Original article

Evaluation of a recombinant enzyme-linked immunosorbent assay for detecting *Chlamydophila psittaci* antibodies in turkey sera

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Abstract – *Chlamydophila psittaci* (formerly *Chlamydia psittaci*) is one of the major pathogens associated with turkey respiratory disease. Devastating outbreaks with high mortality rates, similar to those of 1950 to 1970 in the USA occasionally occur, but respiratory signs without or with low mortality mostly characterize outbreaks now a day. Accurate diagnostic methods should be made available. The present study examined the sensitivity and specificity of a recombinant ELISA (rMOMP ELISA) for detecting *Cp. psittaci* major outer membrane specific antibodies in turkey sera. Test results were compared to those of immunoblotting and of a competitive ELISA (Chlamydia-psittaci-AK-EIA, Röhm Pharma, Germany) and an indirect ELISA (LPS/LGP) detecting antibodies to the lipopolysaccharide/lipoglycoprotein complex. The rMOMP ELISA was most sensitive as determined on serial dilutions of positive control sera originating from experimentally infected SPF turkeys. The competitive ELISA gave false positives since three negative controls reacted positive. For conventional sera, the sensitivities of the competitive ELISA, immunoblotting and the indirect ELISA were found to be 99.4, 93.1 and 82.2%, respectively, as compared to the rMOMP ELISA (100%). The specificities of the rMOMP ELISA, immunoblotting and the indirect ELISA were found to be 100% while the specificity of the competitive ELISA was only 2.7%. The rMOMP ELISA was chosen to compare the prevalence of chlamydiosis in 2002 with the one from 1992. In 2002, 188 on 200 (94%) turkey sera reacted positive compared to 175 on 200 (87.5%) in 1992 and like 10 years ago all examined farms were seropositive at slaughter. Interestingly, Belgian as well as French farms were seropositive.

Chlamydophila psittaci / antibody / recombinant ELISA / turkey

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1. INTRODUCTION

The importance of poultry farming is rising. Between 1999 and 2003, turkey meat production increased worldwide with 4% to a total of 4 894 000 ton. In 2003, 2 283 000 and 1 583 000 tons of turkey meat were consumed in the USA and the European Union, respectively (Poultry International).

Worldwide, turkey production suffers from the negative economical impact of respiratory disease. Nearly all turkey flocks experience one to multiple periods of respiratory disease leading to expensive medical treatment in preventing mortality, loss of weight and carcass condemnation at slaughter. In Europe, influenza virus type A, avian paramyxovirus 1, 2 and 3 (PMV-1, PMV-2, PMV-3), avian pneumovirus (APV), *Ornithobacterium rhinotracheale* (ORT), *Mycoplasma* sp. and *Chlamydophila psittaci* (formerly *Chlamydia psittaci*) are currently regarded as the major pathogens associated with respiratory disease [16, 27, 33]. At present, *Cp. psittaci* is nearly endemic in Belgian and German turkeys [10, 33]. Devastating outbreaks with high mortality rates, similar to those of 1950 to 1970 in the USA occasionally occur, but respiratory signs without mortality mostly characterize outbreaks now a day [1]. Nevertheless, *Cp. psittaci* causes important economical loss and is a threat to public health since this zoonotic agent is able to infect poultry workers [1].

Considering the widespread occurrence of chlamydiosis in turkeys and the economic and public health significance, accurate diagnostic methods should be made available. Serology can be used to monitor the *Cp. psittaci* status of turkey flocks. The modified direct complement fixation test [5] and the latex agglutination test [22] as well as several enzyme-linked immunosorbent assays (ELISA) originally developed for the detection of *Chlamydia trachomatis* antibodies in human sera have been used [26]. The lack of sensitivity and specificity of these tests lead to the development of ELISA using *Cp. psittaci* target antigens.

At present, we evaluated the sensitivity and specificity of a self-made recombinant ELISA for detecting *Cp. psittaci* antibodies in turkey sera. In the ELISA, the recombinant major outer membrane protein of *Cp. psittaci* strain 92/1293 (genotype D) was used as the test antigen. The test results were compared to those of immunoblotting and to those of two other ELISA. The first one being a species independent commercially available competitive ELISA (Chlamydia-psittaci-AK-EIA, Röhm Pharma, Germany) based on the detection of chlamydial lipopolysaccharide (LPS)-specific antibodies and the second one being another in house made ELISA, based on the detection of antibodies against the chlamydial lipopolysaccharide/lipoglycoprotein complex [29]. The most sensitive and specific assay was chosen to compare the prevalence of chlamydiosis in turkeys raised in 1992 and 2002.

