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Comparison of Five Commercial Enzyme-Linked Immunosorbent Assays for Detection of Antibodies to *Bordetella pertussis*

KATRIN KÖSTERS,[1]* MARION RIFFELMANN,[1] BRIGITTE DOHRN,[2] AND CARL HEINZ WIRSING VON KÖNIG[1]

Institut für Hygiene und Laboratoriumsmedizin, Klinikum Krefeld, Krefeld,[1] and Abteilung für Pädiatrie, Klinikum Wuppertal, Wuppertal,[2] Germany

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Measuring antibodies to *Bordetella pertussis* antigens is mostly done by enzyme-linked immunosorbent assays (ELISAs). We compared the performance of five commercially available ELISA kits with the help of 65 serum specimens which were repetitively tested for evaluation of the kits. The specimens contained 20 paired serum samples from patients with clinical pertussis, 15 samples were from children vaccinated with a diphtheria-tetanus-acellular pertussis vaccine, seven specimens were taken from an interlaboratory comparison of ELISAs, and there were three reference preparations from the Food and Drug Administration's (FDA's) Laboratory of Pertussis and from our laboratory. Reference values were obtained from the FDA or from results obtained with an in-house ELISA. Commercial ELISAs were compared with respect to their reproducibility and variability, their ability to detect significant titer rises in paired serum samples, their ability to detect an immune response after vaccination, and the comparability of semiquantitative and quantitative results. Reproducibility was generally good (>89%), intra-assay variation ranged from 2.4 to 28.7%, and indeterminate results were recorded in up to 18.5% of all specimens. Most kits correctly identified the antibody response to an acellular pertussis vaccine. None of the commercial kits identified all cases of pertussis correctly, and the sensitivity ranged between 60 and 95%. All five commercial ELISAs showed great discrepancies when comparing semiquantitative results and contained obviously different antigen preparations. Our data suggest that the five commercial ELISAs tested here need further improvement and standardization.

According to the World Health Organization case definition, the diagnosis of pertussis is based on clinical symptoms (≥21 days of paroxysmal cough) in combination with the isolation of *Bordetella pertussis* and/or a positive serology and/or contact with a culture-confirmed case of pertussis (28). Enzyme-linked immunosorbent assays (ELISAs) currently are the method of choice for detection of antibodies to *B. pertussis* antigens (16). Various ELISA formats with different antigens have been developed (5, 8, 12, 14, 23, 24, 29, 30) and were evaluated intensively in vaccine trials (7, 11, 18, 27).

In addition to vaccine trials, serology plays a key role in the diagnosis of pertussis in adolescents and adults (3, 26), as well as for epidemiologic surveys (1, 6, 17, 19). Furthermore, the diagnosis of pertussis based on a single serum sample using age-specific reference values for different populations is increasingly being used (25).

In 1995 a total of 33 research laboratories and vaccine producers participated in an international collaborative study for the evaluation of ELISAs to measure antibodies to *B. pertussis* antigens which showed differences between different noncommercial assays of similar format (15). However, the results of this study also indicated that results from different laboratories can be compared when a common reference serum is used, when the antigen preparations are similar, and when comparable techniques are employed. Given the broad use of commercially available ELISAs for detecting antibodies to *B. pertussis* antigens in Germany, we decided to compare five commercially available ELISAs with an in-house ELISA, which has been extensively evaluated. ELISA kits were selected according to their market share in private laboratories, which was evaluated in a telephone poll by one of us (C.H.W.V.K.).

We compared the reproducibility and variability of the tests, as well as their ability to detect significant titer rises in paired serum samples and to detect an immune response after vaccination with a diphtheria-tetanus-acellular pertussis (DTaP) vaccine and the comparability of semiquantitative and quantitative results.

MATERIALS AND METHODS

Serum specimens. Specimens included 20 paired serum samples from a recent pertussis vaccine trial (20, 27), 15 samples from an immunogenicity study (Hib 032, kindly provided by SB Biologicals, Rixensart, Belgium), 7 samples from an international collaborative study for the detection of antibodies to *B. pertussis* antigens (15) (kindly provided by the Laboratory of Pertussis, Center for Biologics Evaluation and Research, U.S. Food and Drug Administration [FDA], Bethesda, Md.), the FDA reference serum lots 3 and 4, and a lyophilized in-house reference preparation (lot 2).

