

Evaluation of Five Immunoassays for Detection of *Chlamydia psittaci* in Cloacal and Conjunctival Specimens from Turkeys

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Five commercially available immunoassays were evaluated for the detection of *Chlamydia psittaci* in cloacal and conjunctival swabs from industrially raised turkeys: IMAGEN (DAKO Diagnostics, Ely, Cambridgeshire, United Kingdom), Chlamydia CEL-VET IF (Cellabs, Brookvale, Australia), IDEIA (DAKO Diagnostics), CELISA (Cellabs), and CLEARVIEW (Unipath, Bedford, United Kingdom). Results were compared with isolation in Buffalo Green Monkey cells as a reference method. For the conjunctival samples, the sensitivities of the IMAGEN test, the Chlamydia CEL-VET IF test, the IDEIA, the CELISA, and the CLEARVIEW test were found to be 100, 66, 0, 0, and 0%, respectively, as compared to the reference test. Also for the conjunctival samples, the specificities of the IMAGEN test, the Chlamydia CEL-VET IF test, and the IDEIA were found to be 100, 11, and 92.8%, respectively. For the cloacal specimens, the sensitivities of the IMAGEN test, the Chlamydia CEL-VET IF test, the IDEIA, the CELISA, and the CLEARVIEW test were found to be 100, 93.3, 26.6, 0, and 53.3%, respectively. Also for the cloacal specimens, the specificities of the IMAGEN test, the Chlamydia CEL-VET IF test, the IDEIA, and the CLEARVIEW test were found to be 92, 12, 100, and 88%, respectively. The IMAGEN test was the most sensitive and specific direct chlamydia antigen detection test for cloacal and conjunctival samples from turkeys.

The genus *Chlamydia* consists of three species: *Chlamydia psittaci*, *Chlamydia trachomatis*, and *Chlamydia pneumoniae*. Recently, a fourth species, *Chlamydia pecorum*, has been proposed (7). *C. trachomatis* is primarily a human pathogen and contains three biovars and 15 serovars. *C. pneumoniae* is also a human pathogen, and it consists of one serovar (TWAR strain). *C. psittaci* has been isolated from a wide range of avian and mammalian hosts. This agent also can infect humans. Outbreaks of *C. psittaci* infection in humans have been attributed mainly to close contact with infected psittacine birds, ducks, and turkeys.

In the United States, the public health and economic importance of *C. psittaci* infections in turkeys has been recognized since 1950. Outbreaks in turkeys are characterized by conjunctivitis, rhinitis, sinusitis, tracheitis, airsacculitis, pneumonia, pericarditis, and enteritis (5, 16, 17, 19, 23). Epidemics of *C. psittaci* infections in turkeys have been registered which were economically devastating to the producers because of carcass condemnation at slaughter, egg production decrease, and/or the expense of antibiotic treatment to reduce mortality and allow marketing of poultry (3, 8, 9, 17, 18). In Europe, however, the significance of *C. psittaci* infections is still a matter of debate (12). In the last 10 years, important outbreaks of rhinotracheitis have been observed in commercial European turkey production units. Several etiological agents have been associated with the rhinotracheitis syndrome in European turkeys, including a paramyxovirus (turkey rhinotracheitis virus) and *Escherichia coli*. Recently, evidence was presented that *C. psittaci* was the primary pathogen in an outbreak of respiratory disease on a large European broiler turkey farm (29, 31).

Considering the economic and public health significance of

C. psittaci infections in birds, accurate diagnostic methods should be made available to veterinarians and public health officers. The diagnosis of *C. psittaci* infections in turkeys can be based on demonstration of the antigen or on serological testing. Isolation of chlamydiae in cultured cells has long been the method of choice for antigen detection. However, because isolation is slow and labor-intensive, a number of direct antigen detection methods have been introduced recently. Most of these tests originally were developed for the detection of *C. trachomatis*. The monoclonal antibodies (MAbs) used in these tests are directed against a genus-specific epitope located on the chlamydial lipopolysaccharide (LPS). Theoretically they should also be suitable for the detection of *C. psittaci* infections. Unfortunately, no data are available concerning the sensitivities and specificities of direct nonculture *C. psittaci* antigen detection tests for turkeys.