2. MATERIALS AND METHODS

2.1. Samples

In 1992, two hundred sera were collected from 17- to 18-week-old male broiler turkeys (British United Turkey, BUT 6 breed) at slaughter. Twenty groups of 10 turkeys were sampled. Each group came from a different Belgian farm. The same procedure was repeated in 2002 examining 200 broiler turkeys, from 8 other Belgian and 2 French farms. Blood samples were incubated overnight at room temperature, centrifuged ($325 \times g$, 10 min, 4 °C) whereafter the serum was collected and frozen at –20 °C.

In a previous experiment, fifteen 7-day-old SPF turkeys (AFSSA, Ploufragan, France) were experimentally infected with *Cp. psittaci* strain 92/1293 (genotype D) [32] and one serum of 10 of these infected turkeys sampled between day 14 and 21 post inoculation, was used as a positive control. Twenty-five sera of 8-week-old Broad Breasted White turkeys from the SPF breeder unit

(AFSSA) were used as negative controls. The protocol used to experimentally infect these turkeys has been described elsewhere [32].

The sera collected in 1992 were examined by immunoblotting and by three different ELISA each using another *Cp. psittaci* antigen preparation. The most sensitive and specific ELISA was chosen for subsequent analysis of 200 additional turkey sera collected in 2002.

2.2. Enzyme immunoassays

The Chlamydia-psittaci-AK-EIA used in this study was a competitive ELISA for the detection of antibodies against *Cp. psittaci* in serum of birds and mammals. The test principle used allowed species independent antibody detection. The test was used according to the specifications of the manufacturer. Briefly, non-inactivated undiluted sera were used in this test. The surface of the plastic microtitre wells was coated with a highly purified *Cp. psittaci* whole antigen. In three wells, foetal calf serum was added as a negative standard for the test. Turkey sera were brought into the other wells. The sera were simultaneously incubated for one hour with an enzyme conjugated monoclonal antibody directed against the chlamydial lipopolysaccharide. After incubating and washing, the substrate urea peroxide and the chromogen TMB (tetramethyl benzidine) were added to the wells. Any bound enzyme conjugate in the wells converted the colorless substrate to a blue product. After 30 min a stop solution (1 N sulfuric acid) was added which changed the blue color to yellow. Absorbances were read at 450 nm (Titertek MultiskanR Plus MKII, TechGen International, Brussels, Belgium). The cut-off value of this test was fixed at an inhibition of 20%, calculated from the mean absorbance values of the three wells in which the negative standard was tested. This means, that an inhibition between 0 and 20% represented a negative result while an inhibition higher than 20%

reflected a positive result. The results were expressed as positive or negative.

Furthermore, a non-commercial indirect ELISA, developed for demonstrating *Cp. psittaci* antibodies in turkey sera, was used [10, 29]. Briefly, a *Cp. psittaci* outer membrane complex (700 kDa) extracted by heat treatment and purified by affinity chromatography on polymixin B-agarose, was used to coat microtiter plates. The purified outer membrane complex has a lipopolysaccharide and a lipoglycoprotein (LPS/LGP) nature. Serum, diluted 1 to 100 was added to the coated wells. After incubating and washing, a peroxidase labeled goat-anti turkey conjugate (Nordic, Tilburg, The Netherlands) diluted 1 to 1000 was added to the wells. All dilutions were made in dilution buffer (Bommeli AG, Bern, Switzerland) supplemented with 2% skimmed milk and 10% foetal calf serum. After incubating and washing, the ABTS substrate (2,2' azino-di-3-ethylbenzothiazoline sulphonate, Bommeli AG) was added to the wells. After 30 min, the absorbances were read at 450 nm (EAR 400 ATC-Photometer, SLT Lab instruments, Crailsheim, Germany). The results were positive if the absorbance exceeded the cut off value of the mean of the negative control sera plus three times the standard deviation.