The vaccine trial was designed as a household contact study to evaluate the efficacy of an acellular pertussis vaccine (20, 21), and sera were taken from the participating individuals with prolonged (>21 days) coughing in the acute phase and after 4 to 14 weeks. Specimens from 3,723 patients were obtained between February 1993 and September 1994 and were assayed twice for the presence of immunoglobulin G (IgG) and IgA antibodies to pertussis toxin (PT), filamentous hemagglutinin (FHA), and pertactin with the in-house ELISA. For the present study 20 paired sera from individuals who were earlier confirmed to have clinical and serologic evidence of pertussis were chosen at random. The patients were 1 to 58 years old, with a median age of 4.5 years. The female/male ratio was 11:9. The 20 specimens were all obtained between May and August 1994 and were stored at −20°C. All samples were retested after thawing.

Samples from the immunogenicity study were taken from infants aged 5 to 6 months at 30 to 35 days after third vaccination with a tricomponent acellular pertussis vaccine (Infanrix) in combination with a *Haemophilus influenzae* type b vaccine. A total of 15 samples from the study were randomly chosen by SB Biologicals, where they were stored at −20°C, to be used in our study.

Specimens (MPI-1 to MPI-7) from an international study to evaluate the comparability of immunoassays for the detection of antibodies to *B. pertussis* antigens were kindly donated by Bruce Meade (FDA). Five of the seven recal-

* Corresponding author. Mailing address: Institut für Hygiene und Laboratoriumsmedizin, Klinikum Krefeld, Lutherplatz 40, 47805 Krefeld, Germany. Phone: 49-2151-322431. Fax: 49-2151-322079. E-mail: koesters_hyg@klinikum-krefeld.de.

cified plasma samples (MPI-1, -2, -4, -5, and -6) had been characterized in the past by the Laboratory of Pertussis, by our in-house ELISA, and by various other laboratories (15). For this study, IgG anti-PT and IgG anti-FHA levels were determined as the mean of the results obtained by the Laboratory of Pertussis and our laboratory. IgA anti-PT and IgA anti-FHA levels for these sera, as well as IgG and IgA anti-PT and anti-FHA for MPI-3 and MPI-7, were determined by 15-fold testing with the in-house assay only.

U.S. Reference Pertussis Antiserum lots 3 and 4 were obtained from the Laboratory of Pertussis with 200, 15, 200, and 100, U of IgG anti-PT, IgA anti-PT, IgG anti-FHA, and IgA anti-FHA, respectively, per ml for lot 3 and 393 U of IgG anti-PT and 270 U of IgG anti-FHA per ml for lot 4. No FDA reference values existed for lot 4 IgA antibodies. These levels were determined with the in-house ELISA (IgA anti-PT, 10 U/ml; IgA anti-FHA, 62 U/ml).

The in-house reference preparation lot 2 consisted of pooled recalcified plasma from healthy blood donors, adjusted to the FDA reference sera. It contained 32 U of IgG anti-PT, 5.5 U of IgA anti-PT, and 123 U of each IgG and IgA anti-FHA per ml as estimated by our ELISA.

All samples were coded so that the technicians performing the tests were blinded. The assays were evaluated independently. Before analysis of the samples they were divided into seven replicate aliquots for single day's use and stored at −20°C.

Each specimen was tested with each commercial kit on two consecutive days (one determination per day), except for the in-house reference 2 which was tested 10 times in order to assess intra-assay variation. Specimens from the vaccine trial and from the immunogenicity study, FDA reference lots 3 and 4 and the in-house reference preparation were tested only once for each antigen and isotype with the in-house ELISA, since results from earlier analyses of the same sera were available for comparison. Sufficient data existed for specimens from the international collaborative study which were not repeated with the in-house ELISA for the present study.

All tests were able to give quantitative (ELISAs 0 and 1) or semiquantitative (ELISAs 2 to 5) results.

Indeterminate results, i.e., neither positive nor negative, were regarded as indeterminate for determination of the assay reproducibility and as negative for the specimens of the immunogenicity study. In all other cases, results were not affected by the number of indeterminate results because semiquantitative or quantitative results were used for evaluation.

