Serotyping of European Isolates of *Chlamydia psittaci* from Poultry and Other Birds

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A panel of five serovar-specific monoclonal antibodies which distinguish the five known avian serovars of *Chlamydia psittaci* was used to serotype 45 European avian *Chlamydia psittaci* isolates. Chlamydial antigen was grown in Buffalo green monkey (BGM) cells or in embryonated chicken eggs and was then inoculated into BGM cells. Serotyping was performed in an indirect immunofluorescence test. The 45 European isolates included 22 isolates from the order Psittaciformes, 9 isolates from the order Columbiformes, 6 isolates from the order Galliformes, 5 isolates from the order Passeriformes, and 3 isolates from the order Anseriformes. All of these were successfully serotyped. No additional serovars were found. One isolate from a duck and two isolates from psittacine birds gave positive immunofluorescences with two monoclonal antibodies considered to be specific for two different serovars. These three isolates were cloned by an agar overlay method. Serotyping of the clones demonstrated that the duck and one psittacine bird each were infected with two different serovars. After cloning, one isolate from a psittacine bird reacted only with serovar A. From these results it was concluded that this serotyping system allows the classification of all isolates tested so far. The results show that similar serovars are prevalent in avian species in Europe and the United States. The results also indicate that birds from a certain order are more susceptible to a distinct serovar. The use of a panel of serovar-specific monoclonal antibodies in the immunofluorescence test provides a reliable method for serotyping avian isolates. Monoclonal antibodies to new avian isolate serovars can easily be added to the panel, which makes the system useful for epidemiological studies.

The genus *Chlamydia* consists of three species: *Chlamydia psittaci*, *Chlamydia trachomatis*, and *Chlamydia pneumoniae*. Recently, a fourth species, *Chlamydia pecorum*, has been proposed (4). *Chlamydia trachomatis* is primarily a human pathogen and contains three biovars and 15 serovars. *C. pneumoniae* is also a human pathogen. It consists of one serovar (TWAR strain). *C. psittaci* strains have been isolated from a wide range of avian and mammalian hosts. This agent can also infect humans. Serotyping of mammalian *C. psittaci* isolates has been reported previously (3, 5, 7, 9, 11–13). Serotyping of avian isolates was carried out only recently (1, 5, 6, 15). Andersen (1) classified 34 American isolates and 1 European isolate of *C. psittaci* from birds into four distinct serovars using a panel of serovar-specific monoclonal antibodies (MAbs) in a microimmunofluorescence test. A fifth avian serovar has recently been discovered (2a). In the studies described here, European avian isolates were serotyped in order to obtain more information about the prevalence of the serovars in Europe and about the host distribution of avian *C. psittaci* serovars.

**MATERIALS AND METHODS**

**Isolates and cell cultures.** In a period of 5 months, 264 dead birds, which died following disease, and 120 live birds were examined for the presence of chlamydiae. Forty-five isolates of *C. psittaci* were obtained from the various avian species examined (see Table 2). Isolates from Belgium (n = 41), The Netherlands (n = 1), and Germany (n = 3) were used in the study. The isolates were either grown directly in Buffalo green monkey (BGM) cells or grown in 6-day-old specific-pathogen-free embryonated chicken eggs and then inoculated into BGM cells (16). Inoculations were performed simultaneously on BGM cells in 25-cm² tissue flasks and on BGM monolayers in Chlamydia Trac bottles (International Medical, Brussels, Belgium). At 6 days postinfection these Chlamydia Trac bottles were scored for the presence of chlamydia by a direct immunofluorescence test (IMAGEN Chlamydia test; Novo Nordisk). When more than 20 chlamydial inclusions per microscopic field were observed, sufficient antigen was present for serotyping. Otherwise, serial passages were made on BGM cells until this number of chlamydial inclusions was obtained. A sucrose-phosphate-glutamate buffer (2.5 ml) was added to the tissue culture flasks. The flasks were stored at −70°C until serotyping was performed.

**Serovar-specific MAbs.** The five serovar-specific MAbs and the one genus-specific MAb used in the present study were those produced as described previously (1, 2b). The genus-specific MAb was designated NJ-1/D3. The five serovar-specific MAbs were designated VS-1 (serovar A specific; psittacine group), CP3 (serovar B specific; pigeon I group), GR-9 (serovar C specific; duck group), NJ-1 (serovar D specific; turkey group), and MP (serovar E specific; pigeon II group).