The objective of the present study was to compare the results of *C. psittaci* detection in field samples from broiler turkeys using different direct antigen detection methods and isolation in cell cultures.

MATERIALS AND METHODS

Samples for evaluation of different antigen detection tests.

For the evaluation of the different tests, cloacal and conjunctival swabs were collected from four groups of 10 male broiler turkeys at the time of slaughter. Each group came from a different farm. All 40 turkeys were slaughtered at the age of 17 to 18 weeks at the end of the summer.

Enzyme immunoassay procedures for chlamydial antigen detection. Two commercial immunoassays were evaluated to test for *C. psittaci* antigen in cloacal and conjunctival swabs of turkeys. The properties of both tests are described in Table 1. The first test (IDEIA, lot 1L3010; DAKO Diagnostics, Ely, Cambridgeshire, United Kingdom) is approved for detection of *C. trachomatis* in human urethral and endocervical swabs and in urine and ophthalmic specimens. The second immuno-

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TABLE 1. Properties of the five commercially available chlamydia antigen detection tests used in this study

Assay	Type	Antigen	Conjugate ^a	Visualization
IMAGEN	Direct immunofluorescence	Chlamydiae in smear	MAb-LPS, FITC	Fluorescence
CEL-VET IF	Direct immunofluorescence	Chlamydiae in smear	MAb-LPS, FITC	Fluorescence
IDEIA	Sandwich ELISA	Chlamydial LPS bound to the coated MAb-LPS	MAb-LPS, AF	NADPH
CELISA	Indirect ELISA	Coated chlamydiae detected by MAb-LPS	Anti-mouse antibody, PO	TMB ^b
CLEARVIEW	Immunochromatography	Chlamydial LPS bound to colored-latex-labeled MAb-LPS	Unlabeled MAb-LPS	Blue line in the result window

^a FITC, fluorescein isothiocyanate; AF, alkaline phosphatase; PO, peroxidase.

^b TMB, 3,3',5,5'-tetramethylbenzidine.

assay (CELISA, lot 92C2010; Cellabs, Brookvale, Australia) is approved for detection of *C. trachomatis* in human urethral and endocervical swabs. Both assays contain a MAb directed against the genus-specific chlamydial LPS antigen. The IDEIA is a sandwich enzyme-linked immunosorbent assay (ELISA), whereas the CELISA is an indirect ELISA. Both assays were performed according to the instructions of the manufacturers. For both ELISAs, samples were collected with aluminum-shafted cotton-tipped swabs (Difco International, Brussels, Belgium). Swabs were immediately placed in chlamydia transport medium supplied by the manufacturer. In both tests, a heating step solubilized any chlamydial LPS which was present.

In the IDEIA, chlamydial antigen present in specimens was bound by a MAb adsorbed to the surface of the plastic wells. Formalin-inactivated chlamydial antigen in buffer solution and transport medium without chlamydial antigen were used as positive and negative controls, respectively. An enzyme-conjugated MAb was allowed to bind to the captured antigen, and subsequently the enzyme catalyzed the conversion of substrate (naphthol phosphate) to product. This product participated in a second enzyme reaction, which resulted in a color change. The color development process was stopped by the addition of sulfuric acid. The A_{492} of each well was read with a spectrophotometer (Titertek Multiskan Plus MKII; TechGen International, Brussels, Belgium).