The (rMOMP ELISA) was performed on turkey sera being pretreated with kaolin to remove background activity [23]. Major outer membrane protein (MOMP)-specific antibody titers were determined using standard protocols and microwell plates coated with rMOMP. Serum, diluted 1 to 100 was added to the coated wells. Recombinant MOMP was produced in pcDNA1::MOMPHis transfected COS7 cells as described previously [34]. Briefly, COS7 cells were cultured in Dulbecco modified Eagle medium supplemented with 3.7 g of sodium bicarbonate/L, 1 mM L-glutamine, and 10% fetal calf serum (Invitrogen, Merelbeke, Belgium). Transfection with plasmid DNA was performed by the DEAE dextran method. Forty-eight hours post transfection,

Table I. Results of immunoblotting and three different ELISA on turkey sera collected in 1992.

Sera	N	Immunoblotting		Competitive ELISA		LPS/LGP ELISA		rMOMP ELISA	
		Positive	Negative	Positive	Negative	Positive	Negative	Positive	Negative
Positive control	10	10	0	10	0	10	0	10	0
Negative control	25	0	25	3	22	0	25	0	25
Slaughterhouse	200	163	37	199	1	144	56	175	25

recombinant MOMP production was monitored by an indirect immunofluorescence staining using serovar and genus-specific monoclonal antibodies [34] where after the tissue culture flasks were stored at –70 °C. His-tag labeled Recombinant MOMP was purified by BD Talon^{TM} (BD Biosciences, Erembodegem, Belgium) affinity chromatography and the protein concentration was determined by the bicinchoninic acid protein assay (Sigma, Bornem, Belgium). For the determination of antibody titers, 1:2000 and 1:4000 dilutions of biotinylated anti-chicken/turkey IgG (H+L) antibody and peroxidase-conjugated streptavidin were used, respectively. The results were positive if the absorbance exceeded the cut off value of the mean of the negative control sera plus three times the standard deviation.

2.3. Immunoblotting

Cp. psittaci strain 92/1293 polypeptides were separated by SDS-polyacrylamide gel electrophoresis (PAGE) and blotted onto a polyvinylidone (PVDF) membrane as described by Vanrompay et al. [33]. The membrane was blocked with PBS (pH 7.4) supplemented with 0.2% Tween 20 and 10% γ-globulin free horse serum, and then probed for chlamydial antigens with turkey sera. An in-house made monoclonal antibody against a family-specific epitope on MOMP (7B6III) was used as the control to show us the exact localization of the MOMP during immunodetection. Serum antibody binding was detected by use of polyclonal peroxidase-labeled anti-turkey

conjugate (Peroxidase-Rabbit Anti-Chicken/Turkey IgY (H+L); Zymed® Laboratories Inc., San Francisco, USA) and amino-ethyl carbazole (AEC). The blots were analyzed with a densitometer (Image Master, Pharmacia).

### 2.4. Evaluation of the specificities and sensitivities of the assays

The following formulas were used for calculation of specificity and sensitivity: sensitivity: = [TP/(TP + FN)] × 100, where TP is the true positive result as determined by the recombinant ELISA and FN is the false-negative result, and specificity = [TN/(TN + FP)] × 100, where TN is the true negative result determined by immunoblotting and confirmed by the more sensitive recombinant ELISA and FP is the false-positive result.

3. RESULTS

The results of immunoblotting and three different ELISA performed on samples from 1992 are presented in Tables I and II. All 10 positive control sera reacted in immunoblotting and were positive in all three ELISA. Immunoblotting revealed a reaction pattern to numerous antigens with molecular weights varying from 10 to 122 kDa (Fig. 1). All 25 negative control sera reacted negative in immunoblotting and were negative in both indirect ELISA. However, 3 of 25 negative control sera reacted positive in the competitive ELISA.

Table II. Results of ELISA on turkey sera collected in 1992 compared to immunoblotting as a reference test.