In-house ELISA (ELISA 0). For this study the in-house ELISA was considered the reference assay due to the amount of data accumulated with this test (27) and its validation in an international collaborative study (15). The procedure has been described elsewhere in detail (25, 27). In brief, the assay consists of absorption of PT and FHA (SB Biologicals) to separate microtiter plates, incubation of patient samples in eight dilutions with both antigens, addition of alkaline phosphatase-conjugated anti-IgG or anti-IgA (Kirkegaard and Perry), incubation with substrate, and cessation of the reaction with NaOH. The absorbance values were transformed to ELISA units per milliliter by using an internal reference (lot 2, adjusted to the FDA reference sera) for constructing the reference line. Reference preparations 3 and 4 of the FDA served as controls. The lower level of detection was arbitrarily set at 2 U/ml.

Interpretation of the assay was performed as for the vaccine trial, i.e., recent contact with *B. pertussis* antigens was assumed when there was an IgG or IgA conversion to ≥8 U of PT, FHA, or both per ml or when IgG or IgA antibodies to PT or FHA increased ≥100% to at least 8 U/ml (27).

Commercial assays. (i) ELISA 1. For the Serion ELISA classic (Institut Virion Serion GmbH, Würzburg, Germany), microtiter plates are coated with purified acellular *B. pertussis* components (FHA and PT). Two ELISAs to determine IgG and IgA antibodies were performed according to the manufacturer's instructions. Interpretation of the results was performed with the help of the Serion Easy Base 4PL-Software, and results were expressed in FDA units/milliliter, although no detailed information about the source of the reference sera was given (IgG, >30 U/ml [positive], <20 U/ml [negative], 20 to 30 U/ml [indeterminate]; IgA, >22 U/ml [positive], <15 U/ml [negative], 15 to 22 U/ml [indeterminate]).

(ii) ELISA 2. For the *B. pertussis* IgG-, IgM-, IgA-Enzym-Immunassay (Innova Labordiagnostik GmbH, Darmstadt, Germany), the antigens used to detect IgG and IgA antibodies to *B. pertussis* are not stated on the test insert. The results were determined by a mathematic conversion supplied by the manufacturer and were expressed as inU/milliliter (IgG, >18 inU/ml [positive], <14 inU/ml [negative], 14 to 18 inU/ml [indeterminate]; IgA, >26 inU/ml [positive], <19 inU/ml [negative], 19 to 26 inU/ml [indeterminate]). No information about the source of reference sera was given for this test.

(iii) ELISA 3. For the *B. pertussis* ELISA (Genzyme Virotech GmbH, Rueselsheim, Germany), the antigens absorbed to the microtiter plates are not specified. The assay was performed according to the manufacturer's instructions, and the results were converted to Virotech units (VE) (IgG and IgA, >11 VE [positive], <9 VE [negative], 9 to 11 VE [indeterminate]). No information about the source of reference sera was given for this test.

(iv) ELISA 4. For the *B. pertussis*/toxin IgA/IgG ELISA (Novum Diagnostica GmbH, Dietzenbach, Germany), the antigens used are PT and FHA. The results were transformed to Novum units (NE) as described in the test protocol. (IgG and IgA, >11 NE [positive], <9 NE [negative], 9 to 11 NE [indeterminate]). No information about the source of reference sera was given for this test.

TABLE 1. Intra-assay variation calculated for 10-fold determination of reference preparation 2

ELISA[a]	% Variation					
	IgG			IgA		
	Day 1	Day 2	Mean	Day 1	Day 2	Mean
1	25.5	21.2	23.4	13.3	20.3	16.8
2	7.5	ND[b]	7.5	14.1	17.0	15.6
3	10.4	6.2	8.3	2.1	2.7	2.4
4	8.8	13.2	11.0	21.5	35.8	28.7
5	9.1	9.3	9.2	8.3	7.3	7.8

[a] For ELISA 0, the percent variations were 7.0 (anti-PT) and 3.8 (anti-FHA) for IgG and 14.0 (anti-PT) and 8.0 (anti-FHA) for IgA.
[b] ND, not determined.

(v) ELISA 5. For the *B. pertussis* IgG/IgA ELISA Test (HRP) (Hycor Biomedical GmbH, Kassel, Germany), the antigens employed are not specified. For the determination of IgG antibodies to *B. pertussis*, the optical densities were converted to arbitrary units (AU) by an ELISA reader software and a standard curve according to the manufacturer's instructions (IgG, >40 AU [positive], <27 AU [negative], 27 to 40 AU [indeterminate]). IgA antibodies were expressed as ratios. Samples that were weakly positive according to the manufacturer were regarded as positive: >45%, positive; <35%, negative; and 35 to 45%, indeterminate. No information about the source of the reference sera was given for this test.