**Microimmunofluorescence test.** Ten milliliters of the stored cell culture suspension was centrifuged (45,000 × g, 45 min). The pellet was suspended in 1 ml of phosphate-buffered saline (PBS; pH 7.3). One hundred microliters of the antigen suspension was mixed with 33.3 μl of a 20% yolk sac preparation in PBS in order to obtain an improved adhesion of the antigen on the glass slides and blocking of nonspecific
binding sites. Antigen dots (2.5 μl) were placed on seven wells (diameter, 6 mm) of a Nutacon glass slide (Nutacon, Schiphol, The Netherlands) and were allowed to air dry for 30 min. Thereafter, the glass slides were fixed in acetone (−20°C, 10 min). Ten microliters of PBS, a genus-specific MAb, and the five serovar-specific MAbs were delivered to wells 1 to 7, respectively. The absence of nonspecific binding of the MAbs was demonstrated by using harvests from a noninfected cell culture in the microimmunofluorescence test. No immunofluorescence was observed in the controls. All MAbs were used at a dilution of 1/100 (PBS, pH 7.3). If a reaction with two different serovar-specific MAbs occurred, the isolates were cloned as described below. The glass slides were incubated in a moist chamber at 37°C for 30 min. Thereafter, they were rinsed in four changes of PBS and then four changes of distilled water and were air dried. Subsequently, 10 μl of a fluorescein isothiocyanate-conjugated rabbit anti-mouse immunoglobulin G (heavy and light chain specific; Nordic Immunological Laboratories), at a dilution of 1/50 containing 0.5% Evans blue as a counterstain, was added to the wells. The glass slides were incubated at 37°C in a moist chamber for 30 min, and thereafter, the slides were rinsed in PBS (two times for 5 min each time) and in distilled water (two times for 30 s each time) and were then air dried. The slides were mounted using glycerin and the fluorescence was observed.

**Agar overlay method.** Two isolates from a psittacine bird and one isolate from a duck that each reacted with two different serovar-specific MAbs were cloned by an agar overlay method. Tenfold dilutions of the three isolates, which reacted with two different serovar-specific MAbs, were inoculated onto BGM cells grown in 24-well dishes. After centrifugation (60 min, 500 × g, 37°C), the inoculum was removed and the monolayers were washed twice with PBS (pH 7.3). Minimum essential medium (MEM; 2x; Gibco, Ghent, Belgium) supplemented with 10% fetal calf serum (GIBCO), 2% vitamins for MEM (GIBCO), 2% t-glutamidine (GIBCO), 20 μl of streptomycin sulfate (1% [wt/vol] streptomycin sulfate; Sigma, Antwerp, Belgium) per ml, 40 μl of vancomycin (0.5% [wt/vol] vancomycin; Eli Lilly, Brussels, Belgium) per ml, 10 mg of glucose (Merck, Brussels, Belgium) per ml, and 4 μg of cycloheximide (Fluka Bio Chema, Leuven, Belgium) per ml was prepared and stored at 4°C for about 30 min. Indubiose (1.2%; IBK, Brussels, Belgium) was heated and added to the refrigerated MEM (2x). One milliliter of indubiose-MEM (2x) was added to the wells of a 24-well dish and allowed to polymerize. The dishes were then incubated at 37°C in 5% CO₂. Dishes were examined each day for the appearance of plaques. The plaques were collected and the clones were grown in BGM cells.

**RESULTS**

The 45 European isolates included 22 isolates from birds of the order Psittaciformes, 9 isolates from the order Columbiformes, 6 isolates from the order Galliformes, 5 isolates from the order Passeriformes, and 3 isolates from the order Anseriformes. They were all successfully serotyped. No additional serovars were found (Table 1).

Of the 45 isolates examined, 42 reacted only with one of the five serovar-specific MAbs at a dilution of 1/100. Of these 42 isolates, 20 isolates from psittacine birds, 3 isolates from pigeons, and 2 isolates from canaries belonged to serovar A (Table 2). Six isolates from pigeons, two isolates from canaries, two isolates from chickens, one isolate from a pheasant, one isolate from a turkey, and one isolate from a Gouldian finch belonged to serovar B (Table 2). One isolate from a duck and one isolate from a swan belonged to serovar C (Table 2). Two isolates from turkeys belonged to serovar D (Table 2). None of the 42 isolates was classified as serovar E.

Three of the 45 isolates examined, namely, one from a duck and two from psittacine birds, reacted strongly with one serovar-specific MAb at a dilution of 1/100 and gave a weak reaction with one other serovar-specific MAb at the same dilution. For these three isolates, cloning was performed. Of four clones obtained from the duck, three reacted with the serovar C-specific MAb and one reacted with the serovar B-specific MAb. Of four clones from isolate 91/1231 from a psittacine bird, two reacted with the serovar A-specific MAb and two reacted with the serovar B-specific MAb. After cloning of the remaining isolate 91/1099 from a psittacine bird, four clones reacted only with the serovar A-specific MAb.

