Chlamydial infections in duck farms associated with human cases of psittacosis in France

Karine Laroucau, Bertille de Barbeyrac, Fabien Vorimore, Maïthé Clerc, Claire Bertin, Taher Harkinezhad, Kristel Verminnen, Françoise Obeniche, Isabelle Capek, Christiane Bébéar, et al.

Title: Chlamydial infections in duck farms associated with human cases of psittacosis in France

Authors: Karine Laroucau, Bertille de Barbeyrac, Fabien Vorimore, Maïthé Clerc, Claire Bertin, Taher Harkinezhad, Kristel Verminnen, Françoise Obeniche, Isabelle Capek, Christiane Bébéar, Benoit Durand, Gina Zanella, Daisy Vanrompay, Bruno Garin-Bastuji, Konrad Sachse

PII: S0378-1135(08)00393-3
DOI: doi:10.1016/j.vetmic.2008.09.048
Reference: VETMIC 4178

To appear in: *VETMIC*

Please cite this article as: Laroucau, K., de Barbeyrac, B., Vorimore, F., Clerc, M., Bertin, C., Harkinezhad, T., Verminnen, K., Obeniche, F., Capek, I., Bébéar, C., Durand, B., Zanella, G., Vanrompay, D., Garin-Bastuji, B., Sachse, K., Chlamydial infections in duck farms associated with human cases of psittacosis in France, *Veterinary Microbiology* (2008), doi:10.1016/j.vetmic.2008.09.048

Chlamydial infections in duck farms associated with human cases of psittacosis in France

Karine Laroucau[(1)*], Bertille de Barbeyrac[(2)], Fabien Vorimore[(1)], Maïthé Clerc[(2)], Claire Bertin[(1)], Taher Harkinezhad[(3)], Kristel Verminnen[(3)], Françoise Obeniche[(2)], Isabelle Capek[(4)], Christiane Bébéar[(2)], Benoit Durand[(5)], Gina Zanella[(5)], Daisy Vanrompay[(3)], Bruno Garin-Bastuji[(1)], Konrad Sachse[(6)].

[(1)] Bacterial Zoonoses Unit, French Food Safety Agency (AFSSA), Maisons-Alfort, France

[(2)] National Reference Centre for *Chlamydiae*, Centre Hospitalier Universitaire, Bordeaux, France

[(3)] University of Ghent, Department of Molecular Biotechnology, 9000 Ghent, Belgium

[(4)] Institut de Veille Sanitaire, Saint-Maurice, France

[(5)] Epidemiology, French Food Safety Agency (AFSSA), Maisons-Alfort, France

[(6)] Institute of Molecular Pathogenesis, Friedrich-Loeffler-Institut (Federal Research Institute for Animal Health), Jena, Germany

[*]**Corresponding author**. Phone: (33) 1 49 77 13 00, Fax: (33) 1 49 77 13 44, E-mail address: k.laroucau@afssa.fr

Abstract

Five severe cases of psittacosis in individuals associated with duck farms were notified in France between January and March 2006. Diagnostic examination included serology and/or molecular detection by PCR from respiratory samples.

As a consequence, we investigated all duck flocks (n=11) that were housed in the three farms where human infections occurred. While serology by complement fixation test was negative for all samples, cloacal and/or tracheal chlamydial excretion was detected by PCR in all three units. Notably, one duck flock was tested strongly positive in 2 of the 3 affected farms, and *Chlamydophila (C.) psittaci* strains were isolated from cloacal and/or tracheal swab samples from both farms.

Human samples and duck isolates exhibited the same PCR-RFLP restriction pattern, which appeared to be an intermediate between genotypes A and B. Analysis of *omp*A gene sequences and comparison to those of the type strains showed that the isolates could not be strictly assigned to any of the generally accepted genotypes of *C. psittaci*. Further analysis by MLVA of the PCR-positive human samples revealed two distinct patterns, which were related to previously isolated *C. psittaci* duck strains.

Keywords: *Chlamydophila psittaci*, psittacosis, duck, genotyping, human infection

List of abbreviations

MLVA : Multiple Locus VNTR Analysis

MOMP: Major Outer Membrane Protein

VNTR: Variable Number of Tandem Repeats

PCR-RFLP: Polymerase Chain Reaction – Restriction Fragment Lengh Polymorphism

1.Introduction

Avian chlamydiosis is a well-recognised zoonotic disease caused by the obligate intracellular bacterium *Chlamydophila* (*C.*) *psittaci*, which occurs worldwide.

It was reported that more than 467 avian species could be affected by chlamydial infection (Kaleta and Taday, 2003). The infection is usually systemic and occasionally fatal in birds. The clinical signs vary greatly in severity and depend on the species and age of the bird, as well as the causative strain involved (Andersen, 1997). Avian strains of *C. psittaci* cover at least 6 serotypes (A to F) as determined by serovar-specific monoclonal antibodies (Andersen, 1991; 1997, Vanrompay *et al*., 1993). Each of these serotypes was assumed to be associated with a particular category of host species, *i.e.* type A and F with psittacines, type B with pigeons, type C with ducks, type D with turkeys, and type E with pigeons and ratites. More recently, molecular typing methods have been developed, such as restriction fragment length polymorphism of PCR products (PCR-RFLP), which identified genotypes that are adequately reflecting the serotypes (Sayada *et al*., 1995, Sudler *et al*., 2004).

As far as the transmission of *C. psittaci* strains from birds to humans is concerned, certain professions like veterinarians, bird breeders, handlers and traders have been reported to face a particular risk (Huminer *et al*., 1988, 1992, Hinton *et al*., 1993, Saito *et al*., 2005). Typical transmission pathways involve inhalation of infectious aerosols during handling of infected animals, carcasses or tissues. Besides, contaminated faeces and feathers may play an essential role in zoonotic transmission. The symptoms of human psittacosis are variable, ranging from severe systemic disease to the complete absence of clinical signs (Andersen and Vanrompay, 2000).

