Comparison of Western Immunoblotting to an Enzyme-Linked Immunosorbent Assay for the Determination of Anti-Bordetella pertussis Antibodies

Stephen D. Merrigan,1* Ryan J. Welch,1 and Christine M. Litwin1,2

Associated Regional and University Pathologists (ARUP) Institute for Clinical and Experimental Pathology,1 and Department of Pathology, University of Utah,2 Salt Lake City, Utah

Received 15 October 2010/Returned for modification 13 November 2010/Accepted 27 January 2011

During Bordetella pertussis infection, it has been established that an increase of anti-pertussis toxin (PT) and anti-filamentous hemagglutinin (FHA) antibodies occurs. Immunoblots from two manufacturers using FHA and PT antigens were compared with an enzyme-linked immunosorbent assay (ELISA) that used both FHA and PT. One manufacturer used two concentrations of PT bands for the IgG immunoblot, calibrated to the World Health Organization standard for PT in international units (IU/ml), 100 IU/ml (PT-100) and 8 IU/ml (PT). The second immunoblot kit measured antibodies to a single calibrated PT band. Both kits measured IgM antibodies, and one additionally measured IgM antibodies. Two of 41 (5%) ELISA IgM positives were confirmed positive by IgM immunoblotting, suggesting poor specificity of the IgM ELISA. The agreements of the IgG and IgA immunoblots with the ELISA ranged from 72.5% to 85.3%, with only 38 to 51% of IgA positives confirmed by immunoblotting and only 61 to 68% of IgG positives confirmed by immunoblotting. The two immunoblots correlated well with each other, with 91.7% and 94.3% agreement for IgG and IgA, respectively. When the FHA band was used with the PT band as the criterion for positivity, significant differences existed in specificity compared to the ELISA (IgG, 84.1% versus 33.3%; IgA, 82.4% versus 71.0%). When the positive IgA immunoblots (evidence of natural recent infection) were compared to the positive PT-100 IgG immunoblots (evidence of recent infection or vaccination), the PT-100 blot showed a 71% sensitivity in detecting natural recent infection. B. pertussis immunoblots, alone or in combination with ELISAs, can aid in the diagnosis of B. pertussis infection.

Bordetella pertussis is a slow-growing Gram-negative coccobacillus, the causative agent of pertussis (whooping cough) and a highly transmissible respiratory infection of the tracheal epithelial cilia (6, 13, 20). Symptoms in unvaccinated persons include fits of coughing, inspiratory whoop, and posttussive vomiting; however, individuals with partial immunity have milder, cold-like symptoms and a chronic cough (5, 13, 19, 20). After the introduction of the pertussis vaccine in the 1940s, the number of cases reached a low in the United States in the late 1970s and early 1980s, with a reported 0.5 to 1.0 cases per 100,000 persons (20). However, the number of reported cases has fluctuated, with a peak incidence of 8.72 per 100,000 persons when 25,616 cases occurred in 2005, according to the CDC (24).

A nasopharyngeal swab culture is considered the “gold standard” for the diagnosis of pertussis infection; however, many factors affect culture results, such as specimen transport and swab material (14). Numerous studies, however, have established that there is an increase in anti-pertussis toxin (PT) and anti-filamentous hemagglutinin (FHA) serum antibodies during Bordetella infection and that the measurement of these antibodies could assist in the laboratory confirmation of pertussis (7, 10–12). Since PT is produced only by B. pertussis, anti-PT antibodies are highly specific for B. pertussis infection (18, 20). Anti-FHA antibodies are less specific than PT antibodies and may be produced following infection with Mycoplasma pneumoniae, Chlamydia pneumoniae, nonencapsulated Haemophilus influenzae, or other Bordetella species, such as B. parapertussis and B. bronchiseptica (7, 16, 18). Adenylate cyclase toxin (ACT) antibodies have also been proposed to be a sensitive marker for Bordetella sp. infection (27). The detection of IgA antibodies against PT and FHA suggests natural infection not immunization, since IgA is rarely if at all detected following immunization (17, 21). However, the detection of IgG to PT and FHA is more sensitive than that of IgA, since not all individuals mount a detectable IgA response following infection (22). An IgM response is only rarely and inconsistently seen following exposure or immunization (25). Additionally, since both PT and FHA are included in the acellular pertussis vaccines, antibodies to these antigens often persist following vaccination, hindering serodiagnosis in vaccinated individuals. Therefore, standardization of the diagnostic threshold would be important for the detection of active infection.