All psittacine isolates examined reacted with the serovar A-specific MAb (psittacine serovar). One of these 22 isolates was shown to consist of a serovar A-specific and a serovar B-specific clone. For the other psittacine isolate, the clones that were obtained reacted only with the serovar A-specific MAb. Six of the nine isolates from the order Columbiformes examined belonged to serovar B (pigeon I serovar). The others belonged to serovar A (psittacine serovar). Three of the five isolates from birds of the order Passeriformes examined belonged to serovar B. The other two isolates belonged to serovar A. Two of the three isolates from birds of the order Anseriformes examined belonged to serovar C (duck serovar). After cloning and serotyping, the remaining isolate from a duck was demonstrated to consist of a serovar B-specific and a serovar C-specific clone. Four of the six isolates from birds of the order Galliformes examined belonged to serovar B (pigeon I serovar). The other two isolates belonged to serovar D (turkey serovar). Of the four isolates from turkeys, two were serotyped as the turkey serovar (serovar D) and two were serotyped as the pigeon I serovar (serovar B).

**DISCUSSION**

A panel of five serovar-specific MAbs against the five known avian serovars was used to classify 45 European avian isolates in an indirect microimmunofluorescence test. Serovar-specific MAbs are useful tools in classifying avian isolates into distinct serovars. When a positive reaction is observed, the specificity is such that one can be confident that the isolate is of the same serovar (2b). The indirect

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**TABLE 1. Results of serotyping of 45 European avian C. psittaci isolates**

<table>
<thead>
<tr>
<th>Order of host</th>
<th>Total no. of isolates</th>
<th>No. of isolates belonging to serovar*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>A</td>
</tr>
<tr>
<td>Psittaciformes</td>
<td>22</td>
<td>22</td>
</tr>
<tr>
<td>Columbiformes</td>
<td>9</td>
<td>3</td>
</tr>
<tr>
<td>Passeriformes</td>
<td>5</td>
<td>2</td>
</tr>
<tr>
<td>Anseriformes</td>
<td>3</td>
<td>1c</td>
</tr>
<tr>
<td>Galliformes</td>
<td>6</td>
<td>4</td>
</tr>
<tr>
<td>Total</td>
<td>45</td>
<td>27</td>
</tr>
</tbody>
</table>

* None of the isolates belonged to serovar E.

b Mixed infection with serovars A and B.

* Mixed infection with serovars B and C.
immunofluorescence test used for serotyping is easy to perform.

Andersen (1) typed several U.S. avian isolates. Results of the present studies indicate that the avian serovars found in Europe are the same as those found in the United States. All isolates could be classified under one of the five known serovars since no isolate was found to react only with the genus-specific MAb and not with a serovar-specific MAb.

Three isolates, two from psittacines and one from a duck, could not be classified into one distinct serovar. Cloning of these three isolates by an agar overlay method followed by serotyping of the clones showed that the duck isolate and one psittacine isolate each consisted of two different serovars. This study demonstrates that one host can be infected simultaneously with two different serovars. Double infections with C. trachomatis in humans have been reported (8, 10). To our knowledge, however, this is the first time that double infections with different C. psittaci serovars were unambiguously proven in animals. The clones of the other psittacine isolate reacted only with one serovar-specific MAb, although before cloning the isolate reacted with two different serovar-specific MAbs. Even though this could not be demonstrated by cloning, this bird was most likely infected with two different serovars.

The results indicate that birds of a certain order are more susceptible to a distinct serovar. This was also demonstrated by Andersen (1). Andersen (1) therefore designated the serovars according to the birds most frequently infected with this serovar. However, results of the present study demonstrate that birds of a certain order can be infected with strains of different serovars. We therefore propose that the serovars be designated A to E. We propose that letters instead of numbers be used because Perez-Martinez and Storz (11) already assigned the mammalian serovars of C. psittaci.
numbers from 1 to 9. Avian serovars definitely form a separate group (2), and thus, they should be labeled as a separate group. Numbers greater than those that Perez-Martinez and Storz (11) used could be given to additional mammalian serovars, which may be found in the future. Also, in the trachomatis group we do have the precedent that the trachoma agents have been given letters, and the lymphogranuloma venereum agents are numbered.

The use of the serotyping system presented here can lead to a better understanding of the epidemiology of *Chlamydia* infections in birds. Serotyping of avian *C. psittaci* strains can be useful not only for the study of the geographical distributions of the serotypes but also for the study of the distributions of serovars in avian, mammalian, or human hosts. The use of a panel of serovar-specific MAbs in diagnostic tests can both demonstrate the presence of *chlamydia* and determine the serovar of the strain involved. This can be important, since serovar-specific epitopes have been correlated with the pathogenicities of *Chlamydia* strains (14).

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**REFERENCES**

2a. **Andersen, A. A.** Unpublished data.