C. psittaci infection in ducks has been reported previously in Europe, Australia and the USA (Chalmers *et al*., 1985, Arzey *et al*., 1990, Hinton *et al*., 1993, Léon *et al*., 2004, Guérin *et al*., 2006). Most of the recent human cases seemed to be linked to domestic poultry rather than psittacines (Heddema *et al*., 2006; Gaede *et al*. 2008).

In France, five cases of severe pneumonia were observed in February and March 2006. As the infected individuals were in close contact with farmed birds, avian influenza had been initially suspected, but was not confirmed by diagnostic findings. Subsequently, samples from patients and animals present at the three farms affected were examined for chlamydial infection.

In the present paper, diagnostic data from real-time PCR, serology and genotyping by PCR-RFLP and multi-locus VNTR analysis (MLVA) are reported to characterise human and animal cases, and the prevailing epidemiological situation is described.

2. Materials and methods

2.1. Humans

2.1.1 Samples

Respiratory samples (throat swabs, tracheal aspirates or bronchoalveolar fluid) from four patients were collected during their hospitalisation, stored at – 80°C and sent to the National Reference Centre (NRC, Bordeaux, France) for chlamydiae in dry ice. Aliquots of early serum from each patient and late serum from two patients were also sent to the NRC (for dates of sampling see Table 1).

2.1.2. Direct detection of *C. psittaci* from human samples

Respiratory samples were analysed for *C. psittaci* using a specific *inc*A real-time PCR protocol (Ménard *et al*., 2006) and by cell culture in order to obtain isolates. For PCR, clinical samples were extracted by using the automated MagNA Pure DNA extraction (Roche Diagnostics, Meylan, France) (De Martino *et al*., 2006). The TaqMan real-time PCR was carried out in a final volume of 25 µl containing 5 µl of extracted DNA using an ABI Prism7000 thermocycler (Applied Biosystems, Courtaboeuf, France).

Cell culture was performed on McCoy cells in minimal essential medium supplemented with 1 mM glutamine, cycloheximide, glucose and 5% foetal bovine serum in a biosafety level 3 laboratory. Monolayers were made on 15 mm round coverslip in 1-dram shell vials and inoculated with 500 µl of sample. After 48 h incubation, the monolayers were fixed and stained with monoclonal antibodies recognising genus-specific chlamydial LPS (ImagenTM Chlamydia, Dako, I2L Elitech, Labarthe Inard, France).

2.1.3. Serology

A commercialised immunofluorescence test was used (Chlamydia MIF, Focus, Eurobio, France). This assay can measure responses to IgM and IgG subclasses. Each well contains four spots, one yolk sac

control, and three individual antigen spots consisting of elementary bodies of *C. psittaci*, *C. trachomatis* and *C. pneumoniae* suspended in a yolk sac matrix. Each run included a positive (murine serum) and negative control (human serum). The reciprocal of the highest serum dilution giving apple-green fluorescence was termed the serum endpoint titre for IgG. For IgM, one dilution was tested (1/16), and the result was assessed qualitatively, *i.e*. positive or negative.

2.2. Ducks

2.2.1. Samples

Samples were collected from duck flocks present in the 3 farms (A, B and C) affected by human cases. While all duck flocks present in plants A (06-0859, 06-0870, 06-0871) and B (06-0885, 06-0886 and 06-0889) were sampled, due to the large size of farm C (60 000 ducks in 15 farm premises), only the 5 flocks related to the two human cases were included in the sampling (06-1018, 06-1019, 06-1020, 06-1021 and 06-1022). In each sampled flock, 20 animals were examined. Ducks were submitted to bleeding, and cloacal and tracheal swabbing. One panel of swabs was stored in conservation buffer SPG (Spencer and Johnson, 1983) at – 80°C until inoculated into chicken eggs. The other panel was stored dry at – 80°C until subjected to DNA extraction. Age and bleed information of the animals examined are summarised in Table 2.

2.2.2. Direct detection of chlamydiae from avian samples

The dry panel of cloacal and tracheal swabs was subjected to DNA extraction using a QIAamp DNA Mini Kit, following the buccal swab protocol (Qiagen, Courtaboeuf, France). DNA was eluted with 150 µl of AE buffer and stored at –20°C before analysis.

A *Chlamydiaceae*-specific real-time PCR targeting the 23S rRNA gene was used in this study (Ehricht *et al*., 2006). The protocol includes primers Ch23S-F (5'-CTGAAACCAGTAGCTTATAAGCGGT-3'), Ch23S-R (5'-ACCTCGCCGTTTAACTTAACTCC-3'), and probe Ch23S-p (FAM-5'-CTCATCATGCAAAAGGCACGCCG-3'-TAMRA). Each reaction mix contained 2 µl sample DNA template, 10 µl of Universal Mastermix 2X (Applied Biosystems), 0.5 µl of each primer (25 µM) and 2 µl of the probe (1 µM), and 5 µl deionized water. The temperature-time profile was 95°C 10 min, 45 cycles of 95°C 15 s, 60°C 60 s.

2.2.3. Inoculation onto chicken eggs

For cell culture, suspensions of cloacal and tracheal swabs stored in conservation buffer at – 80°C were thawed, transferred into sterile Eppendorf tubes and centrifuged at 10,000 rpm for 5 min. Supernatant was transferred into a new sterile tube. Antibiotic solution containing 0.1 mg of vancomycine, 0.1 mg of streptomycin, 0.1 mg of kanamycin and 100 U of nystatin was added to the supernatant and the pellet suspensions, which were then incubated at 37°C for 2 h before inoculation. Yolk sacs of 7 day-old embryonated eggs were inoculated with 0.2 ml per egg, and 5 eggs per sample. For each set of inoculation, 3 eggs were inoculated with *C. psittaci* Loth strain as positive control, and 3 other eggs were kept separately as non-infected controls. Eggs were incubated at 38°C and observed daily.