Statistical analyses. Data were tabulated and ranked. Data were analyzed by the linear regression method (Sigma-Plot; Jandel Scientific, Erkrath, Germany) and by ranking specimens according to antibody levels.

RESULTS

Intra-assay variation. The intra-assay variation of tests was estimated twice for each commercial assay, except for ELISA 2, IgG, by 10-fold determination of standard serum 2. For the in-house assay a variation from 3.8% (anti-FHA IgA) to 14.0% (anti-PT IgA) was found. Intra-assay variation for the commercial tests ranged from 2.4% (ELISA 3, IgA) to 28.7% (ELISA 4, IgA) (Table 1).

Reproducibility. Reproducibility of the test results on two consecutive days with respect to their interpretation, i.e., positive, negative, and indeterminate, is shown in Table 2. The number of discrepant results ranged from 1 (1.5%) (ELISA 1, IgG) to 7 (10.8%) (ELISA 2, IgG) for 65 specimens.

Indeterminate results. The number of indeterminate results ranged from 5 (7.8%) (ELISA 1) to 12 (18.5%) (ELISA 5) for 65 specimens (Table 3).

Paired serum samples from pertussis children. According to the case definition (27), all 20 paired sera showed evidence of recent contact with *B. pertussis* when analyzed with the in-house ELISA in 1998 as well as in 1995. A total of 11 samples showed a significant titer increase or seroconversion of IgG and IgA antibodies; 8 were identified due to the rise of isotype IgG, and 1 was identified due to the increase of isotype IgA only.

TABLE 2. Rate of discrepant results for 65 serum samples each assayed on two consecutive days

ELISA	Discrepant results (%)	
	IgG	IgA
1	1.5	3.1
2	10.8	7.7
3	4.6	9.2
4	9.2	4.6
5	6.2	9.2

TABLE 3. Indeterminate results for 65 specimens obtained on day 1

ELISA	No. of indeterminate results		Total (%)
	IgG	IgA	
1	2	3	7.8
2	3	7	15.4
3	5	4	13.8
4	9	2	16.9
5	6	6	18.5

TABLE 5. Detection of IgG and IgA antibody responses after vaccination with acellular pertussis vaccine

ELISA	No. of samples positive for IgG antibodies/no. of samples positive with ELISA 0	No. of samples negative for IgA antibodies/no. of samples negative with ELISA 0
1	15/15	15/15
2	15/15	15/15
3	15/15	15/15
4	14/14[a]	15/15
5	11/14[a]	15/15

[a] One sample was not evaluable due to discrepant results on duplicate testing.

None of the five commercial assays gave any rules as to how to interpret the results obtained. Thus, we applied the following criteria: recent contact with *B. pertussis* antigens was assumed when levels of at least one antibody isotype (IgG and/or IgA) increased ≥100% to a level above the cutoff value or when there was an IgG and/or IgA seroconversion from negative to positive. An increase of ≥100% but not to a level above the cutoff value or seroconversion from indeterminate to positive without a ≥100% increase in antibody levels was regarded as no evidence of recent contact with *B. pertussis* antigens. The results are presented in Table 4. Overall sensitivity for the five commercial assays ranged from 60 to 95%.

The most sensitive method for the detection of recent contact with pertussis in patients presenting with cough ≥21 days duration was ELISA 1. Of 20 serum pairs, 19 were correctly identified as showing evidence of recent contact to *B. pertussis* on both days: 11 serum pairs were regarded as positive because of a significant rise in the titer of IgG antibodies only, 7 were regarded as positive on day 1 (8 on day 2) due to a combined rise of IgG and IgA, and 1 (0 on day 2) serum pair was regarded as positive due to a rise in isotype IgA only (Table 4). ELISA 1 was unable to detect a significant rise in titer of the same paired specimen on both days. This sample pair showed seroconversion for IgA anti-PT and anti-FHA only with the in-house ELISA.

ELISA 2 was able to detect 19 of 20 significant rises in titer on day 1 and 18 on day 2. Most serum pairs showed a significant rise in IgG isotype only, whereas five pairs on day 1 and four on day 2 were diagnosed by a titer rise for IgA and IgG.

ELISA 3 judged only 16 pairs—the same pairs on duplicate testing—as positive for contact with *B. pertussis* antigens.