The World Health Organization (WHO) accepts significant increases in IgG or IgA antibodies to PT or FHA as an indication of pertussis, even though other Bordetella species produce FHA (14, 20). The WHO human pertussis antiserum reference reagent became available in January 2009, and an international collaborative study established that 1 ELISA unit (EU) of IgG anti-PT is approximately equal to 1 international unit (IU) of IgG anti-PT (28). The criteria, however, for de-
fining the diagnostic threshold have not been established by international and national health organizations. Baughman et al. used mixture modeling of data from a population of more than 6,000 U.S. residents of ages 6 to 49 years to establish an anti-PT IgG level of >94 EU/ml as the diagnostic cutoff for recent infection, with a lower value of >49 EU/ml as an intermediate cutoff (3, 4). Other diagnostic thresholds have been developed by others, including the Massachusetts State Laboratory, which uses a cutoff value of 200 EU/ml, and the Netherlands, which uses a value of 125 EU/ml (9, 18).

Multiple indirect methods for serological detection of antibodies to B. pertussis have been manufactured, the most common being ELISA (13–15, 23). Immunoblot assays may prove to be a useful tool for distinguishing between B. pertussis infections and infections from other Bordetella species (25). In this study we analyzed the results for 250 patient samples submitted for IgG, IgA, and IgM B. pertussis antibody testing using two commercially available immunoblot assays that use different combinations of PT, FHA, and ACT antigens. The goal of this study was to assess the utility of immunoblot assays for anti-B. pertussis antibody testing using the ELISA method as the reference standard in the absence of culture results.

MATERIALS AND METHODS

Serum collection. A total of 250 sequential human serum samples sent to ARUP Laboratories for Bordetella pertussis antibody testing were collected and deidentified. The mean (± standard deviation [SD]) age of the patients used in the study was 44.0 ± 30.2 years, and 62% of the patients were female. The age range was from less than 1 to 96 years of age. The procedures were followed in accordance with the ethical standards established by the University of Utah and are in accordance with the Helsinki Declaration of 1975. All patient samples included in this study were deidentified according to a University of Utah Institutional Review Board-approved protocol to meet the Health Information Portability and Accountability Act (HIPPA) patient confidentiality guidelines. Specimens were stored at −20°C until testing commenced and were then stored at 2 to 8°C while all evaluations were performed.

ELISA testing. All 250 samples were tested for three classes of antibodies to Bordetella pertussis with the Savanon SeroPertussis Bordetella pertussis enzyme-linked immunosorbent assay (ELISA) test kit (Ashdod, Israel). Testing for IgG, IgA, and IgM B. pertussis antibodies was conducted as specified by the manufacturer’s protocol.

In brief, the patient serum was diluted 1/100, and 50 μl of diluted specimen, cutoff control, negative control, and positive control were added to wells coated with a combination of specific immunodominant B. pertussis proteins, including PT and FHA. The plate was then covered and incubated for 1 h at 37°C in a humid chamber. Following 3 washes, 50 μl of 1/300-diluted class-specific horseradish peroxidase (HRP) conjugate was added to each well, and the plate was covered and incubated for 1 h at 37°C in a humid chamber. Following another 3 washes, 100 μl of tetramethylbenzidine (TMB) substrate was added to each well. The plate was then covered to incubate at room temperature (RT) for 15 min. One hundred microliters of stop solution was then added to each well, and the plate was read at an absorbance of 450 nm with a background filter of 620 nm. Samples were considered ELISA positive if the index value (IV) was >2.4 for IgG and >1.1 for IgA and IgM. Analysis of IgM-positive samples was repeated for confirmation.

Immunoblot testing. The positive and negative ELISA samples were tested with two immunoblot assays: the Viramed Bordetella pertussis ViraStrip test kit, IgG, IgA, and IgM (Viralab Inc., Oceanside, CA), and the Virotech B. pertussis CatACT+LINE immunoablot. IgG and IgA (Genzyme Virotech GmbH, Rüsselsheim, Germany). Both the Viramed test kit and the Virotest kit contain the PT and FHA bands. The IgG Viramed immunoblot differs from the Viramed IgA and IgM immunoblot and the Virotech IgG and IgA immunoblot in that it contains two PT bands calibrated with the WHO standards for PT in IU/ml. The PT-100 IgG and PT bands correlate with 100 IU/ml and 8 IU/ml, respectively. These two calibrated bands allow the differentiation of high (acute infection or recent vaccination, <36 months) and low (past infection or vaccination) specific PT IgG antibody levels. The Virotech immunoblot differs from the Viramed immunoblot in that it contains a third antigen, CatACT, which contains a catalytic domain of the adenylate cyclase toxin specific for Bordetella. Equivocal ELISA results were not analyzed by immunoblotting.