Vitellus membranes were collected, then analysed by a MIF test using a direct immunofluorescence assay (Chlamydia direct IF, BioMérieux, Marcy l'Etoile, France).

2.2.4. Complement fixation test

A microtiter complement fixation test (CFT) for chlamydial antibodies was performed according to the national standard (Collective, 2000). Sera were treated at 59°C for 30 min before testing. Guinea pig complement was used at 2 units, and a commercial ornithosis antigen (Dade Behring) was used at the recommended dilution. The presence of 50% of haemolysis starting from the 1/8 dilution of sera was considered positive.

2.2.5 MOMP ELISA test

The recombinant major outer membrane protein (rMOMP) ELISA was performed on duck sera being pre-treated with kaolin to remove background activity (Novak *et al*., 1993). Major outer membrane protein-specific antibody titers were determined using standard protocols and microwell plates coated with rMOMP of the genotype D *C. psittaci* strain 92/1293 expressing *Chlamydiaceae* family-specific epitopes. rMOMP was produced in pcDNA1::MOMPHis transfected COS7 cells as described previously (Vanrompay *et al*., 1998). 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% foetal calf serum (Invitrogen, Merelbeke, Belgium). Transfection with plasmid DNA was performed by the DEAE dextran method. Forty-eight hours post transfection, rMOMP production was monitored by an indirect

immunofluorescence staining using serovar D and family-specific monoclonal antibodies (Vanrompay *et al*., 1998) where after the tissue culture flasks were stored at –70 °C. His-tag labelled rMOMP was purified by BD TalonTM (BD Biosciences, Erembodegem, Belgium) affinity chromatography and the protein concentration was determined by the bicinchoninic acid protein assay (Sigma, Bornem, Belgium). MOMP-specific antibody titres were determined using two-fold dilutions of duck sera (starting at a dilution of 1 : 100). For the determination of antibody titers, 1:1000 and 1:2000 dilutions of biotinylated rabbit anti-duck IgG (H+L) antibody (Nordic Immunology) and peroxidase-conjugated streptavidin (Invitrogen) 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. Genotyping

2.3.1. PCR-RFLP analysis

*Alu*I and *Mbo*II-based PCR-RFLP analysis was performed as previously described (Sayada *et al*. 1995). Fragments were separated on a 2% Nusieve agarose gel. Representative chlamydial strains of all known avian serovars were included in the study *i.e.* VS1 (genotype A), CP3 (genotype B), GR9 (genotype C), NJ1 (genotype D), Cal10 (genotype E) and VS225 (genotype F) *C. psittaci* reference strains.

2.3.2. Sequencing

Partial *omp*A gene fragments were amplified from human samples and animal strains as described previously using ML-genoI and ML-genoII primers (Geens *et al*., 2005). DNA sequencing of these products was done at MWG (Biotech France, Roissy, France).

2.3.3. Multi-locus VNTR analysis (MLVA)

DNA extracted from human samples and duck isolates were analysed according to a MLVA procedure (Laroucau *et al*., 2008). Briefly, PCR was performed in a total volume of 15 µl containing 5-10 ng of DNA, 1× PCR reaction buffer, 1 U of Hot start Taq DNA polymerase (Qiagen), 200 µM of each deoxynucleotide triphosphate, and 0.3 µM of each flanking primer (ChlaPsi_280, ChlaPsi_480, ChlaPsi_605, ChlaPsi_810, ChlaPsi_222, ChlaPsi_281, ChlaPsi_929 or ChlaPsi_1778). The initial

denaturation step at 95°C for 15 min was followed by 40 cycles consisting of denaturation at 95°C for 30 s, primer annealing at 57°C or 60°C for 30 s depending on the primers, and elongation at 72°C for 45 s. The final extension step was at 72°C for 10 min. Five microlitres of amplification product were loaded onto a 4% Metaphor/standard (50-50) agarose. Gels stained with ethidium bromide were visualized under UV light, and photographed. The size marker used was a 100-bp ladder (Mbi, Euromedex, France).

3. Results

3.1. Description of human cases

Between February and March of 2006, four persons working with poultry were admitted to hospital reporting signs of respiratory distress. After avian influenza was ruled out, psittacosis was suspected. A summary of diagnostic data and information on the patients is given in Table 1. Cases 1 and 2 were a married couple working at farm A. The husband had been involved in duck breeding activities for years, and his wife was an occasional assistant worker. While the woman was admitted to resuscitation, the husband was hospitalised for pneumonia. Tracheal aspiration samples collected from the woman proved positive by PCR and culture. Serological analysis of the man's sera revealed an increase of antibody level to chlamydiae. Case 3 was the wife of a duck farm worker (farm B). As she developed respiratory failure, she was transferred to the intensive-care unit for mechanical ventilation. The tracheal aspiration sample was tested positive by PCR. Cases 4 and 5 represent a woman and a man who were working at the intensive breeding farm C. While the fourth case was diagnostically confirmed by PCR, case number 5, which was not hospitalised, could not be finally confirmed as samples for PCR diagnosis were not available. Nevertheless, the latter can be regarded as a likely case of psittacosis because of the symptoms, the increase in antibody titers and the close link with case number 4.

3.2. Investigation in duck farms associated to cases of psittacosis

All bird flocks present at farms A and B were sampled and examined. While serological testing by CFT was negative for all animals examined, cloacal and/or tracheal chlamydial excretion was detected by PCR in all 6 flocks (Table 1, Figure 1 and data not shown). In each farm, one flock showed a high level of chlamydial excretion (flocks 06-0859 and 06-0889) in both cloacal and conjunctival swabs.

The other flocks appeared to be less strongly infected, and chlamydiae were only detected in a few ducks, and mainly from cloacal swabs.

As farm C was in an intensive breeding farm, sampling was restricted to the flocks to which the diseased persons were supposed to have had close contact. Chlamydial excretion was detected only in very few animals, and at a low level in 4/5 flocks (Table 1 and Figure 1). Serological testing by CFT was negative for all animals examined (data not shown).

