

Correlation of IgA, IgM and IgG antibody-detecting assays based on filamentous haemagglutinin, pertussis toxin and *Bordetella pertussis* sonicate in a strictly adult population

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(Accepted 6 September 2004)

SUMMARY

Pertussis diagnosis among adults is primarily based on serology. In this study, receiver operation characteristics (ROC) analysis was utilized to evaluate three IgA, IgM and IgG ELISAs based on *B. pertussis* whole-cell sonicate (WC), pertussis toxin (PT), or combined PT-filamentous haemagglutinin (FHA) antigens in a healthy adult population and in adults suffering from symptoms consistent with pertussis. The assays were compared to a PT and FHA reference ELISA. The reference assay showed high correlation with PT- and PT-FHA-based ELISAs but low correlation was found with the WC-based ELISA. However, ROC analysis indicated that all the assays enabled fine differentiation between healthy subjects and subjects suffering from symptoms suggestive of pertussis. The WC IgA ELISA showed the best diagnostic performance [highest specificity (98·8%) with highest sensitivity (53·8%)]. We conclude that despite a low correlation between the various assays they all carry good diagnostic capability for an adult population.

INTRODUCTION

The role of pertussis as a cause of prolonged cough among adults is well documented; several studies found high prevalence (ranging from 12·4% to 26%) of *Bordetella pertussis* infection among adults suffering from prolonged cough [1–7]. These findings emphasize the need to utilize a specific and sensitive method for the diagnosis of this disease; a challenging yet frustrating goal since *B. pertussis* is a highly fastidious organism and, therefore, is difficult to culture. Furthermore, the sensitivity of culture decreases with increasing age and with time elapsed from onset of clinical signs [8]. Molecular analysis by PCR also has

a low sensitivity in the late stages of the disease (i.e. paroxysmal and convalescent stages) [9, 10].

Adults are more likely to be encountered by the physician in the paroxysmal stage, which usually starts at least 1 week from the onset of clinical signs and is characterized by the most significant morbidity [11]. Therefore, culture and PCR are not optimal for the diagnosis of pertussis in this population and the most sensitive method for diagnosing adults appears to be serology. Most of the studies conducted to evaluate the prevalence of pertussis among adults suffering from prolonged cough are based on diagnosis of the disease by ELISA [1–7]; in a recent Canadian study, only four patients out of 442 (0·9%) adolescents and adults who had cough-related illness showed positive results in nasopharyngeal culture or PCR, while 84 patients (19·1%) were diagnosed by serology analysis of a single serum sample [6].

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Whole-cell sonicates (WC) of *B. pertussis* were used as antigens for ELISAs in the 1980s [3, 12–14] but were gradually replaced by purified antigens: pertussis toxin (PT), filamentous haemagglutinin (FHA), pertactin (PTN) and fimbrial proteins (FIM). PT and FHA ELISAs were utilized in serosurveys to assess population immunity and exposure to *B. pertussis* [15–19] and the presence of PT antibodies has been correlated with protection against pertussis [20–22]. Assays for IgG, IgM and IgA to PT and FHA have been used extensively during vaccine trials in which they were thoroughly validated [23–27] and showed high specificity for the detection of recent infections by *B. pertussis* [28, 29]. However, since most of the healthy adults have antibodies to these antigens [30], diagnosis is based on the presence of levels higher than a specified cut-off, thereby lowering the sensitivity. Furthermore, the use of ELISA for the detection of antibodies to each antigen separately is expensive and laborious. Thus, utilization of WC and/or combined FHA-PT ELISAs as an alternative for these assays might be advantageous. Attempts have been made to systematically standardize different ELISAs performed in different laboratories so that vaccines, populations and proposed vaccine combinations could be compared despite the use of different assays [31, 32]. Thus, the aim of this study was to assess the correlation between WC, combined PT-FHA, and single-antigen ELISAs, and to evaluate their diagnostic performance. To our knowledge, correlation between the results of these assays has not yet been reported in a solely adult population.

