Detection of anti-pertussis toxin IgG in oral fluids for use in diagnosis and surveillance of *Bordetella pertussis* infection in children and young adults

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Bordetella pertussis infection is being increasingly recognized as a cause of prolonged, distressing cough (without whooping symptoms) in children and young adults. Diagnosis of infection in this population is important for treatment and surveillance purposes, and may also prove useful in reducing transmission to unvaccinated babies, for whom disease can be fatal. Serum IgG titres against pertussis toxin (PT) are routinely used as a marker of recent or persisting *B. pertussis* infection. However, collection of serum from young children is difficult, and compliance amongst these subjects to give samples is low. To circumvent these problems, an IgG-capture ELISA capable of detecting anti-PT IgG in oral fluid was devised. The assay was evaluated by comparison to a serum ELISA, using 187 matched serum and oral fluid samples from children (aged 5–16 years) with a history of prolonged coughing, whose serum anti-PT titre had already been determined (69 seropositive, 118 seronegative). The results showed that, using a cutoff of 70 arbitrary units (AU), the oral fluid assay detected seropositive subjects with a sensitivity of 79·7 % [95 % confidence interval (CI) 68·3–88·4] and a specificity of 96·6 % (95 % CI 91·5–99·1). Thus, oral fluid titres of ≥ 70 AU would possess a positive predictive value of 76·2–93·2 % for pertussis amongst children with chronic coughs when used as a surrogate for the serum ELISA (assuming disease prevalence of 12–37 %). This oral fluid ELISA will greatly assist in the convenience of *B. pertussis* disease diagnosis and surveillance.

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INTRODUCTION

The bacterium *Bordetella pertussis* causes potentially life-threatening whooping cough in humans. Childhood immunization with whole-cell or acellular vaccines, however, has dramatically reduced the incidence of severe disease and it is generally only seen in unvaccinated infants (Cherry, 1996; Heininger, 2001). Nevertheless, it has become increasingly recognized that vaccine-derived immunity wanes over 5–10 years and that *B. pertussis* also causes a prolonged cough with milder symptoms in previously vaccinated older children and adults (Cherry, 1999; von König *et al.*, 2002). Indeed, siblings and parents with coughing symptoms are an important source of infection for unvaccinated babies (Orenstein, 1999; Crowcroft *et al.*, 2003). Diagnosis of infection in this older population is important for

surveillance purposes, and may help prevent transmission to unvaccinated babies, with their attendant high morbidity and mortality.

Elevated serum IgG titres against pertussis toxin (PT) are widely accepted as a reliable marker of recent or persisting infection (Hallander, 1999; Yih *et al.*, 2000; de Melker *et al.*, 2000; Giammanco *et al.*, 2003). In the UK, the Health Protection Agency (HPA) Centre for Infections employs an indirect ELISA for diagnostic and surveillance purposes in which a serum anti-PT IgG titre of ≥ 100 eU ml⁻¹ is consistent with recent or current (≥ 2 weeks) infection (Giammanco *et al.*, 2003). A recent study using this serum ELISA estimated the prevalence of *B. pertussis* infection amongst children aged 5–16 years with chronic coughs lasting at least 2 weeks as 37 % (Harnden *et al.*, 2006). Other studies in Europe and the USA, using serology in combination with culture and PCR detection, have estimated infection at between 12 and 32 % in coughing subjects (Birkebaek *et al.*, 1999; Cherry, 1999; Gilberg *et al.*, 2002; Miller *et al.*, 2000; Senzilet *et al.*, 2001) and between

Abbreviations: GACELISA, IgG antibody-capture ELISA; HPA, UK Health Protection Agency; HRP, horseradish peroxidase; NPA, nasopharyngeal aspirate; PPV, positive predictive value; PT, pertussis toxin; TMB, 3,3',5,5'-tetramethylbenzidine.

0.3 and 2.8 % in the general population (Miller *et al.*, 2000; Nardone *et al.*, 2004; Strebel *et al.*, 2001).

There is a growing interest in studying anti-PT serum IgG titres for diagnosis and surveillance of pertussis disease, particularly amongst children and adults with prolonged coughing symptoms. However, taking blood can be distressing to patients, especially children. In order to avoid the distress and risks associated with sampling blood, we have devised an IgG antibody-capture ELISA (GACELISA) capable of detecting anti-PT IgG in oral fluid. Oral fluid, collected from the gingival crevice using an absorbent device, contains ratios of specific to total IgG representative of those present in serum (Mortimer & Parry, 1988; McKie *et al.*, 2002). We present data on the sensitivity and specificity of the oral fluid assay when used as a surrogate for the serum ELISA currently used in our laboratory.