Duck sera from these 3 farms were tested by ELISA in order to specifically screen for MOMP antibodies. Positive responses were observed for some samples and the highest titres were obtained for farm C (Table 1 and Figure 2).

In the first cell culture trial, PCR-positive samples were inoculated into embryonated chicken eggs. Isolates were successfully cultivated from flocks 06-0859, 06-0871, 06-0886, 06-0889 (farms A and B). In a second trial, PCR-negative samples from flocks 06-0870 and 06-0885 were inoculated as PCR-positive samples failed to produce isolates. One isolate was successfully cultured from flock 06-0885. No strain was isolated from flock 06-870, as well as from any cultivated sample from farm C (Table 2).

3.3. Genotyping of isolates

Isolates from ducks were first analysed by PCR-RFLP using primers CTU/CTL. Based on restriction patterns generated by enzymes *Alu*I and *Mbo*II, isolates 06-0859, 06-0871, 06-0885, 06-0886, 06-0889 showed similar patterns. The *Alu*I RFLP profile was identical to a genotype B profile (CP3), whereas their *Mbo*II RFLP profile was identical to those of a genotype A strain (VS1) (Figure 3 and data not shown).

In order to verify the genotyping results, *omp*A sequencing was performed on samples collected from the confirmed human cases 1, 3 and 5 (one from each farm), as well as on all the duck isolates obtained. All sequences were identical, but not completely homologous to the *C. psittaci* sequences deposited in public databases. The sequence of the isolate obtained from flock 06-859 has been deposited under GenBank acc.no. EU159263.

Furthermore, human samples and duck strains were analysed by MLVA using primers ChlaPsi_280, ChlaPsi_480, ChlaPsi_605, ChlaPsi_810, ChlaPsi_222, ChlaPsi_281, ChlaPsi_929 and ChlaPsi_1778. With these 8 primers, similar patterns (i.e. 1:3:2:-:6:3:-:4) were obtained for all duck

strains and human samples from farms A and B, whereas a 2:3:2:-:6:3:-:4 pattern was obtained for the human sample of case 4 from farm C (Figure 4 and data not shown).

4. Discussion

The present investigation conducted around severe human cases of respiratory distress led to the confirmation of psittacosis by PCR in three cases and the demonstration of the presence of *C. psittaci* in all three duck farms concerned.

In the past, significant morbidity and mortality rates were found in infected duck flocks, which apparently depended on age and the presence of concurrent infection with salmonellae. Henceforth, disease signs in outbreaks which occurred in European duck farms in recent decades appeared to be minimal or entirely absent (Andersen *et al*., 1997), as observed in our study. However, in this case, chlamydial excretion was clearly observed in many of the ducks. The use of real-time PCR allowed evaluating the level of excretion and, consequently, the level of exposure for the personnel. In two of the farms (A and B), a single flock was identified as strongly excreting, whereas in the third farm, the level of excretion was much lower and confined to only few animals. Considering this third farm, the link between bacterial load and human infection was less evident, but artificial insemination activities performed on that farm imply close contact between animals and humans. In this farm, the first clinical signs in the fifth patient appeared at the end of the December, whereas the earliest symptoms from the other patient emerged about 60 days later. As for the 2 other farms, no clinical signs were observed with the animals of farm C. It can be hypothesised that these animals were continuous low-level shedders, which would mean a regular exposition of the workers. In future studies, it would be interesting to test environmental samples such as air, ventilation system, air filters, cages etc. in order to assess the contamination level of the premises.

The apparent variation in pathogenicity of *C. psittaci* strains has been attributed to either a change in virulence or improved control of other synergistic pathogens (Andersen *et al*., 1997). Another possible explanation could be the presence in the flocks of different chlamydial genotypes that induce different pathology in ducks. Within the species of *C. psittaci*, avian strains can be differentiated by serological (Andersen, 1991, 1997) or molecular tools (Sayada *et al*., 1995) and classified into serotypes or genotypes, respectively. Until now, very few isolates from European outbreaks related to ducks have been serotyped, all of them being serotype C, one of them occurring in a mixed infection with serovar

B (Vanrompay and Andersen, 1993). In the present study, molecular genotyping performed on human samples and animal isolates led to inconclusive results from PCR-RFLP, since the pattern observed was an intermediate between genotypes A (*Alu*I) and B (*Mbo*II). Sequences of the *omp*A gene from duck and human samples were identical (GenBank acc.no. EU159263), but at the same time unique in that they were not completely homologous to any other sequence deposited in GenBank. Multiple sequence alignment and DNA microarray analysis revealed that the respective strain belongs to genotype E/B, where it represents a hitherto unknown subtype designated EB-859 (Sachse *et al*., 2008). When the MLVA, which is based on the detection of tandem repeats dispersed throughout the genome, was applied to these samples (from humans and ducks), it led to identification of two distinct patterns which were also found in other duck isolates showing different PCR-RFLP patterns (E, C or inconclusive) (Laroucau *et al*., 2008). These two patterns were also observed in a study conducted on chlamydia-positive specimens from ducks sampled in a major breeding area (Laroucau, unpublished data). This means that, despite the identity of *omp*A sequences, different *C. psittaci* isolates occurred in these duck flocks. It seems that only more sophisticated tests of high discriminatory capacity and more detailed screening could reveal the entire genetic diversity of *C. psittaci* strains present.

The confirmed human cases reported here concerned either persons having had occasional contact with ducks or individuals working at artificial insemination. Regarding other studies carried out in France, it appears that chlamydial infections in duck breeding are very widespread (Léon *et al*., 2004; Guérin *et al*., 2006). Nevertheless, these serious human cases seem to be exceptional and could be explained by the late stage of ongoing antibiotic treatment of the patients, as avian flu was initially suspected. Nevertheless, owing to the fact that psittacosis is not a notifiable disease in France, it is also probable that other cases have remained undiagnosed. The present human cases were notified to the National Institute for Public Health Surveillance (InVS) when avian flu alert was activated, and cases of pneumonia in relation with poultry had to be declared.

