations, but in our cases, NPA PCR remained positive occasionally for very prolonged periods (median of 2 weeks, range of 1 day to 12 weeks). Our experience suggests that, although the IS 481 PCR target used in these studies is not specific for *B. pertussis*, nonpertussis *Bordetella* species are exceedingly rare in our community (1.13% of 80 *Bordetella* isolates over

10 years). The sole *B. bronchiseptica* strain isolated over the study period tested negative in our IS 481 PCR assay, and these data suggesting a positive result in that test are highly likely to be due to true *B. pertussis* infection. The infrequency of nonpertussis *Bordetella* isolates in Australia has recently been confirmed by another study [22, 23].

B. pertussis-specific NPA mucosal IgA antibodies are described as appearing after the first week of infection and may persist for many months [7]. Similar findings are now reported from this cohort (mean cough duration of an IgA positive patient was 3.56 weeks; SD, 2.93). Many of our cases did not present until a number of weeks after onset of symptoms, especially in the adult group, when PCR assays are more likely to be negative. It would seem advisable, therefore, to use a testing strategy which combines both assays to identify patients presenting early and late in the disease course. Collecting an NPA sample gives the laboratory the optimum specimen to perform both assays. This is easily performed in an outpatient setting and is well tolerated by patients, even very young children, in our experience. Such specimens are also suitable for diagnosing alternative causes of prolonged cough syndromes, such as *Mycoplasma* and respiratory viruses. The recent vogue for using flocked swabs only yields a suitable specimen for PCR assays [24], but not mucosal IgA, and is therefore likely to be a suboptimal sample for Pertussis diagnosis.

The Australian laboratory notifiable criteria until the last year of this study specified whole-cell (WC) IgA serology (on blood or NPA specimens) as the only serological criteria for pertussis cases, and this technique is still the commonest one utilized in Australia by routine diagnostic laboratories. A European expert advisory group (Pertstrain) recently reviewed a published data on pertussis blood serology and concluded that IgG immunoassays should ideally use nondetoxified pertussis toxin (PT) as antigen, but this should not be used if vaccine has been administered within a year of testing (as vaccine induces IgG antibodies) [23]. WC IgA assays were not recommended because of a perceived lack of specificity, and IgA anti-PT was considered appropriate if IgG anti-PT levels were indeterminate or if a follow-up second specimen could not be obtained. These riders attached to the Pertstrain guidance would severely limit the role of IgG anti-PT in Australia because of our high vaccination rates. In addition, convalescent samples are rarely received by our laboratories (despite frequent requests to doctors) as part of the pertussis workup (i.e., 22/1086 [2%] of initial pertussis serological requests had follow-up blood serology specimens during this period). Certainly, our experience would suggest that specificity of WC IgA is unlikely to be a major issue because of the low prevalence of nonpertussis *Bordetella* strains in our community. A field study of 90 pertussis cases from Australia, in fact, found a WC IgA assay to be more specific than IgA-PT (87.5 vs. 58.3%) [26]. A prospective community study in Australia utilising multiple serum samples and in-house IgG-PT and IgA-PT assays showed similar specificity for both

tests, but superior sensitivity for IgG-PT [27]. Until very recently, the lack of a commercial licensed IgG-PT assay in Australia and the difficulty of diagnostic laboratories obtaining multiple serum samples rendered Pertstrain recommendations unhelpful.

Pertstrain guidelines, however, do not have direct applicability for NPA serology. A single report of an unlicensed IgG anti-PT assay used in oral fluids (not NPA) from 197 patients was endorsed by Pertstrain because of a sensitivity of 79.7% and specificity of 96.6%, but there was neither a comparison with IgA anti-PT or WC IgA nor with NPA specimens [28]. Pertstrain did acknowledge that no commercial kit using this assay was licensed, rendering its advice of no current value to Australian diagnostic laboratories assaying NPA fluids. After the completion of our study, a licensed Pertussis Toxin IgA assay became available. A pilot study in our lab found 87% concordance between WC-IgA and PT-IgA assays performed on 259 archived positive and negative NPA samples. This would suggest that concerns about specificity of WC-IgA on NPA samples may be misguided.

Our cohort study identified 1700 definite positive cases of pertussis (1647 from WA and 53 NT) over 10 seasons in the west and north of Australia. Over this time period, 17,763 cases (14,713 from WA and 2050 NT) of pertussis were notified in WA and NT [29]; hence, 9.6% of these cases (11.2% of total pertussis notifications from WA and 2.6% from NT) were diagnosed by NPA in our laboratories. The above data show that if PCR was the only investigation performed in suspected pertussis cases, 43.1% of the definite cases would have been missed in this cohort. The percentage of cases missed by a PCR-only strategy was statistically lower ($p < 0.0005$) in the pediatric group (38.7%) than the proportion in the adult group (47.7%). Few other laboratories in our jurisdictions use NPA for diagnosis of pertussis, so it is likely that this method is currently underutilized in Australia and total cases of pertussis are underestimated. Culture is generally not a viable option in our region as specimens may take 48 h or more to reach the laboratory from remote settlements. As with other reported cohorts of pertussis, the majority of our patients were adults [4]. 56.9% of our cases had detectable NA by PCR, and 67.1% had detectable *B. pertussis*-specific NPA IgA. An age-related effect was apparent with higher PCR positivity in the pediatric group compared to the adult group. These observations probably reflect the tendency for children to present to their doctors earlier, during the period when PCR is more likely to be positive.

Interestingly, the positivity rate of different pertussis tests fluctuated markedly in different years (Table 3). For PCR in particular, the rate ranged from a low of 5.6% in 2006 to a high of 85.9% in 2010. The rate for NPA IgA positivity was more stable and only lower than the PCR rate in 3 years (2008, 2009, and 2010). Although transient technical problems in laboratory assays might conceivably produce a period of performance below historical data, the correlation with cough duration (which also has a seasonal fluctuation, $r = 0.55$ for PCR and 0.51 for IgA)

would argue against this likelihood. Poor PCR diagnostic rates occurred in years where the recorded duration of cough in patients was unusually long and overall case numbers were low. This phenomenon may have been due to a less virulent circulating strain of *Bordetella* or lower community awareness of Pertussis due to lower incidence which may have resulted in late presentations and, hence, reduced utility of PCR testing. Interestingly, the lower positivity rates for NPA IgA correlated with years where the recorded duration of cough in patients was unusually short, which may possibly reflect seasons with more virulent circulating strains. Clinicians should be aware that individual test performance may vary significantly from season to season, and this underlines the need to use both PCR and NPA IgA serology to reliably diagnose pertussis. Our data suggest that PCR is more likely to be positive in cases during epidemic years than interepidemic years, whereas NPA IgA positivity does not significantly vary over seasons.

The common practice in our community is of doctors only submitting a nose or throat swab for PCR would result in many pertussis cases being missed, perhaps because of a misplaced belief that NPA collection was a traumatic procedure and, hence, be lost opportunities for public health intervention. NPA collection using soft catheters was well tolerated in our experience, including in very young children (specimens were obtained from 97 children below the age of 2 years, and 15 below 1 month). This experience has also been confirmed by controlled clinical trial, with NPA being shown to be better tolerated than nasopharyngeal swab collection [30]. The relative performance of NPA testing in our communities needs to be formally compared to more commonly requested methods such as respiratory swabs and blood serology.

We conclude that NPA samples for PCR and NPA IgA analysis contribute a significant number of laboratory-notified cases in our jurisdictions and are probably underutilized.

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Conflict of Interest Statement

The authors declare that they have no conflict of interest.

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