Only 12 pairs were identified as having had recent contact with *B. pertussis* antigens using ELISA 4. On the second day two other serum pairs were classified as positive for contact with pertussis than on the first day.

Seventeen serum pairs on the first day were shown to be positive for recent contact with *B. pertussis* antigens when assayed with ELISA 5. On the second day two sera which showed a significant increase of IgG antibodies only on the first run gave no evidence of recent contact with *B. pertussis*.

Table 4 also indicates that ELISAs 4 and 5 had more problems in diagnosing titer increases than seroconversions.

Samples from an immunogenicity study. Sensitivity for detecting IgG antibodies to *B. pertussis* after three injections of an acellular three-component pertussis vaccine ranged from 79% (ELISA 5) to 100% (ELISAs 1, 2, 3, and 4) (Table 5). With both ELISAs 4 and 5, one serum was excluded from evaluation because it showed discrepant results on the two occasions it was tested. One equivocal sample (ELISA 5, IgG) was regarded as negative. None of the assays detected IgA antibodies in these samples.

Serum samples from an interlaboratory study, FDA references 3 and 4. For each ELISA the antibody levels (IgG and IgA) were plotted against IgG anti-PT and anti-FHA or IgA anti-PT and anti-FHA, respectively, as determined by the FDA and the in-house ELISA. Regression coefficients ranged between 0.87 (ELISA 1, IgG versus IgG-PT) and 0.02 (ELISA 5, IgA versus IgA-anti-PT) with a median of 0.21 (mean, 0.33), indicating that no correlation was found between the different commercial ELISAs measuring IgA or IgG to *B. pertussis* antigens and the reference values obtained by ELISA 0, which distinguished between antibodies to PT and FHA.

In a second step, sera were ranked according to their antibody level. Results for IgG assays were compared to the IgG anti-PT and IgG anti-FHA levels established as above. Table 6 shows that ELISAs 1, 2, and 4 rank the sera according to their IgG anti-*B. pertussis* antigen content relatively homogeneously, and the results were comparable to the ranking obtained by measuring IgG anti-PT; ELISAs 3 and 5, however, ranked the sera in a different order. Table 7 displays the same ranking now compared with the IgG anti-FHA content. Here, all commercial ELISAs display a different ranking compared to ELISA 0 and the Laboratory of Pertussis. Similar results were obtained

TABLE 4. Sensitivity for detection of serologic evidence of contact to *B. pertussis* antigens in 20 serum pairs

ELISA	No. of serum pairs with serologic evidence of contact with *B. pertussis* antigens (n = 20)	Sensitivity (%)	No. of positive serum pairs on day 2/total no. positive serum pairs[a]	Sensitivity (%)	No. of positive serum pairs on day 2/total no. positive serum pairs[b]	Sensitivity (%)
1	19	95	13/14	95	6/6	100
2	19, 18[c]	95, 90[c]	12/14	86	6/6	100
3	16	80	11/14	79	5/6	83
4	12	60	11/14	79	1/6	17
5	17, 15[c]	85, 75[c]	12/14	86	3/6	50

[a] As defined by seroconversion with ELISA 0 (n = 14) as determined on day 2.
[b] As defined by titer increase with ELISA 0 (n = 6) as determined on day 2.
[c] On days 1 and 2, respectively.

TABLE 6. Ranking of serum samples according to IgG level (1 = low, 9 = high) with the mean of IgG anti-PT levels established by the Laboratory of Pertussis and the in-house ELISA as a reference

ELISA	Ranking								
	MPI-1	MPI-5	MPI-4	MPI-2	MPI-6	ref3	MPI-3	ref4	MPI-7
In-house–FDA IgG anti-PT	1	2	3	4	5	6	7	8	9
1	1	2	3	6	7	4	8	5	9
2	1	2	4	6	7	3	9	5	8
3	1	2	6	8	7	3	5	4	9
4	1	2	3	4	7	5	5	8	9
5	2	6	9	4	3	1	8	5	7

when IgA anti-PT and IgA anti-FHA were ranked according to the results of ELISA 0.

DISCUSSION

In recent years, several vaccine efficacy trials and one immunogenicity study with acellular pertussis vaccines were performed (4, 9, 10, 13, 18, 20). As a by-result from these studies, serological tests measuring antibodies to *Bordetella* antigens have been well standardized under the conditions of controlled clinical trials, and a comparison among various laboratories that did these tests has helped to set performance criteria for ELISAs measuring antibodies to pertussis antigens (15).