It is not surprising, as was previously observed (Arzey *et al.,* 1990), that all duck sera were negative by CFT serology, although intense excretion was clearly demonstrated in this study. The lack of sensitivity of CFT prompted us to use a *C. psittaci* MOMP ELISA (Verminnen *et al*., 2006). Samples generating the highest positive responses were those from farm C, in which less/no excretor animals were detected. The most important serological responses were observed in the oldest flocks (25 week-old versus 4-14 week-old), which were also of a different breed (Pekin instead of mallard). In this

context, breed, age, breeding techniques could have an impact on the chlamydial infection. It is possible that in farm C, animals were old enough to control the infection. No information is available to find out whether farm C had gone through a similar excretion patter as farms A and B beforehand. In farm C, culture was negative, but chlamydial DNA and high antibody titers were still detectable in 25 week-old ducks. It is possible that the other two farms (A and B) were dealing with a more recent, acute infection. Except for flock 06-0886, all flocks had at least one IgG positive sample, but in most of the positive flocks (except 06-0859) only a few animals showed IgG titres. It is known that the onset of antibody production in the host is deferred after primary contact with the pathogen. In turkeys, depending on the infective dose and the strain, the cloaca becomes positive at 5 to 8 days post infection (Vanrompay *et al*., 1995) and IgM antibodies appear at 7 days post infection, IgG antibodies appear later on between 10 and 14 days post infection, reaching their highest level at 3 weeks post infection (Van Loock *et al*., 2006). The conjugate should detect IgG and IgM in theory, because it detects the heavy and light chains. Thus, either no antibodies at all or predominantly IgM were present in flocks of farm A (except 06-0859) and B, and, therefore, the ELISA was negative for most animals even though chlamydial DNA could be detected and in some cases isolates could be grown. These discrepancies require further studies.

The data of the present study confirm that epidemiological investigation of psittacosis in ducks cannot be done using serology alone as no correlation between antibody presence and excretion was observed, but direct DNA-based or antigen-based detection of the chlamydiae and/or isolation are highly desirable.

In conclusion, the findings of the present study demonstrate that chlamydial infection of duck flocks is a public health concern. It can be recommended that efficient measures be taken to ensure a high health status of duck flocks, and chlamydia monitoring be conducted on a more regular basis. This work is an example of cooperation between veterinary and human medicine being fully complementary and, at the same time, indispensable to improve our knowledge on avian chlamydiosis.

Conflict of interest statement

None of the authors (KL, BdB, FV, MC, CB, TH, KV, FO, IC, CB, BD, GZ, DV, BGB, KS) has a financial or personal relationship with other people or organisations that could inappropriately influence or bias this paper.

Acknowledgements

We are grateful to R. Goffette, R. Delavergne, F. Pouilly, A. Thébault from French veterinary services and to C. Larsen of the National Institute for Public Health Surveillance (InVS) for their precious help. We are also grateful to D. Toquin (AFSSA Ploufragan) for providing EOPS duck sera.

The study is an integral part of the European COST Action 855, "Animal chlamydioses and the zoonotic implications".

References

Andersen, A.A. 1991. Serotyping of *Chlamydia psittaci* isolates using serovar-specific monoclonal antibodies with the microimmunofluorescence test. J. Clin. Microbiol. 29: 707-711.

Andersen, A.A. 1997. Two new serovars of *Chlamydia psittaci* from North American birds. J. Vet. Diag. Invest. 9: 159-164.

Andersen, A.A., Grimesn, J.E., Wyrick, P.B. 1997. Chlamydiosis (psittacosis, ornithosis) *in* diseases of poultry, 10th edition, Iowa state university press, Ames, Iowa, USA. p 333-349.

Andersen, A.A., Vanrompay, D. 2000. Avian chlamydiosis. Rev. Sci. Tech. 19: 396-404.

Arzey, K.E., Arzey, G.G., Reece, R.L. 1990. Chlamydiosis in commercial ducks. Aust. Vet. J. 67(9): 333-334.

Vanrompay, D., Andersen, A.A., Ducatelle, R., Haesebrouck, F. 1993. Serotyping of European isolates of *Chlamydia psittaci* from poultry and other birds. J. Clin. Microbiol. 31: 134-137.

Chalmers, W.S., Farmer, H., Woolcock, P.R. 1985. Duck hepatitis virus and *Chlamydia psittaci* outbreak. Vet. Rec. 116(8): 223.

368 Collective, 2000. Méthodes d'analyse en santé animale : recherche d'anticorps contre la chlamydiose
369 chez les oiseaux par la technique de fixation du complément. Norme Française AFNOR NF U-
370 47-007.
371 De Martino, S.J., de Barbeyrac, B., Piémont, Y., Barthel, C., Monteil, H., Jaulhac, B. 2006. Detection
372 of *Chlamydia trachomatis* DNA using MagNA Pure DNA extraction and Cobas Amplicor
373 CT/NG amplification. Clin. Microbiol. Infect.12: 576-579.
374 Ehricht, R., Slickers, P., Goellner, S., Hotzel, H., Sachse, K. 2006. Optimized DNA microarray assay
375 allows detection and genotyping of single PCR-amplifiable target copies. Mol. Cell. Probes
376 20:60-63
377 Gaede, W., Reckling, K.F., Dresenkamp, B., Kenklies, S., Schubert, E., Noack, U., Irmscher, H.M.,
378 Ludwig, C., Hotzel, H., Sachse, K. 2008. *Chlamydophila psittaci* infections in humans during
379 an outbreak of psittacosis from poultry in Germany. Zoonoses & Public Health. 55(4):184-188.
380 Geens, T., Desplanques, A., Van Loock, M., Bonner, B.M., Kaleta, E.F., Magnino, S., Andersen, A.A.,
381 Everett, K.D., Vanrompay, D. 2005. Sequencing of the *Chlamydophila psittaci omp*A gene
382 reveals a new genotype, E/B, and the need for a rapid discriminatory genotyping method. J.
383 Clin. Microbiol. 43(5): 2456-2461.
384 Guérin, J.L., Ballot, A., Sraka, B., Léon, O. 2006. Portage de *Chlamydophila psittaci* dans la filière
385 canard mulard : évaluation du portage chez les reproducteurs et incidence sur le statut du
386 caneton. Proceedings des 7èmes Journées de la recherche sur les palmipèdes à foie gras, 18-
387 19 octobre 2006, Arcachon, France. p 37-40.
388 Heddema, E.R., van Hannen, E.J., Duim, B., Vandenbroucke-Grauls, C.M., Pannekoek, Y. 2006.
389 Genotyping of *Chlamydophila psittaci* in human samples. Emerg. Infect. Dis. 12(12): 1989-
390 1990.
391 Hinton, D.G., Shipley, A., Galvin, J.W., Harkin, J.T., Brunton, R.A. 1993. Chlamydiosis in workers at a
392 duck farm and processing plant. Aust. Vet. J. 70(5): 174-176.
393 Huminer, D., Pitlik, S., Kitayin, D., Weissman, Y., Samra, Z. 1992. Prevalence of *Chlamydia psittaci*
394 infection among persons who work with birds. Isr. J. Med. Sci. 28(10): 739-741.
395 Huminer, D., Samra, Z., Weisman, Y., Pitlik, S. 1988. Family outbreaks of psittacosis in Israel. Lancet.
396 2(8611): 615-618.