METHODS

Subjects and samples. One hundred and eighty-seven matched oral fluid and serum samples were taken from 154 children (i.e. 33 children gave two samples at different times) aged 5–16 years when presenting to a general practitioner with a cough of at least 2 weeks' duration (Harnden *et al.*, 2006). These samples were collected, with informed consent, following approval of the Oxfordshire research ethics committee. For comparison, oral fluid samples were taken from 220 control children, also aged 5–16 years, who had been suspected of having measles, mumps or rubella infection, but whose oral fluid scored negative for antibodies to these viruses. These samples were selected to provide an approximate age match to the 187 samples described above, and were collected anonymously from those submitted for measles, mumps or rubella testing to the HPA Enteric, Respiratory and Neurological Virus Laboratory. Serum was not available for the control subjects.

Oral fluid was collected using an Oracol swab (Malvern Medical Developments) and sent to the laboratory by mail. The fluid was eluted from each swab using 1 ml transport medium, as described earlier (Sheppard *et al.*, 2001), and stored at -20°C . Blood was collected into Vacutainer tubes (Becton Dickinson) and sent to the laboratory by mail. Serum was extracted by centrifugation and stored at 4°C .

Indirect ELISA for anti-PT IgG in serum. Serum anti-PT IgG titres were determined using an indirect ELISA, essentially as described previously (Giammanco *et al.*, 2003). Any titres $<4\text{ eU ml}^{-1}$ were scored as $=4\text{ eU ml}^{-1}$ for data analysis.

Preparation of reagents for oral fluid ELISA. Rabbit anti-human IgG (Dako Cytomation) was absorbed using an immunosorbent consisting of sheep serum proteins linked to Sepharose 4B by standard procedures (Hudson & Hay, 1980). IgG fraction of sheep anti-PT serum 97/572 (National Institute for Biological Standards and Control) was obtained after anion-exchange chromatography using DEAE-cellulose (Sigma) and conjugated to horseradish peroxidase (HRP), as described by Wilson & Nakane (1978).

GACELISA for anti-PT IgG in oral fluid. All steps contained 100 μl per well unless stated otherwise. Microtitre plate wells (Maxisorp, Nunc) were coated overnight at 4°C with absorbed rabbit anti-human IgG (described above) that had been diluted to $2\text{ }\mu\text{g ml}^{-1}$ in 0.05 M carbonate buffer, pH 9.6. Wells were washed (four times) with PBST (PBS + 0.05 %, v/v, Tween 20) and incubated with 200 μl per well of blocking solution (5 %, w/v, Solupro; Microimmune) at 37°C for 2 h.

During the blocking step, oral fluid samples were prepared by mixing them 1 : 1 with serum diluent (PBS containing 1 %, w/v, skimmed milk and 0.05 %, v/v, Tween 20). Reference sera were diluted 1/2000 in serum diluent. The lyophilized human anti-PT serum 89/530 (National Institute for Biological Standards and Control) was hydrated in PBS to one-tenth of the recommended concentration and then diluted 1/80 in serum diluent (resulting in a final total IgG concentration of $5\text{ }\mu\text{g ml}^{-1}$). This was defined as producing 1000 arbitrary units (AU) in the ELISA and was diluted serially in a negative serum (also diluted in serum diluent to produce an IgG concentration of $5\text{ }\mu\text{g ml}^{-1}$) in order to generate a standard curve on each plate that contained a consistent concentration of total IgG in every well.

Following the blocking step, wells were washed with PBST and the oral fluid and serum samples incubated in the wells for 2 h at 37°C . Wells were washed again and incubated with purified PT (produced by GlaxoSmithKline, available from the National Institute for Biological Standards and Control) at a concentration of $1\text{ }\mu\text{g ml}^{-1}$ in PBS containing 3 % (w/v) BSA and 0.2 % (v/v) Tween 20, and incubated for 1 h at 37°C . Following another wash step, sheep anti-PT-HRP conjugate antibodies (see above) were diluted in serum diluent and added to the wells for 1 h at 37°C . Wells were washed a final time and incubated with 3,3',5,5'-tetramethylbenzidine (TMB; Microimmune) for 10 min. The reaction was stopped by adding 50 μl 2 M H_2SO_4 per well. Absorbance at 620 nm was subtracted from that at 450 nm for each well using a Multiskan EX microtitre plate reader and Genesis software (Thermo Labsystems). The titre of PT-specific IgG was calculated (in AU) for each sample by comparison to a four-parameter standard curve fitted to the serially diluted standard serum. Titres $>1000\text{ AU}$ were scored as $=1000\text{ AU}$ for data analysis.