For the Viramed IgG, IgA, and IgM assays, the manufacturer’s protocol was followed. In brief, the strips are placed in individual channels in trays and soaked in 1.5 ml wash buffer for 5 min. Next, 20 μl of patient serum or 100 μl of control is added to the 1.5 ml buffer and incubated for 30 min on a platform rocker at RT. The liquid is decanted from each well and washed with 1.5 ml of buffer during rocking for 5 min, three times. Following washing, 1.5 ml of class-specific goat anti-human alkaline phosphatase conjugate is added and incubated for 15 min on the platform rocker at RT. The strips are washed another three times with wash buffer and once with distilled water before 1.5 ml of 5-bromo-4-chloro-3-indolyphosphate (BCIP) nitroblue tetrazolium (NBT) substrate is added and allowed to develop for 1 h at 37°C in a humid chamber. The liquid was decanted from each well and washed with 1.5 ml of buffer while rocking for 5 min, three times. Following washing, 1.5 ml of class-specific anti-human conjugate was added and incubated for 30 min on the platform rocker at RT. The strips were washed an additional three times with wash buffer and once with distilled water before 1.5 ml of chromogen substrate was added and incubated for 10 ± 3 min until the control band was distinctly visible. The reaction was then stopped by washing three times with distilled water. Samples were considered to be positive for specific B. pertussis antibodies for the IgA and IgM immunoblot if the PT band had a stronger intensity than the cutoff control band and were considered equivocal if the PT band was of an intensity equal to that of the cutoff band. Samples were considered positive for Bordetella spp. if the PT band was negative but the FHA band had a stronger intensity than the cutoff control band and equivocal if the intensity was equal to that of the control band. Samples were considered negative if the intensity of the band was weaker than that of the cutoff control band.

For the Viramed IgG immunoblot, samples were considered to be positive for past infection or vaccination if only the PT band corresponding to 8 IU/ml was positive and were considered positive for acute infection or recent vaccination if the PT-100 band was positive. Samples were considered equivocal for past infection or immunization if the PT band was of an intensity equal to that of the cutoff band or equivocal for acute infection or recent immunization if the PT-100 band had an intensity equal to that of the cutoff band. Samples were considered positive for Bordetella species if both the PT-100 and PT bands were negative but the FHA band had a stronger intensity than the cutoff control band and equivocal if the intensity was equal to that of the cutoff band.

For the Virotech IgG and IgA assay, the manufacturer’s protocol was followed. In brief, the strips were placed in individual channels in the trays and soaked in 1.5 ml wash buffer for 5 min. Next, 15 μl of patient serum or 100 μl of control was added to the 1.5 ml buffer and incubated for 30 min on a platform rocker at RT. The liquid was decanted from each well and washed with 1.5 ml of buffer while rocking for 5 min, three times. Following washing, 1.5 ml of class-specific anti-human conjugate was added and incubated for 30 min on the platform rocker at RT. The strips were washed an additional three times with wash buffer and once with distilled water before 1.5 ml of chromogen substrate was added and incubated for 10 ± 3 min until the control band was distinctly visible. The reaction was then stopped by washing three times with distilled water. Samples were considered to be positive for specific B. pertussis antibodies for the IgA and IgG immunoblot if the PT band had a stronger intensity than the cutoff control band and were considered equivocal if the PT band had an intensity equal to that of the cutoff band. The manufacturer does not give clear guidelines for the use of FHA in the interpretation of Bordetella pertussis or Bordetella species infection. The CatACT band was also not used in the determination of positive and negative results; however, previous studies have shown the utility of this marker, and therefore CatACT band data were analyzed.

Statistical analysis. Two-by-two contingency table analysis with a Yates-corrected Chi-squared test was used to determine the overall agreement, clinical sensitivity, clinical specificity, and 95% confidence intervals (CI) for sensitivity and specificity (26). Equivocal results for the ELISAs and immunoblots were excluded from all agreement, sensitivity, and specificity calculations.

RESULTS

Immunoblot and ELISAs for Bordetella pertussis antibody detection. Of the 250 sequential serum samples sent to ARUP Laboratories for Bordetella pertussis antibody testing by ELISA, all 250 were tested in immunoblot assays from two different manufacturers. A total of 115 (46.0%), 69 (27.6%), and 66 (26.4%) samples were IgG ELISA positive, negative, and equivocal, respectively. Also, 45 (18%), 193 (77.2%), and 12 (4.8%) samples were IgA ELISA positive, negative, and equivocal, respectively. For the IgM ELISA, 41 (16.4%), 201 (80.4%), and 8 (3.2%) samples were positive, negative, and
equivocal, respectively. Equivocal ELISA results were not analyzed by immunoblotting.

The Virotech immunoblot assay tests two classes of antibodies (IgG and IgA) for a response to the FHA, CatACT, and PT antigens, and the Viramed immunoblot assay tests three classes of antibodies (IgG, IgA, and IgM) for a response to the FHA and PT antigens, with an additional PT-100 antigen band on the IgG immunoblot to detect higher levels of antibodies (100 IU/ml).