Kaleta, E.F., Taday, E.M. 2003. Avian host range of *Chlamydophila* spp. based on isolation, antigen detection and serology. Avian Pathol. 32(5): 435-461.

Laroucau, K., Thierry, S., Vorimore, F., Blanco, K., Kaleta, E., Hoop, R., Magnino, S., Vanrompay, D., Sachse, K., Myers, G., Bavoil, P., Vergnaud, G., Pourcel, C. 2008. High resolution typing of *Chlamydophila psittaci* by Multilocus VNTR Analysis (MLVA). Infect Genet Evol. 8(2): 171-181.

Léon, O., Sraka, B., Ballot, A., Armand, C., Guérin, J.L. 2004. Evaluation du portage de *Chlamydophila psittaci* au sein de la filière canards gras : implications pour la santé publique. Proceedings des 6èmes Journées de la recherche sur les palmipèdes à foie gras, 7-8 octobre 2004, Arcachon, France.

Loock, M.V., Loots, K., Zande, S.V., Heerden, M.V., Nauwynck, H., Goddeeris, B.M., Vanrompay, D. 2006. Pathogenic interactions between *Chlamydophila psittaci* and avian pneumovirus infections in turkeys. Vet Microbiol. 112(1):53-63.

Ménard, A., Clerc, M., Subtil, A., Megraud, F., Bebear, C., de Barbeyrac, B. 2006. Development of a real-time PCR for the detection of *Chlamydia psittaci*. J. Med. Microbiol. 55: 471-473.

Novak, M., Moldoveanu, Z., Schafer, D.P., Mestecky, J., Compans, R.W. 1993. Murine model for evaluation of protective immunity to influenza virus. Vaccine 11: 55–60.

Sachse, K., Laroucau, K., Hotzel, H., Schubert, E., Ehricht, R., Slickers, P. 2008. Genotyping of *Chlamydophila psittaci* using a new DNA microarray assay based on sequence analysis of *omp*A genes. BMC Microbiology 8, 63 (17Apr2008).

Saito, T., Ohnishi, J., Mori, Y., Iinuma, Y., Ichiyama, S., Kohi, F. 2005. Infection by *Chlamydophilia avium* in an elderly couple working in a pet shop. J. Clin. Microbiol. 43(6): 3011-3013.

Sayada, C., Andersen, A.A., Storey, C., Milon, A., Eb, F., Hashimoto, N., Hirai, K., Elion, J., Denamur, E. 1995. Usefulness of *omp*1 restriction mapping for avian *Chlamydia psittaci* isolate differentiation. Res. Microbiol. 146(2): 155-165.

Spencer, W., Johnson, F. 1983. Simple transport medium for the isolation of *Chlamydia psittaci* from clinical material. Vet. Rec. 113: 535-536.

Sudler, C., Hoelzle, L.E., Schiller, I., Hoop, R.K. 2004. Molecular characterisation of chlamydial isolates from birds. Vet. Microbiol. 98(3-4): 235-241.

425 Vanrompay, D., Mast, J., Ducatelle, R., Haesebrouck, F., Goddeeris, B. 1995. *Chlamydia psittaci* in
426 turkeys: pathogenesis of infections in avian serovars A, B and D. Vet Microbiol. 47(3-4):245-
427 256
428 Vanrompay, D., Cox, E., Mast, J., Goddeeris, B., Volckaert, G. 1998. High level expression of
429 *Chlamydia psittaci* major outer membrane protein in COS cells and in skeletal muscles of
430 turkeys. Infect. Immun. 66: 5494–5500.
431 Verminnen, K., Van Loock, M., Hafez, H.M., Ducatelle, R., Haesebrouck, F., Vanrompay, D. 2006.
432 Evaluation of a recombinant enzyme-linked immunosorbent assay for detecting
433 *Chlamydophila psittaci* antibodies in turkey sera. Vet Res. 37(4):623-632.

Figure 1

Monitoring of cloacal (C) and tracheal (T) chlamydial excretion in ducks from all flocks under investigation using quantitative real-time PCR: average Ct values and standard deviations for each studied flocks.