For the CELISA, the LPS antigen was allowed to bind to the wells of a microtiter plate during the initial incubation step. Formalin-inactivated chlamydial antigen in buffer solution and transport medium without antigen were used as positive and negative controls, respectively. Then a MAb was added which would attach to any chlamydial LPS present. After a washing step, a peroxidase-conjugated anti-mouse antibody was added which would bind to any antigen-antibody complexes present in the microwell. After a washing step to remove unbound conjugate, the substrate 3,3',5,5'-tetramethylbenzidine was added. During incubation with the substrate, a blue product was produced in positive wells. After addition of the stopping solution, the blue product changed to yellow and A_{450} were read (Titertek Multiskan Plus MKII). For both assays, the intensity of the color was proportional to the amount of chlamydial antigen present in the specimen.

Immunofluorescence assays for chlamydial antigen detection. One commercially available direct immunofluorescence assay (IMAGEN, lot 232413; DAKO Diagnostics) approved for detection of *C. trachomatis* in human urogenital and ophthalmic specimens and for the confirmation of chlamydiae in cell culture and one commercially available direct immunofluorescence test (Chlamydia CEL-VET IF test, lots KC206A2 and 921105C; Cellabs) approved for detection of *C. psittaci* in animal specimens were evaluated for detection of *C. psittaci* in

cloacal and conjunctival swabs of turkeys. The properties of both tests are described in Table 1. Aluminum-shafted, cotton-tipped swabs (Difco International) were used. Transport medium was not used in these assays. Both tests used a fluorescein isothiocyanate-conjugated MAb directed against the genus-specific epitope located on the chlamydial LPS. Tests were performed as recommended by the manufacturers. In short, for both tests swabs were applied to microscope slides by rolling the swabs back and forth over a small area. Specimens were allowed to air dry, and thereafter they were fixed in fresh acetone for 5 min. Immediately after being air dried, the specimens were tested. For the IMAGEN test, 25 μ l of the conjugate diluted 1/10 in phosphate-buffered saline (PBS) (pH 7.3) was applied to the slides. For the Chlamydia CEL-VET IF, 25 μ l of undiluted conjugate was used. Both conjugates contained Evans Blue as the counterstain and sodium azide as a preservative. IMAGEN slides were allowed to incubate for 45 min (37°C) in a moist chamber. Chlamydia CEL-VET IF slides were incubated for 30 min (37°C) in a moist chamber. Afterwards, slides were rinsed twice in PBS (5 min each time) and twice in distilled water (30 s each time). Slides were air dried and mounted. In both assays, slides of unstained fixed cells containing elementary and reticulated bodies were used as a positive control.

The presence of chlamydiae was confirmed under water immersion (Leitz Wetzlar fluorescence microscope; magnification, $\times 500$). Both immunofluorescence assays were scored. For each sample, the number of chlamydiae was determined in five microscopic fields (magnification, $\times 500$). The mean was calculated and scored as follows: 0, no antigen present; 1, mean of 1 to 5; 2, mean of 5 to 10; 3, mean of >10 .

Immunochromatographic test for chlamydial antigen detection. The commercially available CLEARVIEW test (lots 2047 and 2093; Unipath Limited, Bedford, United Kingdom), approved for the direct detection of *C. trachomatis* antigen in endocervical swab specimens, was evaluated for the detection of *C. psittaci* in cloacal and conjunctival swabs of turkeys. The properties of the test are described in Table 1. The test uses a MAb directed against the genus-specific epitope located on the chlamydial LPS. The test was performed as recommended by the manufacturer. In short, for sampling, special swabs made available by the manufacturer were used. These swabs contained no chlamydial transport medium. Chlamydial antigen was extracted from the specimen by heating the swab at 80°C in the extraction buffer supplied by the manufacturer. Following extraction of the antigen, the extract was added to the sample window of the CLEARVIEW chlamydia test unit. The absorbent pad in the sample window contained a colored-latex-labeled murine MAb directed against a genus-specific LPS epitope of chlamydiae. The swab extract rehydrated the la-

TABLE 2. Sensitivities and specificities of five commercially available immunoassays for detection of *C. psittaci* in conjunctival specimens from turkeys