Immunoblotting	N	Competitive ELISA		LPS/LGP ELISA		rMOMP ELISA	
		Positive	Negative	Positive	Negative	Positive	Negative
Negatives	37	36	1	1	36	12	25
Positives	163	163	0	143	20	163	0
Total	200	199	1	144	56	175	25
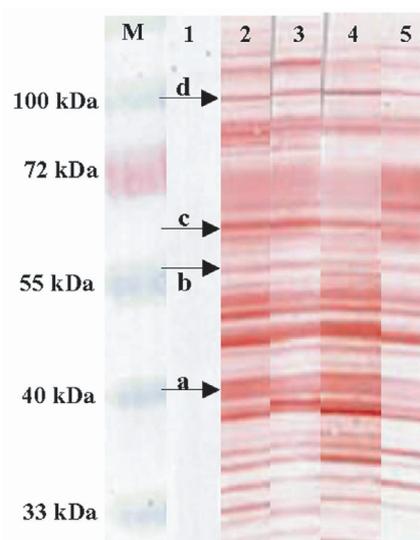
Figure 1. Immunoblotting of conventional turkey sera obtained in 1992 (line 2 to 5). The molecular weight markers are indicated in lane M. Lane 1 is a negative control lane. Arrow a: 40 kDa (MOMP); arrow b: 57 kDa protein; arrow c: 60-62 kDa proteins; arrow d: 97.4 kDa protein.

Using immunoblotting, antibodies were demonstrated in 163 of 200 (81.5%) examined slaughterhouse turkey sera. With most sera, a variable number of bands were obtained analogous to those observed for the positive control sera. However, antibodies to antigens with molecular weights of 40 kDa, 57 kDa, 60 kDa, 62 kDa and 97.4 kDa were predominantly present. All positive sera (100%) showed MOMP-specific antibodies as shown by the reaction of a monoclonal control antibody against a family-specific epitope on MOMP on the same blots. One hundred and thirty-eight of 163 immunoblotting positive sera (85%) showed antibodies to a 97.4 kDa protein.

Using the competitive ELISA, antibodies were demonstrated in all but one (99.5%) examined turkey sera. However, immunoblotting and two indirect ELISA could not confirm this one negative result. Using the competitive ELISA, all 163 immunoblotting positives could be confirmed. However, 36 of 37 immunoblotting negatives gave positive results in the competitive ELISA. The percent of inhibition in these 36 samples ranged from 30 to 80%.

Using the indirect LPS/LGP ELISA, antibodies were demonstrated in 144 of 200 (72%) slaughterhouse turkey sera, while the remaining 56 samples were negative. Thus, immunoblotting revealed 19 additional positives as compared to the LPS/LGP ELISA demonstrating the presence of antibodies to 40 kDa and 94.7 kDa proteins. One sample, negative by immunoblotting gave a positive result in the LPS/LGP ELISA. Using the LPS/LGP ELISA only 143 of 163 immunoblotting positives could be confirmed while the remaining 20 immunoblotting positives, reacted negative. In three of those twenty LPS/LGP ELISA negatives, optical density readings just below the calculated cut-off value were obtained. In fourteen of twenty LPS/LGP ELISA negatives, including the three sera with low extinction values, antibodies to a 40 kDa, 57 kDa, 60 kDa, 62 kDa and a 97.4 kDa antigen were present. In 3 of the 20 LPS/LGP ELISA negatives, only antibodies to a 97.4 kDa antigen were present while in the

Table III. Sensitivities and specificities of serological assay detecting *Cp. psittaci* antibodies in conventional turkeys.

Assay	No. of true positives[a] ($n = 175$) diagnosed as		No. of true negatives[b] ($n = 37$) diagnosed as		Sensitivity (%)	Specificity (%)
	Positive	Negative	Positive	Negative		
Immunoblotting	163	12	0	37	93.1	100
Recombinant ELISA	175	0	0	37	100	100
Indirect ELISA	144	31	0	37	82.2	100
Competitive ELISA	174	1	36	1	99.4	2.7

[a] Recombinant ELISA positive.
[b] Immunoblotting and recombinant ELISA (more sensitive than immunoblotting) negative.

remaining 3 sera only antibodies to a 60, 62 and 32 kDa protein were observed.

One hundred seventy-five of 200 (87.5%) examined turkey sera revealed to be positive by use of the rMOMP ELISA, while the remaining 25 gave an O.D. value of at least 0.015 to 0.020 below the cut off value (0.100). All immunoblotting positives could be confirmed in the rMOMP ELISA. Additionally, 12 of 37 immunoblotting negatives gave a positive result in the rMOMP ELISA. Among them was the only immunoblotting negative serum sample found to be positive in the LPS/LGP ELISA.