METHODS

Study population

Sera were collected from 26 sporadic and epidemic cases (age range 18–31 years) admitting to an army unit clinic during the years 2001–2002 and complaining of prolonged cough (≥ 14 days long) accompanied by post-tussive emesis (which was chosen as an indication of a significant ‘unusual’ cough). These subjects were selected for evaluating the assays’ performances in a population suffering from clinical symptoms suggestive of pertussis and to test the correlations between the assays. The specificities of the assays were evaluated by analysis of sera obtained from a sample of 86 healthy subjects (age range 18–19 years) that did not have clinical indications suggesting possible recent infection with *B. pertussis* and were

not known to be in contact with subjects who recently suffered from pertussis.

Exact information on vaccination status of subjects from both groups was not available. However, most of the subjects were born in Israel (20 out of 26 in the sick group 81 out of 86 in the healthy group), and thus, were probably vaccinated four times during their first year of life.

Laboratory methods

As a reference method for serological evaluation of a population suspected to be recently infected with *B. pertussis* ($n=26$) we chose to use a commercial ELISA kit (Pertuscan 2 + 2[®], Euro-Diagnostica Ltd, Malmö, Sweden) (ELISA no. 1). The European Sero-Epidemiology Network reference centre uses an in-house PT ELISA to standardize various ELISAs for the detection of anti-PT antibodies [32]. ELISA no. 1 was recently shown to have high correlation with this in-house assay (Giammanco A., personal communication). In another recent study it was found to be superior to other commercial ELISAs [33]. Briefly, each sample was tested separately for IgA or IgG against highly purified PT or FHA. A positive result by either IgG to PT (≥ 2) or FHA (≥ 1.2) or IgA to PT (≥ 0.3) or FHA (≥ 0.6) was considered indicative of recent pertussis infection. The assay was performed in duplicates according to the manufacturer’s recommendations.

Three ELISAs (nos. 2–4) were evaluated by comparison to the reference assay, ELISA no. 1. The assays were performed as follows:

ELISA no. 2

A commercial ELISA kit (PanBio Ltd, East Brisbane, Australia) based on WC of *B. pertussis*. The analysis for the presence of specific IgA, IgM or IgG was performed according to the manufacturer’s recommendations. Results are presented as ELISA units obtained by calculating the ratio between the sample absorbance and a cut-off calibrator absorbance. For simplicity, this value was not multiplied by 10 as recommended by the manufacturer.

ELISA no. 3

A commercial ELISA kit (Savyon Diagnostics Ltd, Ashdod, Israel) based on an enriched fraction of *B. pertussis* toxin and FHA (FHA-PT). The analysis for IgA, IgM or IgG was performed in accordance

with the manufacturer's recommendations. Results for IgA are presented as in ELISA no. 2. Results for IgG are presented as arbitrary binding units (BU) that were calculated by interpolation of the sample value from a best-fitted line drawn on the basis of three calibrator samples.

ELISA no. 4

An in-house ELISA (modified from Cohen et al. [34]) designed to analyse IgA or IgG antibodies against PT. The assay was performed in flat-bottomed 96-well microtitre plates (Costar[®], High Binding, Corning, NY, USA). A total of 100 μ l of highly purified PT (List Biologicals Ltd, Campbell, CA, USA, 0.25 μ g/ml in PBS) were incubated in each well for 2.5 h at 37 °C in humid chamber. The plates were blocked for 1 h at 37 °C with a buffer containing 0.05 M phosphate-buffered saline (PBS) supplemented with 5 g/l of casein and bovine serum albumin. Serum samples (100 μ l) diluted 1:400 in blocking buffer were added to each well and incubated overnight at 4 °C. The sera were tested in duplicates. A total of 100 μ l of alkaline phosphatase conjugated anti-human IgG or anti-human IgA (KPL, Gaithersburg, MD, USA), diluted 1:400 in blocking buffer were added and incubated for 2 h at 37 °C in a humid chamber. Substrate was then added at room temperature and the reactions were terminated after 15 min (IgG) or 40 min (IgA) with 50 μ l of stop solution (3 M NaOH). Absorbance was read at 405 nm. Blanks (eight wells without samples) were run in each plate and the mean OD was subtracted from samples. Results were rejected if the mean OD of the blanks was greater than 0.1. The results were calculated as in ELISA no. 2.