Comparison of Viramed IgG immunoblot kit with the Savyon IgG ELISA. Of 115 IgG ELISA positives, 59 were confirmed to be positive, 16 were equivocal, and one sample showed nonspecific staining on the Viramed IgG immunoblot when the PT-100 band was used as the sole criterion for positivity (see Table 2). Five samples were positive by immunoblot analysis of the 69 that were negative by ELISA (see Table 2), and 6 were equivocal, with 1 sample showing nonspecific staining. Agreement, sensitivity, and specificity, calculated by comparing the Viramed IgG immunoblot PT-100 band to the Savyon IgG ELISA and excluding equivocal results, were 75.2%, 68.0% (62.7–71.8%), and 91.9% (84.1–96.4%), respectively (Table 1). No FHA IgM-positive bands were detected on any of the samples.

Comparison of Viramed IgA immunoblot kit with the Savyon IgA ELISA. When the PT band (noncalibrated to 100 IU/ml (acute infection or recent vaccination)).

Comparison of Virotech IgG immunoblot kit with the Savyon IgG ELISA. Using the PT band as the sole criterion for positivity, only 2 samples of 41 IgM ELISA positives were confirmed to be positive on the IgM immunoblot, and none were equivocal. One sample of 201 IgM-negative ELISA samples was positive by IgM immunoblotting, with 1 being equivocal. Agreement, sensitivity, and specificity, calculated by comparing the Viramed IgA immunoblot PT band to the Savyon IgA ELISA and excluding equivocal results, were 83.4%, 4.9% (95% CI, 1.5 to 6.9%), and 99.5% (95% CI, 98.8 to 99.9%), respectively (Table 1). No FHA IgM-positive bands were detected on any of the samples.

Comparison of Virotech IgG immunoblot kit with the Savyon IgG ELISA. With the Virotech IgG immunoblot assay, using only the PT band (calibrated) as the sole criterion for positivity, only 66 samples of 115 IgG ELISA positives were confirmed to be positive (Table 2), with 18 equivocal samples. Of the 69 samples that were negative by ELISA IgG, 8 were positive and 9 were equivocal by immunoblotting (Table 2). Agreement, sensitivity, and specificity, calculated by comparing the Virotech IgG immunoblot PT band to the Savyon IgG ELISA and excluding equivocal results, were 75.2%, 68.0% (95% CI, 62.7 to 71.8%), and 86.7% (95% CI, 78.0 to 92.7%), respectively (Table 1). Using the PT band as the sole criterion for positivity, the sensitivity and specificity of the Virotech IgG immunoblot did not statistically differ from those of the Viramed IgG immunoblot.

When either the PT band or the FHA band was used as the criterion for positivity, instead of exclusively the PT band, there was an increase in sensitivity and a slight decrease in specificity (though this was not statistically different from when the PT band was used alone). This, however, differs from the Viramed IgG immunoblot, where the addition of the FHA band in the positivity criteria decreased the specificity to a very low 33.3%. When the PT, FHA, or ACT band was used as the criterion for positivity, only 66 samples of 115 IgG ELISA positives were confirmed to be positive (Table 2), with 18 equivocal samples. Of the 69 samples that were negative by ELISA IgG, 8 were positive and 9 were equivocal by immunoblotting (Table 2). Agreement, sensitivity, and specificity, calculated by comparing the Virotech IgG immunoblot PT band to the Savyon IgG ELISA and excluding equivocal results, were 75.2%, 68.0% (95% CI, 62.7 to 71.8%), and 86.7% (95% CI, 78.0 to 92.7%), respectively (Table 1). Using the PT band as the sole criterion for positivity, the sensitivity and specificity of the Virotech IgG immunoblot did not statistically differ from those of the Viramed IgG immunoblot.

When either the PT band or the FHA band was used as the criterion for positivity, instead of exclusively the PT band, there was an increase in sensitivity and a slight decrease in specificity (though this was not statistically different from when the PT band was used alone). This, however, differs from the Viramed IgG immunoblot, where the addition of the FHA band in the positivity criteria decreased the specificity to a very low 33.3%. When the PT, FHA, or ACT band was used as the