Assay	No. of true positives ^a (n = 12) diagnosed as:		No. of true negatives ^b (n = 28) diagnosed as:		Sensitivity ^c (%)	Specificity ^d (%)
	Positive	Negative	Positive	Negative		
	IMAGEN	12	0	0		
CEL-VET IF	8	4	25	3	66	11
IDEIA	0	12	2	26	0	92.8
CELISA	0	12	0	28	0	
CLEAR-VIEW	0	12	0	28	0	

^a Culture positive.^b Culture negative.^c (Number of positives detected in true-positive group/total number of true positives) × 100.^d (Number of negatives detected in true-negative group/total number of true negatives) × 100.

beled antibody, and the extracted antigen reacted with the antibody to form a complex. The pad was in contact with a test strip which contained a region of immobilized unlabeled anti-LPS MAb in the result window. The extract-latex mixture moved by capillary action along the strip. The appearance of a line in the result window indicated the presence of chlamydial antigen in the case of a positive result.

Isolation. Samples were collected with aluminum-shafted, cotton-tipped swabs (Difco International) provided with chlamydia transport medium (30). The isolation of *C. psittaci* in cell culture was performed in Buffalo Green Monkey (BGM) cells. Two passages were done. For chlamydial identification in cell culture, the IMAGEN test was used, because in a previous study the IMAGEN test was demonstrated to be more sensitive and specific than the modified Giménez staining (30). In the present study, isolation in cell culture was used as a confirmation assay. Results were scored. For each sample, the number of chlamydiae was determined in five microscopic fields (magnification, ×500). The mean was calculated and scored as follows: 0, no antigen present; 1, mean of 1 to 5; 2, mean of 5 to 10; 3, mean of >10.

Evaluation of specificities and sensitivities of the assays. The following formulas (15, 32) were used for calculation of specificity and sensitivity: sensitivity = [TP/(TP + FN)] × 100, where TP is the true-positive result (as determined by isolation) and FN is the false-negative result, and specificity = [TN/(TN + FP)] × 100, where TN is the true-negative result and FP is the false-positive result.

RESULTS

Evaluation of chlamydial antigen detection tests. The results of the chlamydial antigen detection tests with turkey conjunctival swabs are presented in Table 2. Chlamydiae were isolated from the conjunctivae of 12 of the 40 examined turkeys. Chlamydial antigen was demonstrated in the conjunctival swabs of the four groups examined. All positive IMAGEN test results were confirmed by isolation. With the CLEARVIEW test and the CELISA, only negative results were obtained. Two positive results were recorded by the IDEIA. However, the two samples positive by the IDEIA were negative by isolation. Of the 12 samples positive by isolation, only 8 were positive in the Chlamydia CEL-VET IF test. The other 25 positive CEL-VET IF results were not confirmed by isolation.

Scores assigned to the results of the isolation attempts, the

TABLE 3. Sensitivities and specificities of five commercially available immunoassays for detection of *C. psittaci* in cloacal specimens from turkeys

Assay	No. of true positives ^a (n = 15) diagnosed as:		No. of true negatives ^b (n = 25) diagnosed as:		Sensitivity ^c (%)	Specificity ^d (%)
	Positive	Negative	Positive	Negative		
	IMAGEN	15	0	1		
CEL-VET IF	14	1	22	3	93.3	12
IDEIA	4	11	0	25	26.6	100
CELISA	0	15	0	25	0	
CLEAR-VIEW	8	7	3	22	53.3	88

^a Culture positive.^b Culture negative.^c (Number of positives detected in true-positive group/total number of true positives) × 100.^d (Number of negatives detected in true-negative group/total number of true negatives) × 100.