Statistical analysis and interpretation of results

Performances of the assays were evaluated by the receiver operation characteristics (ROC) procedure [35]; ROC curves depict the sensitivity of the assay against a false-positive rate (calculated by 1-specificity). Higher sensitivity will lead to a higher curve and higher specificity will push the curve to the left. The area under the curve (AUC) is a global (i.e. based on all possible cut-off values), summary statistic of diagnostic accuracy and represents the ability of the evaluated test (i.e. the various ELISAs) to discriminate between subjects with symptoms suggestive of pertussis and healthy subjects. Thus, an AUC of 0.5 represents an assay that has no discriminating ability and its results are actually random. An AUC

of 1 represents a perfect assay as it implies that at least one cut-off value exists with both perfect (i.e. 100%) sensitivity and specificity. Correlations between ELISA no. 1 IgG or IgA assays to the respective IgG and IgA ELISA nos. 2–4 were calculated by the squared Pearson correlation coefficient. Statistical analysis was performed by using SPSS 10.0 for Windows (SPSS Inc., Chicago, IL, USA). Microsoft Excel (Office 2000) was used to design the scatter plots.

RESULTS

Out of the 26 samples of adults who showed clinical signs that were suggestive of pertussis, 10 subjects were considered positive by ELISA no. 1 (positive for IgG or IgA to either FHA or PT). By defining ELISA no. 1 as the reference assay, all three ELISAs had a high AUC of more than 0.97 (for IgG and IgA). However, when all 26 samples of subjects with suggestive clinical signs were considered as indicative of recent pertussis morbidity, the differences between the assays were more apparent (Fig. 1*a–c* and Table). The WC ELISA (no. 2) showed the highest AUC for both IgG and IgA (0.859 and 0.774 respectively) and the FHA-PT ELISA (no. 3) showed the highest AUC for IgM (0.832).

Cut-off values for ELISA nos. 2–4 were defined according to the ROC curves such that each assay had maximum sensitivity with specificity of at least 95%. For WC ELISA (no. 2) these cut-offs showed markedly higher specificity than the specificity for the cut-off value suggested by the manufacturer (95.3% vs. 33.7% respectively). The same was true for FHA-PT IgG and IgM ELISAs (no. 3) (95.3% vs. 81.4%) (Table 1, Fig. 1). When clinical signs suggestive of pertussis were used as indication for morbidity, WC IgA ELISA (no. 2) had the highest sensitivity (53.8%) and the highest specificity (98.8%) and allowed positive identification of 37.5% of the patients who suffered from symptoms suggestive of pertussis and were negative by ELISA no. 1. Combining the results of WC (no. 2) and in-house PT IgA (no. 4) assays (samples that were positive for either *B. pertussis* sonicate or purified PT) conferred a sensitivity of 65.4% with a specificity of 94.2%. When a positive result in ELISA no. 1 on either IgA or IgG to FHA or PT was considered as an indication of morbidity, all the three assays showed relatively high sensitivity ranging between 80% for WC IgA (no. 2) and 100% for in-house PT IgA assay (no. 4) (Table 1). The IgM