### Table 1. Agreement, sensitivity, and specificity of immunoblots compared to ELISA

| Immunoblot            | Band criterion for positivity | Isotype | % agreement | % sensitivity (95% CI) | % specificity (95% CI) |
|-----------------------|-------------------------------|---------|-------------|------------------------|------------------------|
| Viramed               | PT-100                        | IgG     | 72.5        | 60.6 (55.2–63.0)       | 91.9 (84.1–96.4)       |
| Virotech             | PT                            | IgG     | 75.2        | 68.0 (62.7–71.8)       | 86.7 (78.0–92.7)       |
| Viramed             | PT-8                          | IgA     | 75.2        | 98.1 (94.7–99.5)       | 31.6 (25.1–34.1)       |
| Virotech             | PT                            | IgA     | 84.3        | 51.4 (38.5–63.0)       | 91.1 (88.5–93.5)       |
| Virotech             | PT                            | IgM     | 85.3        | 38.2 (26.1–49.5)       | 94.0 (91.7–96.1)       |
| Viramed             | PT                            | IgM     | 83.4        | 4.9 (1.5–6.9)          | 99.5 (98.8–99.9)       |
| Viramed (Viramed), PT (Virotech), or FHA | PT-100 (Viramed) | IgG     | 74.5        | 99.1 (96.0–99.8)       | 33.3 (28.1–34.5)       |
| Virotech             | PT or FHA                     | IgA     | 79.3        | 76.5 (71.6–80.2)       | 84.1 (75.9–90.1)       |
| Virotech             | PT, FHA, or ACT               | IgG     | 79.4        | 75.6 (62.9–85.2)       | 82.4 (79.5–84.9)       |
| Virotech             | PT, FHA, or ACT               | IgA     | 79.3        | 66.7 (54.2–77.3)       | 66.7 (58.1–74.0)       |
| Virotech             | PT, FHA, or ACT               | IgG     | 76.6        | 82.6 (77.5–87.0)       | 66.7 (58.1–74.0)       |
| Virotech             | PT, FHA, or ACT               | IgA     | 68.3        | 68.9 (55.9–79.7)       | 67.9 (64.9–70.4)       |

* PT band calibrated to detect 100 IU/ml (acute infection or recent vaccination).
* PT band calibrated to detect acute infection or recent vaccination, IU/ml not specified.
* Virotech value not significantly different from Viramed value.
* PT band calibrated to detect 8 IU/ml (past exposure or vaccination).
* Virotech value significantly different from Viramed value.
TABLE 2. Comparison of IgG Western immunoblot results to IgG ELISA results

| Immunoblot          | No. of samples confirmed positive by immunoblotting after Savyon Bordetella pertussis IgG ELISA result (n)  \\
|---------------------|---------------------------------------------------------------------------------------------------------|
|                     | Positive (115)                                      | Negative (69)                                      |
| Viramed IgG blot    | 59 (17)                                               | 5 (7)                                              |
| PT-100              | 106 (7)                                               | 39 (12)                                            |
| FHA                 | 114 (1)                                               | 46 (14)                                            |
| FHA alone b         | 3 (0)                                                 | 9 (3)                                              |
| No bands present    | 0 (NA a)                                              | 7 (NA)                                             |
| Virotech IgG blot   | 66 (18)                                               | 8 (9)                                              |
| PT                  | 72 (14)                                               | 5 (9)                                              |
| FHA                 | 8 (2)                                                 | 1 (3)                                              |
| FHA alone b         | 45 (11)                                               | 13 (10)                                            |
| CatACT              | 4 (0)                                                 | 8 (5)                                              |
| No bands present    | 11 (NA)                                               | 35 (NA)                                            |

a n, no. of samples with Savyon Bordetella pertussis IgG ELISA result. Parenthetical numbers are numbers of immunoblot results that were equivocal or had nonspecific staining for the indicated band.

b FHA antigen band positive; all other antigen bands negative.

c CatACT antigen band positive; all other antigen bands negative.

TABLE 3. Comparison of IgA Western immunoblot results and IgA ELISA results

| Immunoblot          | No. of samples confirmed positive by immunoblotting after Savyon Bordetella pertussis IgA ELISA result (n)  \\
|---------------------|---------------------------------------------------------------------------------------------------------|
|                     | Positive (45)                                      | Negative (193)                                     |
| Viramed IgA blot    | 19 (8)                                               | 16 (13)                                            |
| PT                  | 33 (2)                                               | 49 (32)                                            |
| FHA                 | 9 (0)                                                 | 35 (25)                                            |
| FHA alone b         | 9 (NA a)                                              | 104 (NA)                                           |
| No bands present    | 9 (NA a)                                              | 104 (NA)                                           |
| Virotech IgA blot   | 13 (11)                                               | 11 (10)                                            |
| PT                  | 29 (2)                                               | 29 (16)                                            |
| FHA                 | 8 (0)                                                 | 13 (8)                                             |
| FHA alone b         | 12 (5)                                               | 37 (13)                                            |
| CatACT present      | 0 (1)                                                 | 24 (7)                                             |
| CatACT alone c      | 10 (NA)                                               | 116 (NA)                                           |

a n, no. of samples with Savyon Bordetella pertussis IgA ELISA result. Parenthetical numbers are numbers of immunoblot results that were equivocal or had nonspecific staining for the indicated band.

b FHA antigen band positive; all other antigen bands negative.

c CatACT antigen band positive; all other antigen bands negative.

d NA, not applicable.