We determined the sensitivities of the assays by testing serial dilutions of our positive control sera originating from experimentally infected SPF turkeys. When testing serial dilutions, the recombinant ELISA was two times more sensitive than immunoblotting. Immunoblotting was as sensitive as the competitive ELISA and both were two times more sensitive than the indirect ELISA. Thus, the recombinant ELISA must be used to determine true positives. We cannot comment on the specificities for testing field samples by using negative control sera from SPF turkeys since conventional turkeys may be infected with "cross-reactive micro-organisms". Indeed, when evaluating the tests on negative control sera all, except for the competitive ELISA reacted 100% specific since no positives were obtained. Thus, when testing the specificity, one must use true negative conventional sera. Consequently, we can only use sera negative in immunoblotting and confirmed negative by the more sensitive recombinant ELISA. For conventional sera (Tab. III), the sensitivities of the competitive ELISA, immunoblotting and the indirect ELISA were found to be 99.4, 93.1 and 82.2%, respectively, as compared to the rMOMP ELISA. The specificities (Tab. III) of the rMOMP ELISA, immunoblotting and the indirect ELISA were found to be 100% while the specificity of the competitive ELISA was only 2.7%.

Thus, the rMOMP ELISA was chosen to compare the field situation in 2002 with the one from 1992. In 2002, 188 of 200 (94%) turkey sera reacted positive and as in 1992, all 10 examined farms were seropositive at slaughter. Interestingly, Belgian as well as French farms were seropositive.

4. DISCUSSION

Firstly, the results of immunoblotting and three different ELISA performed on samples from 1992 are discussed. Using immunoblotting, antigens of 10 kDa, 40 kDa, 57 kDa, 60 kDa, 62 kDa and 97.4 kDa, as determined by the imaging densitometer, were predominantly recognized by antibodies in positive control sera from SPF turkeys experimentally infected with an avian serovar D *Cp. psittaci* strain and in positive slaughterhouse turkey sera. The

presence of anti-chlamydial antibodies to antigens with these molecular weights has already been described in several papers concerning the analysis of the humoral immune response to *Cp. psittaci*, *C. trachomatis* or *Cp. pneumoniae* [7, 8, 11, 12, 21]. These antigens have been characterized as the following: a chlamydial LPS of 10 kDa [5]; a 40 kDa MOMP [6, 21]; a 57 kDa outer membrane protein believed to be a member of the family of the heat shock proteins [18–20]; a 60 kDa surface exposed heat shock protein (hsp60) [20, 21, 35]; a 60 kDa cysteine-rich outer membrane protein (crp60) [9, 25]; a 62 kDa surface exposed protein [21], and finally a 97.4 kDa outer membrane protein [12, 13]. The presence of epitopes, cross-reactive with other Gram-negative bacteria such as *Escherichia coli, Salmonella typhimurium*, *Neisseria gonorrhoeae* and with *Coxiella burnetii* has been described only for the chlamydial LPS (10 kDa) and the hsp60 [35].

Using the competitive ELISA, three negative control sera reacted positive. These sera were negative in immunoblotting and in the indirect ELISA, indicating that the competitive ELISA gave false positive results. The occurrence of false positives for this test has been described by Hafez and Sting [10], who found several specific pathogen free chicken sera to react positive in the competitive ELISA. Furthermore, they tested a negative control serum of a commercial indirect ELISA (Fa. Roche, Munich, Germany) in the commercial competitive ELISA and found a positive result. All the 200 slaughterhouse sera, except for one, were found positive by the competitive ELISA, while only 163 and 144 of these 200 sera were found positive by immunoblotting and by the indirect ELISA, respectively. The higher number of positives in the competitive ELISA cannot be due to the higher sensitivity of the test since the sensitivity of the competitive ELISA and the immunoblotting was comparable as demonstrated by testing two-fold dilutions of three known positive control sera obtained from experimentally infected turkeys[1]. Therefore, for