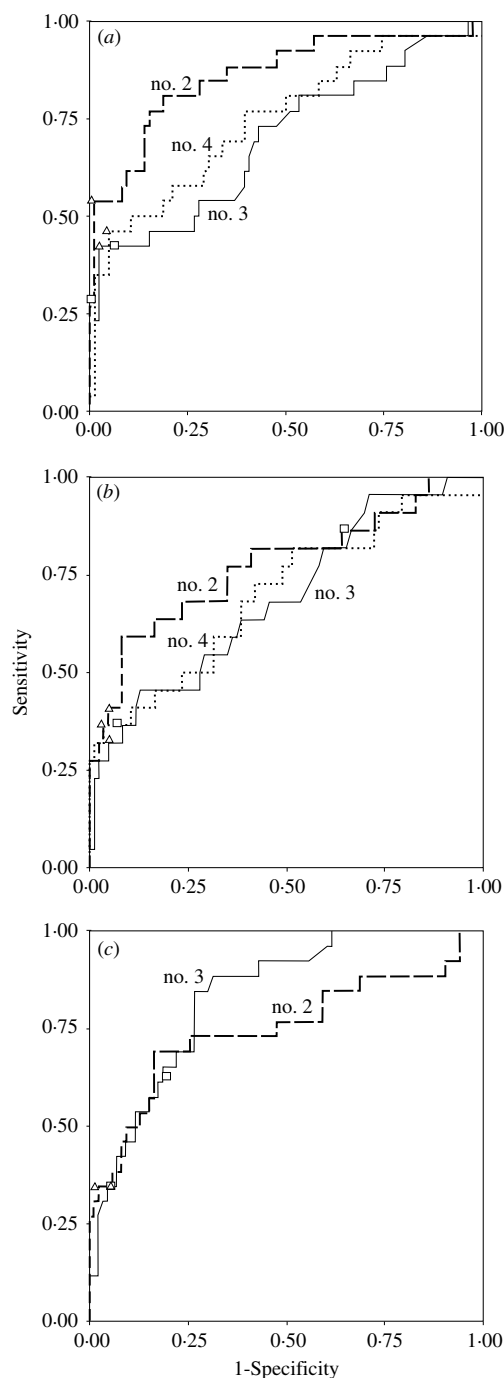


Fig. 1. ROC curves, depicting the sensitivity (i.e. rate of subjects positive by each ELISA out of the 26 subjects who suffered from clinical signs suggestive of pertussis) of the three assays [---, ELISA no. 2 (WC); —, ELISA no. 3 (FHA-PT); ····, ELISA no. 4 (PT)] against their false-positive rates, represented by 1-specificity (i.e. rate of subjects positive by each ELISA out of the 86 healthy subjects) along all possible cut-off values. Diagonal segments are produced by ties. (a) IgA assays. (b) IgG assays. (c) IgM assays (assay nos. 2 and 3 only). Triangles represent cut-off values determined in the study, and squares represent cut-off values recommended by the manufacturer (Table).

and IgG assays showed AUCs comparable to those of the IgA assays, however, they had lower sensitivity when cut-offs corresponding to specificity higher than 95% were chosen.

Correlations between the reference assay (ELISA no. 1) and the other three assays are depicted in Figure 2. PT ELISA (no. 1) showed the highest correlation with PT ELISA (no. 4) for both IgG and IgA ($R^2=0.96$ and 0.74 respectively). FHA ELISA (no. 1) showed the highest correlation with FHA-PT ELISA (no. 3) for both IgG and IgA ($R^2=0.92$ and 0.75 respectively). All IgA assays had lower correlations with the parallel ELISA no. 1 compared to the IgG assays. Of the three tested assays, WC ELISA (no. 2) had the lowest correlation with ELISA no. 1 in all categories.

DISCUSSION

The aim of this study was to assess the correlation between WC and combined PT-FHA ELISAs and the separate PT and FHA ELISAs, and to evaluate the diagnostic performance of the IgA, IgG and IgM assays based on these antigens. To our knowledge, it is the first study to compare these methods in a strictly adult population. The use of ROC analysis provided a valuable tool that allowed quantitative comparisons between the performances of the different assays and determination of cut-off values with increased specificity and comparable sensitivity to the values determined by the manufacturers. The main limitation of this study was the use of a sample of subjects in whom the diagnosis of pertussis was not confirmed by a gold-standard laboratory method, but rather had clinical symptoms suggestive of pertussis. This approach, however, was previously adopted in studies conducted by Poynten et al. [36] and by de Melker et al. [28]. Since the sensitivity of culture (which is considered as the gold standard) is extremely low in adults [8], samples of culture-proven adults are very rare. Indeed, diagnosis of pertussis among adults mostly relies on serology [1–7]. Nevertheless, this study clearly demonstrates the ability of the assays to identify subjects with illness suspected to be caused by *B. pertussis*.