Comparison of Virotech IgA immunoblot kit with the Savyon IgA ELISA. With the Virotech IgA immunoblot assay, using only the PT band as the sole criterion for positivity, only 13 samples of 45 IgA ELISA positives were confirmed to be positive (Table 3), with 10 equivocal samples and 1 sample with nonspecific staining. Of the 193 ELISA IgA negatives, 11 were positive by the Virotech immunoblot and 9 were equivocal, with 1 sample showing nonspecific staining (Table 3). Agreement, sensitivity, and specificity, calculated by comparing the Virotech IgA immunoblot PT band to the Savyon IgA ELISA and excluding equivocal results, were 81.0%, 83.2% (95% CI, 61.6 to 95.5%), and 94.0% (95% CI, 91.7 to 96.1%), respectively (Table 1). Using the PT band as the sole criterion for positivity, the sensitivity and specificity of the Virotech IgA immunoblot were not statistically different from those of the Viramed IgA immunoblot.

If either the PT or the FHA band of the Virotech IgA immunoblot assay was used as the criterion for positivity, the sensitivity significantly increased to 66.7%, but the specificity significantly decreased to 82.4%. Using the PT or FHA band as the sole criterion for positivity, the Virotech IgA immunoblot sensitivity was not significantly different from that of the Viramed IgA immunoblot; however, the specificity of the Virotech IgA immunoblot was significantly higher. When using the ACT, PT, or FHA band for the criterion for positivity for the Virotech IgA immunoblot, the specificity remained the same since there were no instances in the IgA-positive ELISA samples where the ACT band was present alone on the immunoblot. However, there was a significant decrease in specificity, to 67.9% (Table 1).

Comparison of two immunoblot assays for Bordetella pertussis antibody detection. When the PT-100 band of the Viramed immunoblot assay was compared with the PT band of the Virotech immunoblot assay, the IgG agreement was 91.7% (Table 4). The sensitivities and specificities, compared with the ELISA, of the two IgG blots were not significantly different from each other. However, the two blots differed greatly in sensitivity and specificity if either the PT or FHA band was used for the criterion for positivity.

Comparing the PT band on the Viramed IgA immunoblot with the PT band on the Virotech immunoblot showed an agreement of 94.3%. The sensitivities and specificities, compared with the ELISA, of the two IgA blots were not significantly different from each other, using positive PT bands as the criteria for positivity. The Virotech IgA immunoblot had a significantly higher specificity than the Viramed IgA immunoblot when the PT or FHA bands were used as the criteria for positivity.
Comparison of IgA immunoblot positivity with IgG Immunoblot PT or PT-100 positivity. Since an IgA-positive immunoblot with the PT band suggests recent natural infection, we compared the results of the Viramed IgA immunoblot with the results of the Viramed IgG immunoblot using the PT-100 band as the criterion for positivity. The PT-100 band on the Viramed IgG immunoblot was positive in 64% of the IgA immunoblots positive for PT. The same comparison was conducted for the Virotech assay, and the IgG PT band was positive in 79% of the IgA immunoblots positive for PT.

DISCUSSION

The availability of sensitive and specific methods to diagnose pertussis infections is quite limited. Two new pertussis immunoblots, which have been calibrated using the WHO human pertussis antiserum reference reagent, are commercially available for diagnostic use. In this study, we compared the Viramed immunoblot with the Virotech immunoblot using an ELISA as a reference method in the absence of patient histories or culture results; however, it has been established that a rise in anti-\textit{B. pertussis} antibodies against PT and FHA is a clear indication of either active disease or vaccination (7, 10–12).

The comparison between the Viramed immunoblot and the Virotech was complicated by the fact that the immunoblots differed with regard to the concentration of antigens used and types of antigens. The Viramed IgG immunoblot uses two PT bands, one calibrated to 100 IU/ml (PT-100) and a lower-concentration PT band (8 IU/ml), along with an FHA band. The Viramed IgA and IgM immunoblot use a single PT band along with an FHA band. The Virotech IgG immunoblot uses one PT band, calibrated to detect acute infection (though the concentration is not listed), along with an FHA band and a CatACT band.

When the IgG assay results were compared, the agreement was 91.7% when the PT-100 band was used for the Viramed assay and the PT band was used for the Virotech assay. This supports the claim that the Virotech single PT band is calibrated similarly to the Viramed immunoblot. A possible advantage of the addition of the lower-concentration PT band on the Viramed immunoblot is the ability to detect low-level antibodies in individuals either vaccinated in the past or having had past infection.

However, when the criteria were changed to include a positive band for either PT (or PT-100 in the case of Viramed) or FHA, the Viramed IgG and IgA immunoblots had significantly lower specificities than the Virotech immunoblots, suggesting a qualitative difference in the concentration of the FHA antigen between the two commercial immunoblots.