In this study we have tried to apply these criteria to commercially available test kits, and we have compared five ELISAs for the detection of antibodies to *Bordetella* antigens with the help of several serum panels characterized by the Laboratory of Pertussis and/or our in-house ELISA.

Day to day reproducibility in respect to interpretation was acceptable with discrepant results ranging between 1.5 and 10.7%. Intra-assay variation was acceptable, i.e., <10% for ELISAs 3 and 5 only. Since all of the assays were performed by the same technician, thereby reducing technologist's influence on precision, intra-assay coefficients of variation are a good reflection of assay precision. Because the protocols for the commercial assays are very similar, variability may be antigen dependent as suspected by Lynn et al. (15) for ELISAs being compared in an international collaborative study.

The number of indeterminate results is important when comparing the suitability of different ELISA kits for laboratory routine. All kits suggest repeat analysis of an equivocal serum together with a second serum sample after 1 to 2 weeks. This makes assays with a significant number of indeterminate result less efficient and impedes final results.

TABLE 7. Ranking of serum samples according to IgG level (1 = low, 9 = high) with the mean of IgG anti-FHA levels established by the Laboratory of Pertussis and the in-house ELISA as a reference

ELISA	Ranking								
	MPI-1	MPI-5	MPI-3	MPI-7	MPI-6	MPI-4	MPI-2	ref3	ref4
In-house–FDA IgG anti-FHA	1	2	3	4	5	6	7	8	9
1	1	2	8	9	7	3	6	4	5
2	1	2	9	8	7	4	6	3	5
3	1	2	5	9	7	6	8	3	4
4	1	2	5	9	7	3	4	5	8
5	2	6	8	7	3	9	4	1	5

The sensitivity and specificity for detecting IgG and IgA antibodies, respectively, after immunization was acceptable. No IgA antibodies were detected with any of the five commercial ELISAs after immunization, thus confirming results with the reference ELISA and supporting the findings that IgA antibodies to *B. pertussis* antigens are evidence of natural infection and not immunization (2). All commercial ELISAs except ELISA 5 were able to detect an IgG antibody response after immunization with DTaP in all 15 serum samples tested. Pending confirmation with larger sample numbers, commercial ELISAs may be a useful tool for immunogenicity studies.

Regarding the sensitivity for detection of IgA, it was noted that ELISA 4 found only one specimen of all 65 samples (1.5%) to contain IgA antibodies, whereas all other commercial assays showed that 23 to 34% of the specimens were IgA positive.

None of the kits was able to identify all cases of pertussis in paired serum samples correctly. This result was based on the assumption that a significant increase in specific IgG or IgA correlated with infection. Sensitivity ranged between 60 and 95%. The lower figure is unacceptable, assuming that the main application of the ELISAs is to play an important role in the diagnosis of pertussis.

In recent years the diagnosis of pertussis based on a single serum sample has gained increasing importance (25). A prerequisite for this kind of testing is a reliable semiquantitative or quantitative method for detecting antibody levels and comparison with an age-matched reference group. So far, the assays we tested showed discrepant results when serum samples were ranked according to antibody level, making diagnosis from just one serum sample dependent on the ELISA used and not on the amount of antibodies to purified antigens.

None of the commercial assay ranked the nine specimens in the same order as the reference ELISAs, although some kits distinguished correctly between specimens with high and low levels of antibodies. It has to be considered though that the results of the commercial assays were compared to a ranking obtained by measuring antibody levels to FHA and PT independently. These results, however, suggest that some kits (ELISAs 1, 2, and 4) preferentially measured anti-PT and none preferentially measured anti-FHA, while ELISAs 3 and 5 seemed to employ a different antigen composition.

None of the commercial assays distinguished between antibodies to different antigens of bordetellae. However, all clinical trials and many other published studies have used ELISAs which could distinguish between antibodies to PT and FHA (7, 22, 25, 31). Thus, it is difficult if not impossible to extrapolate from their results and conclusions on the interpretation of the assays investigated in this study.

Overall, our results show that the commercial ELISAs tested here still need further improvement and efforts should be made to standardize performance, antigen contents, and reference material in order to guarantee accuracy of diagnosis and interlaboratory comparability for serodiagnosis of *Bordetella* infections.

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