According to the ROC analysis, the whole cell lysate (WC) assay (no. 2), gained the highest scores when the criteria for case definition were based on clinical symptoms suggestive of *B. pertussis* infection, although its correlations with both PT and FHA assays (no. 1) were low. By considering clinical signs

Table. Comparison of the sensitivity and specificity of three ELISAs based on PT + FHA, PT, and B. pertussis sonicate

	IgA				IgG				IgM										
	WC (no. 2)		FHA + PT (no. 3)		PT (no. 4)	PT + WC (nos. 2 + 4)		WC (no. 2)		FHA + PT (no. 3)		WC (no. 2)		FHA + PT (no. 3)					
AUC* ...	0.859		0.696		0.747	n.a.		0.774		0.69		0.7		n.a.		0.75		0.832	
Cut-off value† ...	†	Manuf.‡	†	Manuf.‡		0.4 (PT)		†	Manuf.‡	†	Manuf.‡		1.15 (PT)	†	Manuf.‡	†	Manuf.‡	†	Manuf.‡
	0.78	1.1	1.93	1.1	0.4	7.8 (WC)		1.94	1.1	91.5	50		1.15	1.94 (WC)	1.27	1.1	1.47	1.1	
Specificity (%) (n = 86)	98.8	98.8	97.7	94.2	95.3	94.2		95.3	33.7	95.3	81.4		96.5	90.7	97.7	95.3	95.3	81.4	
Sensitivity (%) (compared to clinical signs) (n = 26)§	53.8	26.9	42.3	42.3	46.2	65.4		40.9	86.4	31.8	45.5		36.4	45.5	34.6	34.6	34.6	61.5	
Sensitivity (%) (compared to ELISA no. 1) (n = 10)	80	40	90	90	100	100		90	100	90	100		90	100	70	70	50	70	
Positive samples (%) out of samples negative by ELISA no. 1 (n = 16)	37.5	25	12.5	12.5	12.5	50		14.3	78.6	0	14.3		7.1	14.3	12.5	12.5	25	62.5	

WC, Whole cell; FHA, filamentous haemagglutinin; PT, pertussis toxin.

* Area under the ROC curve when suggestive clinical signs were considered as indicative of pertussis morbidity.