IMAGEN test, and the Chlamydia CEL-VET IF assay, together with the extinction values of the CELISA and the IDEIA, helped us to compare further the sensitivities of these tests. Scores of the IMAGEN test and isolation attempts were equally high. For conjunctival specimens, positive scores of the CEL-VET IF were always 1, while in the IMAGEN test scores of 1, 2, and 3 were noted. The spectrophotometric absorbance of one of the positive IDEIA results (0.195) was only slightly above the calculated cut-off value (0.176), while the spectrophotometric absorbance of the other positive IDEIA result (0.405) was higher than those found for true-negative specimens but lower than the absorbance of the positive control.

The results of the chlamydial antigen detection tests with turkey cloacal swabs are presented in Table 3. Chlamydiae were isolated from the cloacae of 15 of the 40 examined turkeys. All positive isolation results were confirmed with the IMAGEN test. One specimen was positive in the IMAGEN test and negative by isolation. Eight of the 15 specimens positive by isolation were positive in the CLEARVIEW test. The other three CLEARVIEW-positive specimens were negative by isolation. Four positive results were recorded in the IDEIA. These specimens were also positive by isolation. Of the 15 samples positive by isolation, 14 were positive in the Chlamydia CEL-VET IF test. The other 22 positive CEL-VET IF results were not confirmed by isolation. In the CELISA, only negative results were obtained.

Scores given to the results of isolation, the IMAGEN test, and the Chlamydia CEL-VET IF assay, together with the extinction values of the CELISA and the IDEIA, helped us to compare further the sensitivities of these tests for examining cloacal specimens. Scores of the IMAGEN test and isolation attempts were equally high. For cloacal specimens, positive scores of the CEL-VET IF were always 1, while those of the isolation attempts were 1, 2, or 3. Three of four positive IDEIA specimens had a score of 3 in the isolation attempts. The other specimen had a score of 2 in the isolation attempts.

The results of the chlamydial antigen detection tests with cloacal and conjunctival swabs of turkeys are compared in Table 4. For all the antigen detection methods applied, chlamydiae were found more often in the cloacal swabs than in the conjunctival swabs. The results in Table 4 show that, of 19 chlamydia-excreting turkeys, the IMAGEN test detected all 19, while the CLEARVIEW test detected 11, the IDEIA detected 5, the CEL-VET IF detected 38, and the CELISA detected 0.

TABLE 4. Numbers of turkeys positive for conjunctiva or cloaca, for the conjunctiva alone, for the cloaca alone, and for both conjunctiva and cloaca

Assay	No. of turkeys (<i>n</i> = 40) positive for <i>C. psittaci</i> in:			
	Conjunctiva	Cloaca	Conjunctiva or cloaca	Conjunctiva and cloaca
Isolation	12	15	19	8
IMAGEN	12	16	20	8
CLEARVIEW	0	11	11	0
IDEIA	2	4	5	1
CEL-VET IF	33	36	38	31
CELISA	0	0	0	0

DISCUSSION

Under optimal conditions, isolation has proven to be the most sensitive and specific diagnostic method for chlamydia infections (1, 13, 15, 21, 27) and therefore was chosen as the confirmation assay in this study. The BGM cell culture has been shown to be the most sensitive artificial host for isolating *C. psittaci* from specimens obtained from birds (2, 30). Therefore, in this study the isolation was performed in BGM cells.

The definition of true positive is based upon testing of specimens cultured in BGM cells and identified with the IMAGEN test. In a previous study (30), we have shown that chlamydiae isolated in cell culture could be revealed by either modified Giménez staining or the IMAGEN test. The latter method, however, proved to be more sensitive when small numbers of chlamydiae were present in the cells. Therefore, in the present paper, the results of the IMAGEN test following isolation are used to define the true positives and true negatives.