If results of the \textit{B. pertussis} ELISAs for IgM, IgA, and IgG are compared to the Western immunoblot results using the immunoblots as the gold standard, the specificities of the ELISAs are poor. Only 5%, 38 to 51%, and 66 to 68% of ELISA positives were confirmed by IgM, IgA, and IgG immunoblots, respectively. The low confirmation rate for the IgM ELISA confirms findings in prior studies that an IgM response is only rarely and inconsistently seen following exposure or immunization (8). The confirmation rates for the IgA and IgG ELISAs were also low, suggesting that the specificities of the ELISAs are poor and that at least the IgG ELISA needs to be calibrated against the WHO standard.

Sensitive and specific ELISA methods to diagnose pertussis in adolescents and adults are not widely available, and none are currently FDA approved. Studies of ELISA kits which had the highest specificity for the diagnosis of pertussis often used single serology cutoffs of 100 EU/mL (1, 9). However, one study using a value of 48 EU/mL had a specificity of approximately 95% (27). The ELISA used in our study was not calibrated against the WHO standard; therefore, correlation with the recommended cutoff of 100 IU/ml could not be made. One study comparing five commercial kits for pertussis antibodies showed great discrepancies when comparing semiquantitative results. The different ELISAs contained different antigen preparations. It was concluded from this study that the five tested commercial ELISAs needed further improvement and standardization (17).

In addition to the PT band, FHA has been used to detect antibodies during acute infection and after \textit{Bordetella} vaccination, since it is presently used in acellular vaccines. Despite antibodies to FHA being present in individuals infected with \textit{Bordetella} spp. other than \textit{B. pertussis}, the detection of FHA antibodies can increase the sensitivity of the detection of pertussis infection. In our study, addition of the FHA band to the positivity criteria for the immunoblots increased the sensitivity of both the Viramed and Virotech immunoblots. However, the two blots differed greatly in specificity when the FHA bands were added in the criteria for positivity, with a statistically significant decrease in the specificity of the Viramed immunoblots. This suggests that the formulation of the Viramed FHA antigen is different from that of the Virotech FHA antigen and that the specificity of this FHA antigen is lower than that of the Virotech preparation.

Studies have suggested that antibodies against the CatACT antigen may be a good marker of natural \textit{B. pertussis} infection since CatACT is not used in current pertussis vaccines and thus is not affected by vaccination status (8, 27). These studies proposed that the addition of CatACT antibody testing could increase the specificity of detection of culture-positive infections. Although the CatACT band was included in the Virotech immunoblot, positive bands were not used in the criteria by the manufacturer for determination of a positive immunoblot. We examined patterns of positivity of the CatACT band to ascertain whether it may be useful in increasing sensitivity or specificity compared to the ELISA. We found that the addition of the CatACT band did not increase the sensitivity of the immunoblot but rather significantly decreased the specificity. It has been observed that antibody responses to CatACT have been detected in healthy individuals, compromising its utility as a diagnostic antigen for pertussis infection (2). In summary, the calibration of the Viramed and Virotech immunoblots with the WHO PT standard should make these serologic tests for \textit{B. pertussis} antibodies more specific. The Viramed and Virotech assays are very similar to each other in sensitivity and specificity with regard to the detection of antibodies to PT compared to ELISA. The Viramed FHA antigen, however, showed lower specificity than the Virotech immunoblot compared to the ELISA. Ultimately these assays may aid in the assessment of \textit{B. pertussis} antibodies, either as standalone assays or in combination with ELISA results.
ACKNOWLEDGMENTS

This study was supported by the ARUP Institute for Clinical and Experimental Pathology. Special thanks go to Gold Standard Diagnostics and Viramed for providing reagents used in this study.