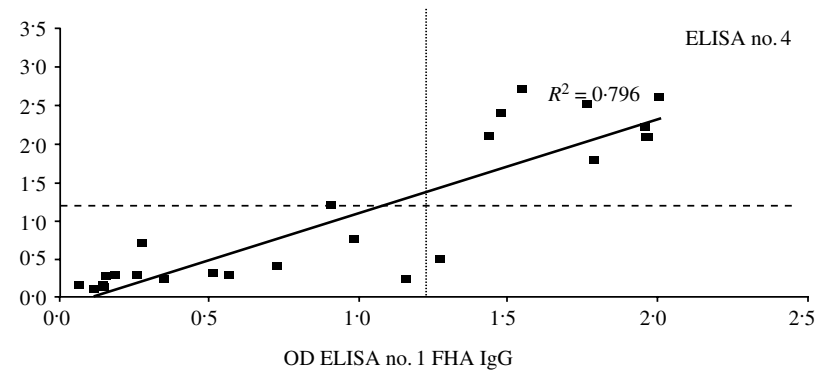
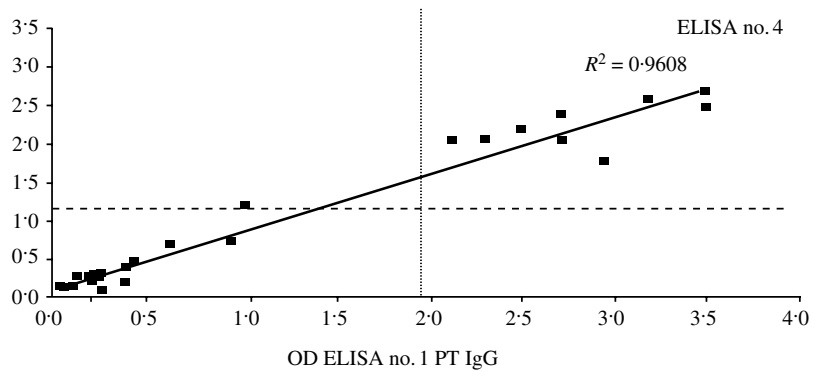
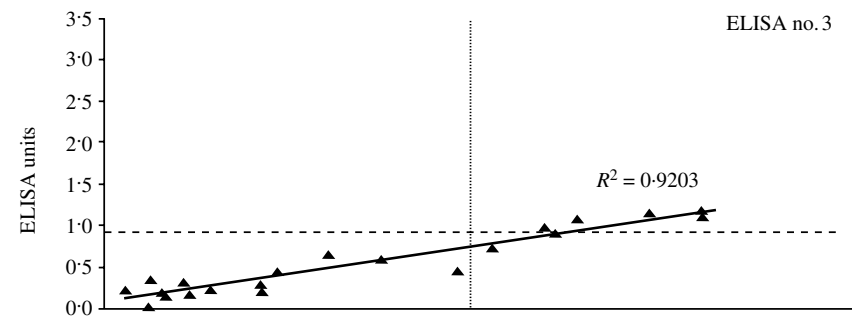
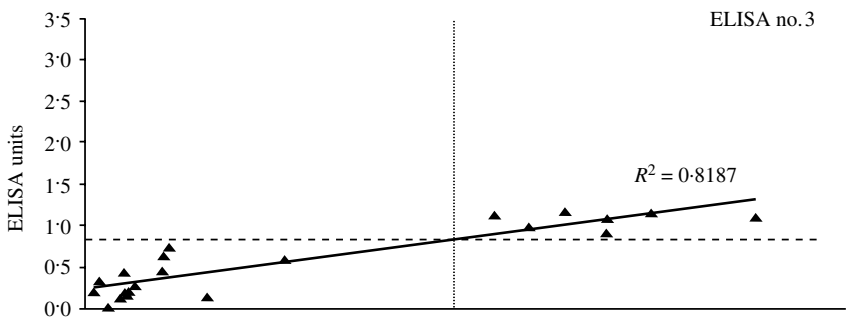
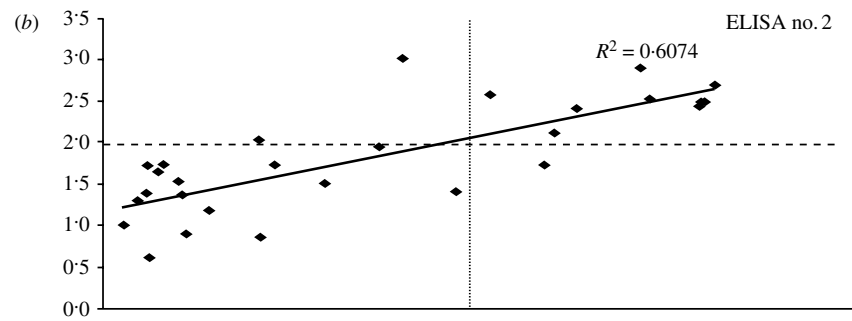
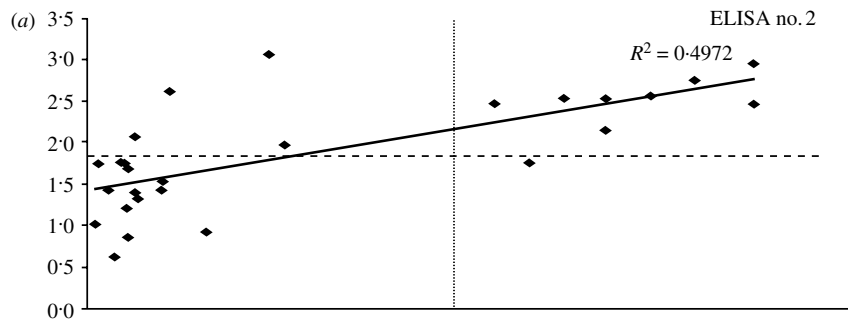
† Cut-off values were chosen according to the ROC curves results in a way that the assay will have maximum sensitivity with a specificity of at least 95%.

‡ Cut-off value determined by manufacturer.