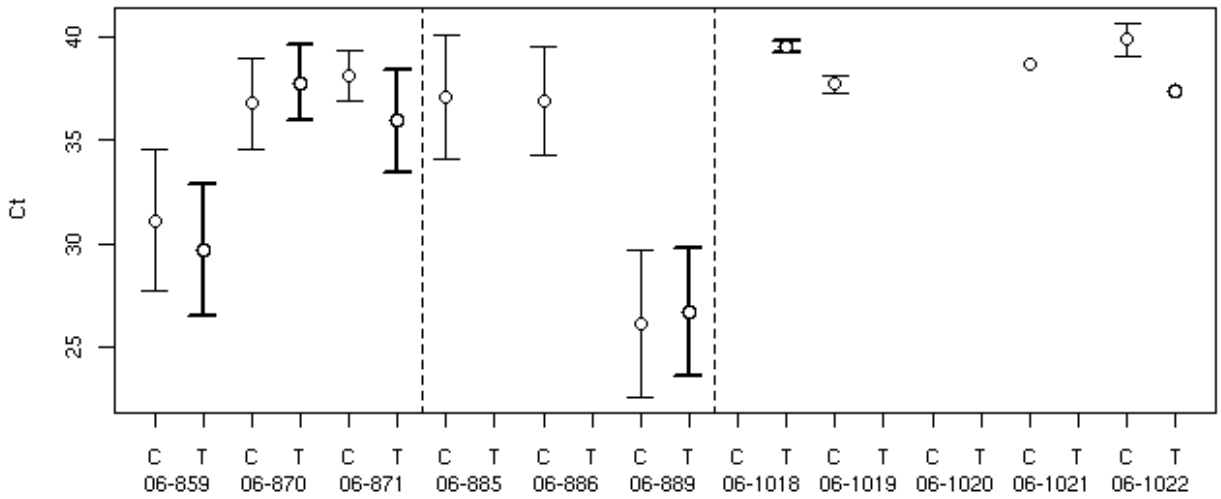

Figure 2

Monitoring of MOMP antibodies in duck sera from all flocks using ELISA: average titre (log10) and standard deviations for each studied flock. The titre corresponds to the final serum dilution being positive.

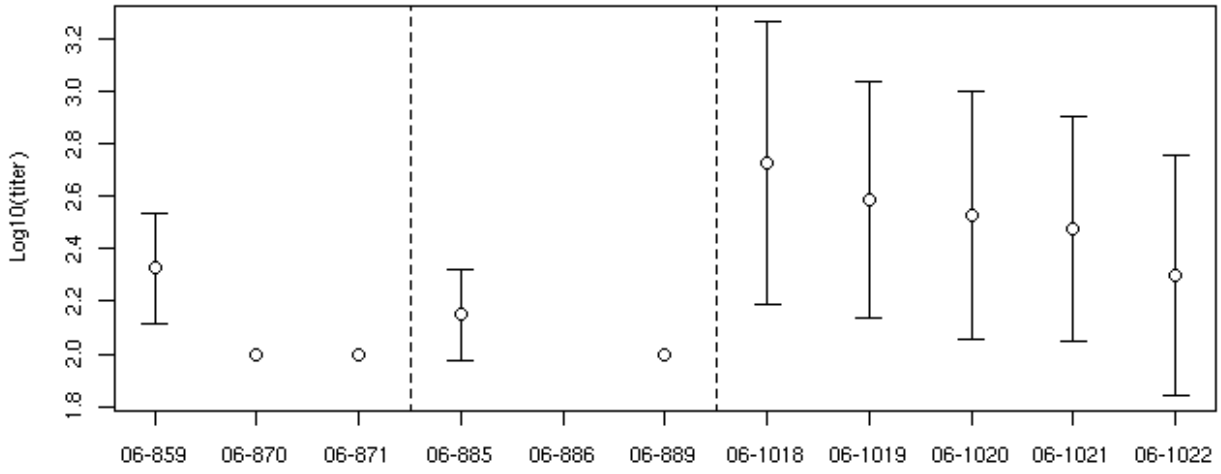

Figure 3

Enzyme restriction profiles of CTU/CTL PCR products for 6 representative avian isolates (VS1, CP3, GR9, NJ1, Cal10 and VS225) (Lanes 1 to 6) and for 2 duck isolates 06-0859 (flock A) and 06-0889 (flock B) (Lanes 7, 8). A 100-bp ladder (Mbi, Euromedex, France) is run on the side of each group of samples.