ELISA for quantifying total IgG. All steps contained 100 μl per well unless stated otherwise. Maxisorp microtitre plate wells (Nunc) were coated overnight at 4°C with rabbit anti-human IgG (DAKO Cytomation) at $2\text{ }\mu\text{g ml}^{-1}$ in carbonate buffer, pH 9.6. Wells were washed (four times) with PBST and then incubated with 200 μl per well of blocking solution (described above) at 37°C for 2 h. During the blocking step, oral fluid or serum samples were diluted to a range of dilutions in serum diluent (described above). A human serum protein calibrator (DAKO Cytomation) was serially diluted in serum diluent to give total IgG concentrations between 800 and 0.78 ng ml^{-1} in order to generate a standard curve for each plate. Following the blocking step, wells were washed with PBST and the oral fluid and serum samples incubated in the wells for 1 h at 37°C . Wells were washed again and an HRP-conjugated rabbit anti-human IgG antibody (Sigma), diluted in PBS containing 3 % (w/v) BSA, 0.05 % (v/v) Tween 20 and 0.05 % Bronidox-L, was incubated in the wells for 1 h at 37°C . Wells were washed a final time and incubated with TMB for 5 min. The reaction was stopped by adding 50 μl 2 M H_2SO_4 per well. Absorbance at 620 nm was subtracted from that at 450 nm for each well using a Multiskan EX microtitre plate reader and Genesis software. The concentration of IgG in each sample was calculated by comparison to a four-parameter standard curve fitted to the serially diluted standard.

RESULTS AND DISCUSSION

Correlation between serum and oral fluid anti-PT IgG titres

The concentration of total IgG in oral fluids collected with an Oracol swab from the 187 children (aged 5–16 years) with prolonged coughs (≥ 2 weeks) used in this study varied between 0.33 and $23.1\text{ }\mu\text{g ml}^{-1}$ (median $2.5\text{ }\mu\text{g ml}^{-1}$). A GACELISA was used to analyse oral fluid IgG, since this

format accommodates samples containing varied concentrations of antibody, on the condition that all samples contain sufficient antibody to saturate the capture antibody layer. Preliminary experiments showed that the anti-IgG capture antibody layer was saturated by IgG concentrations in oral fluid above $\sim 0.75 \mu\text{g ml}^{-1}$ (results not shown). GACELISA has previously proven useful in the surveillance of other infections, such as measles, mumps and rubella (Perry *et al.*, 1993), and is less prone to non-specific interactions than indirect ELISA (D. J. Litt and others, unpublished results). In this particular assay, a capture antibody is used to immobilize IgG from the oral fluid, after which soluble PT is added, which binds to any specific antibody present. Finally, a PT-specific HRP conjugate is added to quantify the amount of immobilized PT.

The anti-PT IgG titres generated by oral fluids from the 187 children with prolonged coughs correlated well with their matched sera (Fig. 1). A line of best fit (based on a power relationship between the variables) generated the correlation coefficient $R^2=0.75$, and suggested that a subject with a serum titre of 100 eU ml^{-1} (i.e. seropositive) would possess an oral fluid titre of 67 AU.

Sensitivity and specificity of the oral fluid GACELISA in children with prolonged coughs

The ability of the oral fluid GACELISA to act as a surrogate for the serum ELISA was tested using matched sera and oral fluids from the same 187 children with prolonged coughs. Of these, 69 were seropositive (serum anti-PT titre $\geq 100 \text{ eU ml}^{-1}$).

Fig. 1. Comparison of anti-PT IgG titres generated by matched serum and oral fluid samples from 187 children with prolonged coughs. A line of best fit (power relationship) and the serum and oral fluid titre values of 100 eU ml^{-1} and 70 AU, respectively, are highlighted.