§ For IgG assays (n = 22).

|| For IgG assays (n = 14).

n.a., Not applicable.



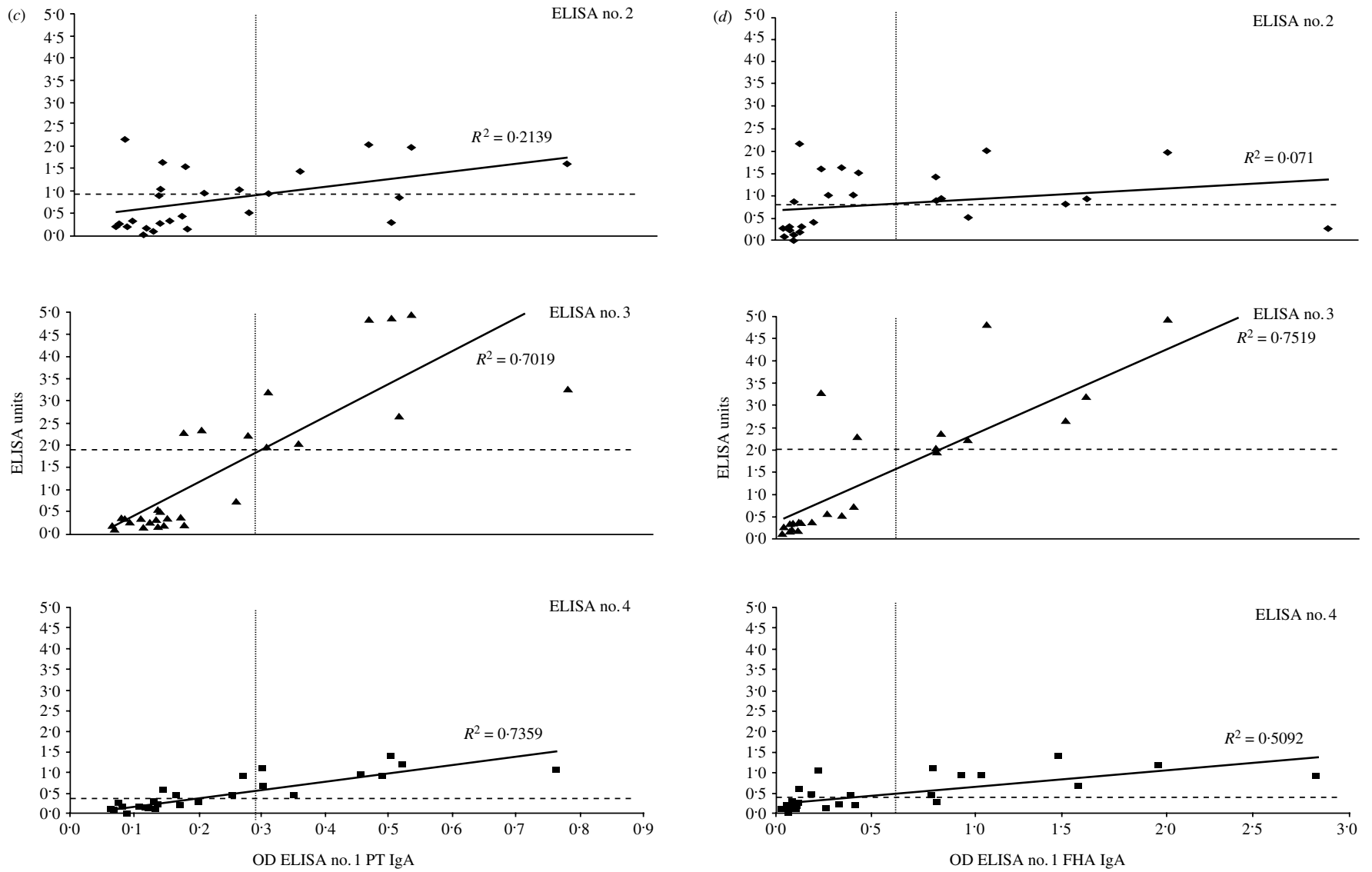


Fig. 2. Correlation between ELISA no. 2 (WC, \blacklozenge), no. 3 (PT-FHA, \blacktriangle), no. 4 (PT, \blacksquare) and ELISA no. 1. (a) IgG to PT; (b) IgG to FHA; (c) IgA to PT; (d) IgA to FHA. R^2 =squared Pearson correlation coefficient. Horizontal dashed lines represent the cut-off values (see text for cut-off determination) of the various assays. Vertical dotted lines represent the cut-off values of ELISA no. 1.