REFERENCES

1. Andre, P., et al. 2008. Comparison of serological and real-time PCR assays to diagnose Bordetella pertussis infection in 2007. J. Clin. Microbiol. 46: 1672–1677.
2. Arciniega, J. L., E. L. Hewlett, K. M. Edwards, and D. L. Burns. 1993. Antibodies to Bordetella pertussis adenylate cyclase toxin in neonatal and maternal sera. FEMS Immunol. Med. Microbiol. 6:325–330.
3. Baughman, A. L., et al. 2004. Establishment of diagnostic cutoff points for levels of serum antibodies to pertussis toxin, filamentous hemagglutinin, and fimbriae in adolescents and adults in the United States. Clin. Diagn. Lab Immunol. 11:1045–1053.
4. Baughman, A. L., K. M. Bisgard, F. Lynn, and B. D. Meade. 2006. Model analysis for establishing a diagnostic cut-off point for pertussis antibody levels. Stat. Med. 25:2894–3010.
5. CDC. 2005. Pertussis—United States, 2001–2003. MMWR Morb. Mortal. Wkly. Rep. 54:1283–1286.
6. CDC. 2008. Prevention of pertussis, tetanus, and diphtheria among pregnant and postpartum women and their infants. MMWR Morb. Mortal. Wkly. Rep. 57:1–47.
7. Cherry, J. D. 2005. The epidemiology of pertussis: a comparison of the epidemiology of the disease pertussis with the epidemiology of Bordetella pertussis infection. Pediatrics 115:1422–1427.
8. Cherry, J. D., et al. 2004. Determination of serum antibody to Bordetella pertussis adenylate cyclase toxin in vaccinated and unvaccinated children and in children and adults with pertussis. Clin. Infect. Dis. 38:502–507.
9. de Melker, H. E., et al. 2000. Specificity and sensitivity of high levels of immunoglobulin G antibodies against pertussis toxin in a single serum sample for diagnosis of infection with Bordetella pertussis. J. Clin. Microbiol. 38:800–808.
10. Fry, N. K., et al. 2004. Laboratory diagnosis of pertussis infections: the role of PCR and serology. J. Med. Microbiol. 53:519–525.
11. Giammanco, A., et al. 2003. European Sero-Epidemiology Network: standardisation of the assay results for pertussis. Vaccine 22:112–120.
12. Giammanco, A., et al. 2008. European Sero-Epidemiology Network 2: standardisation of immunoassay results for pertussis requires homogeneity in the antigenic preparations. Vaccine 26:4486–4493.
13. Gustafsson, B., and P. Askelof. 1989. Rapid detection of Bordetella pertussis by a monoclonal antibody-based colony blot assay. J. Clin. Microbiol. 27: 628–631.
14. Hallander, H. O. 1999. Microbiological and serological diagnosis of pertussis. Clin. Infect. Dis. 28(Suppl. 2):S99–S106.
15. Hewlett, E. L., V. M. Gordon, J. D. McCaffrey, W. M. Sutherland, and M. C. Gray, 1989. Adenylate cyclase toxin from Bordetella pertussis. Identification and purification of the holotoxin molecule. J. Biol. Chem. 264:19379–19384.
16. Isacson, J., B. Trollfors, J. Taranger, and T. Lagergard. 1995. Acquisition of IgG serum antibodies against two Bordetella antigens (filamentous hemagglutinin and pertactin) in children with no symptoms of pertussis. Pediatr. Infect. Dis. J. 14:517–521.
17. Kosters, K., M. Riffelmann, B. Dohrn, and C. H. von Konig. 2000. Comparison of five commercial enzyme-linked immunosorbent assays for detection of antibodies to Bordetella pertussis. Clin. Diagn. Lab. Immunol. 7:422–426.
18. Marchant, C. D., et al. 1994. Pertussis in Massachusetts, 1981–1991: incidence, serologic diagnosis, and vaccine effectiveness. J. Infect. Dis. 169:1297–1305.
19. Mattoo, S., and J. D. Cherry. 2005. Molecular pathogenesis, epidemiology, and clinical manifestations of respiratory infections due to Bordetella pertussis and other Bordetella subspecies. Clin. Microbiol. Rev. 18:326–382.
20. Mendell, G. L., J. E. Bennett, R. Dolin. 2010. Mendell, Douglas, and Bennett’s principles and practice of infectious diseases, 7th ed. Churchill Livingstone Elsevier, Philadelphia, PA.
21. Mink, C. M., C. H. O’Brien, S. Washillak, A. Deforest, and B. D. Meade, 1994. Isotype and antigen specificity of pertussis agglutinins following whole-cell pertussis vaccination and infection with Bordetella pertussis. Infect. Immun. 62:1118–1120.
22. Muller, F. M., J. E. Hoppe, and C. H. Wirsing von Konig. 1997. Laboratory diagnosis of pertussis: state of the art in 1997. J. Clin. Microbiol. 35:2435–2443.
23. Muyldermans, G., et al. 2004. Simple algorithm for identification of Bordetella pertussis pertactin gene variants. J. Clin. Microbiol. 42:1614–1619.
24. National Center for Health Statistics. 2010. Health, United States, 2009: with special feature on medical technology. National Center for Health Statistics, Hyattsville, MD.
25. Redd, S. C., et al. 1988. Immunoblot analysis of humoral immune responses following infection with Bordetella pertussis or immunization with diphteria-tetanus-pertussis vaccine. J. Clin. Microbiol. 26:1373–1377.
26. Rosner, B. 2006. Fundamentals of biostatistics, 6th ed. Thomson-Brooks/Cole, Belmont, CA.
27. Watanabe, M., B. Connelly, and A. A. Weiss. 2004. Simple algorithm for identification of Borde- tella pertussis pertactin gene variants. J. Clin. Microbiol. 42:1614–1619.
28. Xing, D., et al. 2009. Characterization of reference materials for human antiserum to pertussis antigens by a monoclonal antibody-based colony blot assay. J. Clin. Microbiol. 27: 628–631.
29. Xing, D., et al. 2009. Establishment of diagnostic cutoff points for pertussis antibodies in human sera. J. Clin. Microbiol. 47:3016–3018.