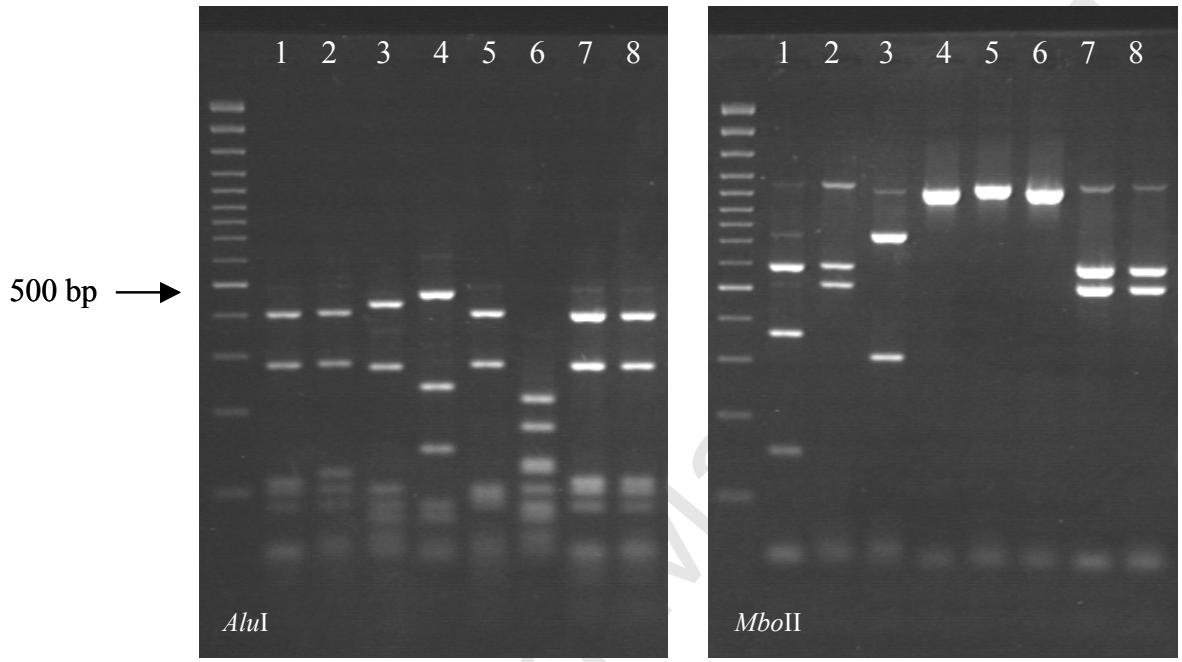

Figure 4

PCR amplification of 8 VNTRs (ChlaPsi_280, ChlaPsi_480, ChlaPsi_605, ChlaPSi_810, ChlaPsi_222, ChlaPsi_281, ChlaPsi_929, ChlaPsi_1778) on human samples confirmed positive by PCR (A: case n°2; B: case n°3 and C: case n°4) (seeTable 1).

A 100-bp ladder (100 to 1,000 bp) is run on both sides of each group of samples. The number of repeat units within each allele is indicated.

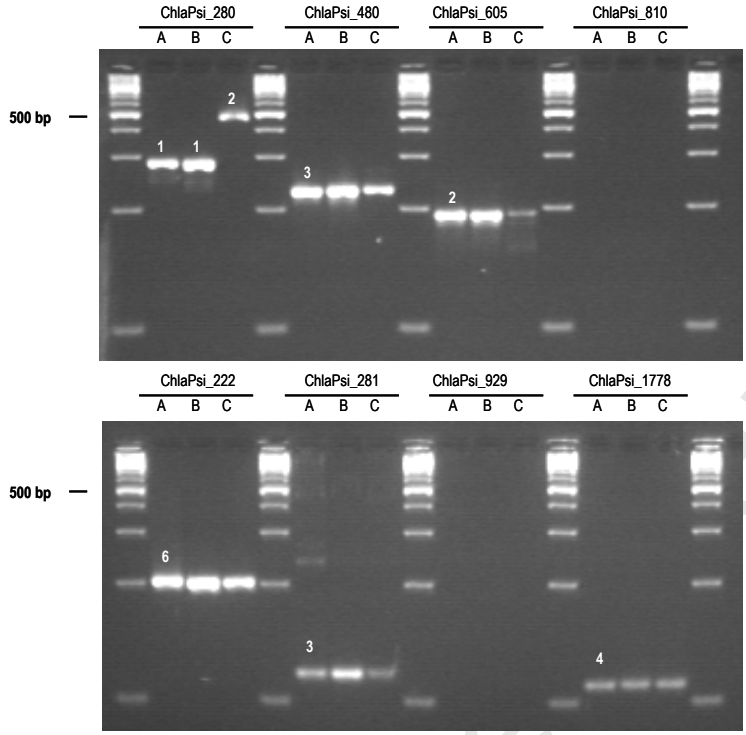

Table 1

Human cases: clinical signs and and diagnostic data

	N° case	Sex	Age (years)	First clinical signs	Hospitalisation	Clinical diagnosis	Serology date	Serology results	Respiratory samples	PCR	Culture
Plant A	1	M	43	19/02/2006	27/02/2006	Pneumonia	01/03/2006	IgG<1/16 IgM positive	Throat swab sample	negative	negative
							03/03/2006	IgG 1/128 IgM positive			
	2	F	46	20/02/2006	26/02/2006	Pneumonia + ARDS	27/02/2006	IgG 1/256 IgM positive	Tracheal aspiration	positive	positive
Plant B	3	F	51	20/02/2006	27/02/2006	Pneumonia + ARDS	03/03/2006	IgG 1/16 IgM negative	BAL	positive	negative
Plant C	4	F	44	01/03/2006	04/03/2006	Pneumonia + ARDS	06/03/2006	IgG < 1/16 IgM negative	Tracheal aspiration	positive	negative
	5	M	38	26/12/2005	-	Pneumonia	05/01/2006	IgG 1/64 IgM negative	ND	ND	ND
							23/02/2006	IgG 1/128 IgM negative			

ARDS: acute respiratory distress syndrome; BAL: broncho-alveolar fluid; ND: not done.

Table 2

Characteristics of investigated duck flocks

					PCR positive samples				culture positive samples		ELISA	
Flock identification	Race	Age (week-old)	Effective	Production	trachea	mean of Ct value (trachea)	cloacae	mean of Ct value (cloacae)	trachea	cloacae	positive samples	mean of the final serum dilution being positive
Plant A						33,0		34,0				
06-0859	mallard	4	4000	fattening	19/20	29,7	20/20	31,1	3/3	nd	11/20	236
06-0870	mallard	8	4500	fattening	6/20	37,8	9/20	36,8	0/6	0/9	2/20	100
06-0871	mallard	11	4700	fattening	12/20	36,0	8/20	38,2	4/4	nd	2/19	100
Plant B						26,7		30,8				
06-0885	mallard	13	400	fattening	0/20	na	4/20	37,1	0/9	1/17	4/20	150
06-0886	mallard	14	1300	fattening	0/20	na	11/20	36,9	nd	2/4	0/19	0
06-0889	mallard	5	1500	fattening	20/20	26,7	20/20	26,1	3/3	nd	1/20	100
Plant C						38,8		38,8				
06-1018	pekin	25	1400	reproduction	2/20	39,6	0/20	na	0/2	0/1	19/20	1015
06-1019	pekin	25	1500	reproduction	0/20	na	3/20	37,7	0/3	0/19	20/20	660
06-1020	pekin	25	900	reproduction	0/20	na	0/20	na	nd	nd	16/20	625
06-1021	pekin	25	-	reproduction	0/20	na	1/20	38,7	nd	0/1	17/20	529
06-1022	mallard	-	6000	fattening	1/20	37,4	3/20	39,9	0/1	0/3	7/20	371