for case definition, the combination of in-house PT (no. 4) and WC (no. 2) IgA assays resulted in a sensitivity of 65.4% and a specificity of 94.2%. Thus, this method can significantly increase the diagnosis of pertussis infections. This finding is in agreement with the finding of Nagel et al. [12] showing that IgA antibodies to WC *B. pertussis* rise after contact with the pathogen but not after vaccination and can, therefore, serve as a good indicator for infection. Poynten et al. [36], however, found that the same IgA WC ELISA we were using here had lower sensitivity and higher specificity than assays based on PT, FHA and pertactin. The low sensitivity found by this group might be attributed to a cut-off point higher than the one used in the present study.

It can be argued that since PT is specific for *B. pertussis*, whereas other antigens (that are presented in the WC ELISA) are shared by other bacteria, diagnosis of pertussis should be based solely on ELISA to PT. However, the analytical specificity of an ELISA based on ultrasonicated formalin-killed *B. pertussis* was previously assessed by Viljanen et al. [14] who tested the ability of other bacteria to inhibit a *B. pertussis*-positive sera. The only bacterium that was able to cause significant inhibition was *B. parapertussis*. This is of little clinical significance since *B. parapertussis* causes a disease that is similar to the one caused by *B. pertussis* [37] and is susceptible to the same antimicrobial agents. Thus, the relevance of non-related cross-reactive antibodies to these antigens is probably overestimated and does not merit clinical significance.

In the present study, the in-house PT IgG ELISA (no. 4) showed excellent correlation with the reference PT ELISA (no. 1) ($R^2=0.96$). High correlation was observed between PT and FHA ELISAs. The combined FHA and PT IgG ELISA (no. 3) had good correlation with both PT ($R^2=0.82$) and FHA ($R^2=0.92$) assays (no. 1), and thus, it can be used both for the purpose of assessment of immunity and for the diagnosis of pertussis. In contrast, WC ELISA (no. 2) showed low correlation with these assays. These findings, however, do not contradict the ability of the WC assay to diagnose morbidity caused by *B. pertussis*; Agglutinin levels, for example, have been shown to be elevated in as much as 85% of *B. pertussis* culture-proven infections despite a poor correlation with rises of FHA and PT [38].

ELISAs for IgA, IgG and IgM that were evaluated in this study elicited comparable results. However, although having AUCs that were similar to those

of the IgG and IgA assays, sensitivities of the IgM assays were low when cut-offs values corresponding to high specificity (>95%) were selected. In these cut-off values IgA showed the best performance for all antigens tested. We have previously shown similar results while analysing an outbreak of pertussis among a group of young adults; in that study we showed that IgA to WC had higher specificity than IgM and IgG [39]. The lower specificity of IgM assays can be explained by the fact that most of the subjects were previously vaccinated against pertussis and thus, when exposed to *B. pertussis*, developed higher titres of IgA and IgG compared to the IgM titres [40].

In summary, this study supports the utilization of all of the assays assessed for the diagnosis of pertussis in adults. Of particular interest is the high diagnostic performance elicited by the WC *B. pertussis* IgA and IgG ELISA and its high added value as an adjunct assay to PT ELISA for diagnosis of pertussis. Poor correlation between WC ELISA (no. 2) and PT/FHA assay (no. 1) suggests that they detect different patients and thus, their combination may contribute significantly to the sensitivity of the serological diagnosis of pertussis. This poor correlation however, renders the WC ELISA (no. 2) unsuitable for sero-surveys intended for immunity assessment. It is, therefore, preferable to use for this purpose, assays that are based on PT, or combinations of PT and FHA.

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