turkey sera, the competitive ELISA is not specific and/or the prescribed cut-off value is too low. False positives in testing conventional sera may be due to the use of a monoclonal antibody directed against the chlamydial LPS. The chlamydial LPS does share antigenic determinants with the LPS of other Gram-negative bacteria. These common antigenic determinants are located on the Lipid A- and the 3- deoxy- D- manno-octulopyranosonic acid (Kdo) fragment of the LPS [4, 6, 24, 30]. The affinity of antibodies for the epitopes by which they are induced is generally higher than for cross-reactive epitopes [3]. In the competitive ELISA, undiluted sera were used and therefore such cross-reacting antibodies could be detected more easily. However, an exact explanation for the occurrence of false positives in the competitive ELISA cannot be given at present.

In one serum, positive by the indirect ELISA, only antibodies to the 60 kDa antigen were detected by immunoblotting. This serum reacted negative in the competitive ELISA. This may be due to the restriction of the test which is only capable of detecting antibodies to the chamydial LPS. Using anti-chlamydial hsp60 monoclonal antibodies in immunoblotting, cross-reactivity to *Escherichia coli*, *Salmonella typhimurium*, *Neisseria gonorrhoeae* and *Coxiella burnetii* has been observed [35]. Therefore, sera presenting only a band to a 60 kDa antigen might contain non-specific antibodies against cross-reactive epitopes located on the chlamydial hsp60.

In three of 19, indirect ELISA negative samples, only antibodies to a 97.4 kDa antigen could be demonstrated. Antibodies to this protein were present in 176 out of 200 turkey sera. The 97.4 kDa antigen has been described as a major chlamydial immunogen in turkeys [12, 13]. In experimentally infected turkeys at 10 days post inoculation, only antibodies to the 97.4 kDa antigen were present and in most turkeys,

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[1] Vanrompay D. et al., unpublished results, 1994.

they were the only ones persisting for at least 142 days [12]. The 97.4 kDa antigen probably belongs to the chlamydial polymorphic outer membrane proteins (POMPS or pmp). The Pmp have already been characterized in *Cp. abortus*, but not yet in *Cp. psittaci* [14, 15].

The indirect ELISA was not as sensitive as the immunoblotting in testing field sera. Possible explanations are the following: (1) the higher serum dilution used in the indirect ELISA, (2) differences in serological responses being detected or (3) the differences in target antigens used in both tests since genus- and serovar-specific monoclonal antibodies against MOMP did not react with the antigen preparation[2]. Thus, since the LPS is only responsible for a genus-specific serological response [6, 24] and reactive epitopes on the immunodominant MOMP appeared to be absent in the antigen preparation, this may explain why the indirect ELISA was less sensitive as compared to immunoblotting. Immunoblotting can detect genus-, species-, and serovar-specific responses against the MOMP. Moreover, the target antigen spectrum being provided in immunoblotting is even larger since whole organisms are being used [2, 17, 28].

Thus, the results on the comparison of immunoblotting and three different ELISA revealed that the competitive ELISA gave false positive results since three negative control sera reacted positive. Therefore, the competitive ELISA is not useful for testing turkey sera. In its present form, the indirect ELISA was not as sensitive as the immunoblotting since immunoblotting revealed the presence of chlamydia-specific antibodies in 163 of the 200 examined slaughterhouse sera, while by the indirect ELISA only 144 of 200 sera were found positive. However, immunoblotting is cumbersome and therefore not suitable for routine diagnosis. The recombinant MOMP ELISA provides a sensitive and specific alternative and was

therefore chosen to compare the field situations of 1992 and 2002. In 2002, all examined farms were seropositive at slaughter, revealing a total of 94% positive animals compared to 87.5% in 1992. The data from 2002 provide additional and more recent information on the prevalence of *Chlamydophila psittaci* infections in the turkey industry and are in fact a further validation of the recombinant ELISA since we also examined pharyngeal swabs of these birds by nested PCR [31]. All nested PCR positive birds (52.5%) showed MOMP antibodies. However, 41.5% of the ELISA positives were negative in our nested PCR. The latter was not unlikely, since *Cp. psittaci* antibodies can remain for several months following an infection. Thus, the results showed no improvement regarding the prevalence of *Chlamydophila psittaci* infections in turkeys.

ACKNOWLEDGEMENTS

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