For conjunctival specimens, the IMAGEN test gave both 100% sensitivity and 100% specificity when tested against isolation. Similar results for the IMAGEN test were found for detecting *C. trachomatis* in ophthalmic specimens from patients with acute follicular conjunctivitis (20). In contrast, the CEL-VET IF gave only 11% specificity. For cloacal specimens, the IMAGEN test gave a sensitivity of 100% and a specificity of 96%, while the specificity of the CEL-VET IF was only 12%. Although both fluorescence assays use a MAb against the chlamydial LPS, a tremendous difference in specificity was found. False-positive results with direct immunofluorescence using smears could be due to nonspecific immunoglobulin binding, cross-reactivity of the MAb with other microorganisms, or incorrect microscopic observation (6, 10, 14, 28). All false positives in the CEL-VET IF presented as scattered punctate staining. Contaminating material, such as organic material, present in the samples was probably stained with this test.

For conjunctival specimens, none of the two ELISA-type assays was able to detect any of the culture-positive specimens. For cloacal specimens, one of the ELISAs, the IDEIA, had a sensitivity of 26.6%, while the other was not able to detect any of the culture-positive specimens. This study revealed both false-positive and false-negative results for the IDEIA. When the IDEIA was used for *C. trachomatis* in endocervical, urine, and urethral human specimens and for *C. psittaci* in avian feces, cloacal swabs, and organs, the occurrence of both false-positive and false-negative results was described (11, 25, 26, 34). False-positive results found in these different studies were due to the presence of high concentrations of *Staphylococcus* spp. False-negative results could be due to the fact that

ELISAs fail to detect small numbers of chlamydial elementary bodies (<10) (25). To detect *C. trachomatis*, Thomas et al. (25) increased the sensitivity of the IDEIA by taking multiple swabs and putting them into one lot of transport medium, thereby increasing the amount of antigen. The CELISA was unable to detect any *C. psittaci*-positive conjunctival or cloacal swabs. The reliance of the CELISA on adsorption of the antigen to the plastic may be a mistake in using specimens from birds that may contain much contaminating material, which will compete for absorption sites on the plastic with the LPS and give a false-negative result.

CLEARVIEW detected 11 of 19 chlamydia-excreting turkeys. This indicates the occurrence of false-negative results with the CLEARVIEW test. *C. psittaci* was not isolated from four cloacal samples that were positive in the CLEARVIEW test. This indicates the occurrence of false-positive results in the CLEARVIEW test in examining cloacal specimens. When the CLEARVIEW test was used to detect *C. trachomatis* in human endocervical specimens (4, 22) and to detect *C. psittaci* in ovine fetal membranes and vaginal swabs (33), in vaginal swabs from koala bears (28), and in avian organs and feces (7a), the occurrence of both false-positive and false-negative results was also described when compared to the tissue culture method. False-negative results can be due to an insufficient extraction of the chlamydial LPS. The sensitivities of the CLEARVIEW test for detecting *C. psittaci* in conjunctival and cloacal samples from turkeys were 0 and 53.3%, respectively, while the specificity for cloacal samples was 88%. In the literature, a sensitivity of 93.5 to 95% and a specificity of 98 to 99% for the CLEARVIEW test for examining human endocervical specimens has been described (4, 22). The CLEARVIEW test thus appears to be less specific and less sensitive for examining turkey conjunctival and cloacal samples than for human samples. Nevertheless, in our study the CLEARVIEW test was more sensitive than the IDEIA. This was also confirmed by Gerbermann (7a).

The comparison of the results of chlamydial detection in conjunctivae and cloacae are presented in Table 3. With the isolation method, 19 turkeys were positive for chlamydiosis. Both conjunctivae and cloacae were positive in only eight of these turkeys. This indicates that, if possible, both conjunctivae and cloacae should be examined in turkeys.

In conclusion, of the different antigen detection tests evaluated, the IMAGEN test was the most specific and sensitive. Thus, a rapid, specific, and sensitive tool is available for diagnosis of *C. psittaci* infection in broiler turkeys. The important differences in specificity and sensitivity of the diagnostic tests observed in this study should constitute a warning to the diagnostic industry. Similar problems were observed in the diagnosis of *C. trachomatis* (24).

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