The oral fluid anti-PT titres showed some overlap between the seropositive and seronegative groups of coughing children (Fig. 2). Using a cutoff of 70 AU, the oral fluid GACELISA showed an overall concordance of 169/187 (90.4%) with the serum ELISA. This comprised a sensitivity of 55/69 [79.7%; 95% confidence interval (CI) 68.3–88.4] and a specificity of 114/118 (96.6%; 95% CI 91.5–99.1) (Table 1).

The 14 false-negative results and four false-positive results generated by the oral fluid assay in this study did not appear to be associated with patients' ages, the time between disease onset and sampling, or the total concentration of IgG in the oral fluid samples (Table 2). Other studies have reported that samples with low IgG concentrations can generate false-negative results in GACELISAs (Nigatu *et al.*, 1999), and this cannot be ruled out as the explanation for some of the false negatives seen in this study. Although the IgG capture antibody layer in the assay is saturated when oral fluids contain more than $\sim 0.75 \mu\text{g total IgG ml}^{-1}$, we did observe that samples containing $< 3 \mu\text{g total IgG ml}^{-1}$ may produce suboptimal absorbance values in the assay (results not shown). Nevertheless, other factors are clearly involved in the generation of discordant results, in particular the two strong false-positive oral fluid titres observed in this group. We hypothesize that these oral fluids contained

Fig. 2. Anti-PT IgG titres in (A) oral fluids from 69 seropositive ($\geq 100 \text{ eU ml}^{-1}$) children with prolonged coughs, (B) 118 seronegative children with prolonged coughs, and (C) 220 age-matched controls. The median of each population is shown with a horizontal bar. The oral fluid titre value of 70 AU is highlighted with a dashed line.

Table 1. Sensitivity, specificity and PPV of different cutoff values in the oral fluid anti-PT GACELISA when distinguishing between 69 seropositive (≥ 100 eU ml⁻¹) and 118 seronegative children with prolonged coughs

Disease prevalence values of 12, 32 and 37 % are representative of those reported in previous studies.

Cutoff value (AU)	Sensitivity (%)	Specificity (%)	PPV		
			12 % prevalence	32 % prevalence	37 % prevalence
10	100.0	25.4	15.5	38.7	44.1
20	100.0	61.9	26.3	55.2	60.6
30	98.6	50.8	21.5	48.5	54.1
40	89.9	83.1	42.0	71.4	75.7
50	84.1	90.7	55.1	80.9	84.1
60	84.1	94.9	69.3	88.6	90.7
70	79.7	96.6	76.2	91.7	93.2
80	75.4	96.6	75.2	91.3	92.9
90	71.0	97.5	79.2	92.9	94.3
100	68.1	98.3	84.6	95.0	95.9
150	55.1	98.3	81.6	93.9	95.0
200	44.9	99.2	87.8	96.1	96.9
300	31.9	99.2	83.7	94.7	95.7
400	24.6	100.0	100.0	100.0	100.0

Table 2. Subjects amongst 187 children with prolonged coughs whose matched sera and oral fluids produced discordant anti-PT IgG titres (seropositive ≥ 100 eU ml⁻¹; oral fluid positive ≥ 70 AU)

'Onset' refers to the onset of coughing symptoms (y, years; m, months).

Subject	Serum titre (eU ml ⁻¹)	Oral fluid titre (AU)	Oral fluid IgG concn (μ g ml ⁻¹)	Time from onset to sampling (days)	Age at onset
False-negative oral fluid result:					
A10	149	33	8.1	99	6 y 9 m
A14	168	34	2.7	32	11 y 2 m
A27	357	69	1.0	46	13 y 6 m
A29	105	44	2.5	96	7 y 11 m
B16	138	24	6.4	33	6 y 7 m
B37	201	65	9.4	58	5 y 11 m
B48	978	43	2.7	64	6 y 10 m
C44	100	46	5.1	33	7 y 10 m
D2	656	39	0.8	44	6 y 4 m
E16	363	68	2.0	45/50*	8 y 5 m
E27	156	39	4.5	30	8 y 9 m
E34	106	35	1.1	120	12 y 6 m
E36	330	40	6.6	29	8 y 7 m
E39/2	167	31	1.1	21	8 y 2 m
False-positive oral fluid result:					
E6	56	91	1.1	43	12 y 4 m
E7	89	80	1.8	28	6 y 6 m
B29	44	320	0.9	38	16 y 6 m
E38	43	186	2.0	35	5 y 2 m

*45 days for serum, 50 days for oral fluid.

uncharacterized substances from their subjects' mouths that interfered with the assay.

Positive predictive value of the oral fluid GACELISA in children with prolonged coughs

It has recently been shown that 37 % of children aged 5–16 in the UK with prolonged coughing symptoms are seropositive for *B. pertussis* infection (Harnden *et al.*, 2006). Using ≥ 70 AU as the definition of positivity, the oral fluid GACELISA exhibited a positive predictive value (PPV) of 93.2 % in this population when used as a surrogate for the serum ELISA (i.e. assuming a disease prevalence of 37 %; Table 1). The corresponding negative predictive value (NPV) was 89.3 %. By raising the cutoff in the oral fluid GACELISA to ≥ 100 AU it would be possible to increase the PPV to 95 % with only a modest reduction in sensitivity (Table 1).

If the upper and lower limits of disease prevalence shown by other studies (not confined to the 5–16 year age group) of 12 and 32 % in subjects with prolonged coughs are assumed, a 70 AU cutoff results in respective PPVs of 76.2 and 91.7 % (Table 1) and NPVs of 97.3 and 91.3 % for the oral fluid GACELISA.

Oral fluid anti-PT IgG titres in control children

Analysis of oral fluids from 220 control children (Fig. 2) revealed that 8/220 (3.6 %) possessed anti-PT IgG titres ≥ 70 AU. There were no matched sera for these oral fluid samples in order to compare the results using the serum ELISA. However, this is similar to the result of a recent study in the UK, which showed that 2.8 % of normal children aged <15 years were seropositive for *B. pertussis* infection (Nardone *et al.*, 2004). It is encouraging that the oral fluid GACELISA detected a similar incidence of infection as the serum ELISA when used in the general population (irrespective of coughing symptoms).

Reproducibility within and between runs

Five oral fluid samples (with titres between 23 and 213 AU), assayed five times each within the same run, produced coefficients of variation ranging between 1.9 and 8.3 % (median 3.8 %). Fifteen oral fluid samples (with titres ranging between 41 and 192 AU), each assayed on eight independent occasions, produced coefficients of variation of between 5.4 and 18.6 % (median 9.4 %).

General comments

Our results demonstrate that the oral fluid anti-PT GACELISA can be used as a surrogate for the serum ELISA, demonstrating high specificity and PPV in subjects with prolonged coughing symptoms. Even though the assay does not appear to be as sensitive as the serum ELISA, it will be a valuable alternative to the serum anti-PT ELISA, especially in epidemiological studies or for diagnosis in young patients reluctant to provide blood samples. Care

would need to be taken when using this ELISA as a surrogate for the serum ELISA in the general population, irrespective of coughing symptoms, however, as the much lower incidence of disease of 0.3–2.8 % in the general population (Miller *et al.*, 2000; Nardone *et al.*, 2004; Strebel *et al.*, 2001) would result in a corresponding PPV of only 6.6–40.3 %.

To our knowledge, this is the first description of an anti-PT IgG antibody capture assay designed for use with oral fluids. Indirect ELISAs have been used to detect PT-specific IgG in saliva and nasopharyngeal aspirates (NPAs), following disease or intranasal vaccination against pertussis, with disappointing results (Granstrom *et al.*, 1988b; Berstad *et al.*, 2000). This is not surprising, since indirect ELISAs are not ideal for use with samples containing the expected low and variable antibody concentrations in oral fluid (Mortimer & Parry, 1988). These earlier studies appear not to have analysed the concentration of IgG in the samples, nor did they study the correlation between the PT-specific IgG titre in mucosal fluid and that in serum.

Evaluation of PT-specific IgA along with IgG in oral fluids may be a means of increasing the sensitivity of this diagnostic method, since rises in anti-PT IgA titre in saliva and NPAs are easier to detect than those of IgG following disease or intranasal immunization (Zackrisson *et al.*, 1990; Granstrom *et al.*, 1988a; Berstad *et al.*, 2000). IgA also offers the advantage over IgG that its levels do not increase very strongly after primary parenteral vaccination against pertussis (Goodman *et al.*, 1981; Thomas *et al.*, 1989; Cherry, 1999). Hence, IgA titres may be helpful in detecting infections during the months following vaccination, when IgG levels are uninformative.

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