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Isolation of a new chlamydial agent from infected domestic poultry coincided with cases of atypical pneumonia among slaughterhouse workers in France

Karine Laroucau [a,*], Fabien Vorimore [a], Rachid Aaziz [a], Angela Berndt [b], Evelyn Schubert [b], Konrad Sachse [b]

[a] Bacterial Zoonoses Unit, French Food Safety Agency (AFSSA), 23 avenue du Général de Gaulle, 94706 Maisons-Alfort, France
[b] Institute of Molecular Pathogenesis, Friedrich-Loeffler-Institut (Federal Research Institute for Animal Health), Jena, Germany

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ABSTRACT

Three cases of atypical pneumonia in individuals working at a poultry slaughterhouse prompted an epidemiological survey in 10 poultry farms that had supplied birds. Using a *Chlamydiaceae*-specific real-time PCR assay, chlamydial agents were detected in 14 of 25 investigated flocks. Rather unexpectedly, *Chlamydophila psittaci* was identified only in one of the positive flocks, whereas ArrayTube DNA microarray testing indicated the presence of a new, so far unclassified member of the genus *Chlamydophila*.

For further characterization of the agent involved, positive cloacal swabs were used to inoculate embryonated chicken eggs and isolates were obtained from 6 different flocks. Sequencing of 16S rRNA genes revealed nearly identical sequences of all samples. Alignment with representative sequences of *Chlamydiaceae* showed the separate position of the present strains outside the currently recognized species of *Chlamydophila*, but clearly within this genus. In contrast, partial *omp*A gene sequences displayed considerable diversity among the isolates, which had already been observed in restriction enzyme analysis of *omp*A PCR products. These data suggest that each farm had been infected with a different strain of this new chlamydial agent, the zoonotic potential and the exact taxonomic status of which have yet to be defined.

1. Introduction

Chlamydial infections leading to outbreaks of avian chlamydiosis in domestic, companion and wild birds are regularly reported from all parts of the world. As their general importance is based on two aspects, i.e. economic losses to the bird owners and potential zoonotic transmission to humans, control measures are obligatory in a number of European countries, where specific state legislation is in force. The most prominent chlamydial agent in *Aves* is *Chlamydophila (C.) psittaci*, which was shown to occur in as many as 465 bird species (Kaleta and Taday, 2003). Following the recent revision of chlamydial taxonomy (Everett et al., 1999), this obligate intracellular bacterium now predominantly comprises avian serovars. The family *Chlamydiaceae* with its two genera *Chlamydia* and *Chlamydophila* currently combines a total of nine species, i.e. *Chlamydia trachomatis*, *Chlamydia suis* and *Chlamydia muridarum*, as well as *C. pneumoniae*, *C. abortus*, *C. caviae*, *C. felis*, *C. pecorum*, and *C. psittaci*, respectively.

The importance of *C. psittaci* as the causative agent of psittacosis or avian chlamydiosis in psittacine birds and domestic fowl has been known for decades. A number of recent reports have confirmed that its zoonotic potential remains significant in the face of regularly occurring outbreaks of disease in domestic fowl (Vanrompay et al., 1995; Gaede et al., 2008; Laroucau et al., 2009). In addition, infections can take a subclinical and/or chronic course (Harkinezhad et al., 2009). However, occasional detections of *C. abortus* (Herrmann et al., 2000; Pantchev et al., 2008) and so far non-classified chlamydial agents (Gaede et al., 2008), as well as genetic evidence on intermediate strains between *C. psittaci* and *C. abortus* (Van Loock et al., 2003) suggest that the spectrum of *Chlamydiaceae* spp. encountered in birds is not confined to a single species.

In this context, it should be noted that laboratory diagnosis of infections involving chlamydiae has undergone a remarkable methodological change in the past two decades (Sachse et al., 2009). While only a few specialized laboratories are still conducting routine isolation of chlamydiae using cell culture or embryonated eggs, DNA-based detection methods have become widely accepted. This implies that specific PCR tests for individual chlamydial species and/or pan-*Chlamydiaceae* assays are conducted, which are capable of detecting even small amounts of a known agent within a working day. However, new and hitherto

Abbreviations: MOMP, major outer membrane protein; RFLP, restriction fragment length polymorphism; rtPCR, real-time PCR.

* Corresponding author. Tel.: +33 1 49 77 13 00; fax: +33 1 49 77 13 44.
E-mail address: k.laroucau@afssa.fr (K. Laroucau).

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non-classified taxa will often be overlooked by this approach, so that the inclusion of a highly parallel screening assay, e.g. a DNA microarray test, is advisable.

Following the occurrence of symptoms of atypical pneumonia in a group of poultry slaughterhouse workers in France early in 2008, a diagnostic investigation in the slaughterhouse and the flocks of the suppliers was undertaken. Initial tests suggested involvement of chlamydiae, so that diagnostic examinations focused on *Chlamydiaceae* spp. In the present paper, we report the results of this study, which led to the identification of so far non-classified avian chlamydial agents.

2. Materials and methods

2.1. History of human infections

In the period from April 25 to May 30 in 2008, three individuals presented to their physicians with atypical pneumonia. Case 1, a 49-year-old woman, had fever and flu-like symptoms and was administered Naxy. Case 2 was a 25-year-old woman suffering from thoracic pain and fever. She had to be hospitalized and was treated with Clamoxyl and Rulide. Case 3, a man aged 62, had fever and complained about weariness. He was administered Zeclar. No diagnostic data from microbiology and serology are available.

2.2. Animal samples

Cloacal swabs were collected from poultry flocks received for slaughter and from birds belonging to the slaughterhouse owner. In each sampled flock, 10 animals were examined. One panel of swabs was stored in 1 ml of conservation buffer SPG (Spencer and Johnson, 1983) at −80 °C until inoculated into chicken eggs, whereas the other panel was stored dry at −80 °C until subjected to DNA extraction. Data on age, breed and flock of the examined birds are contained in Table 1.

2.3. DNA extraction

Dry cloacal swabs were subjected to DNA extraction using the QIAamp DNA Mini Kit (QIAGEN, Courtaboeuf, France) following the buccal swab protocol. For DNA extraction from aliquots of chlamydial cell culture and vitellus membranes, the tissue protocol of the same kit was used. Finally, DNA was eluted with 150 μl of AE buffer and stored at −20 °C until examination.

2.4. Direct detection of chlamydiae by real-time PCR

A *Chlamydiaceae*-specific real-time PCR targeting the 23S rRNA gene (23S-rtPCR) was used in this study (Ehricht et al., 2006). The protocol includes primers Ch23S-F (5′-CTGAAACCAGTAGCTTA-TAAGCGGT-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 2× (Applied Biosystems, Courtaboeuf, France), 0.5 μl of each primer (25 μM), 2 μl of the probe (1 μM), and 5 μl deionized water. The temperature–time profile was 95 °C for 10 min, 45 cycles of 95 °C for 15 s and 60 °C for 60 s.

The *omp*A-based real-time PCR assay specific for *C. psittaci* was conducted as recently described (Pantchev et al., 2008).

2.5. Culture on embryonated chicken eggs

For culture, suspensions of cloacal swabs stored in conservation buffer at −80 °C were thawed, transferred into sterile Eppendorf

Table 1
Characteristics of investigated poultry flocks and results of diagnostic testing.

Flock ID	Breeder no.	Flock effective	Breed	Age at time of sampling (weeks)	23S-rtPCR *Chlamydiaceae*		*ompA*-rtPCR *C. psittaci*	Cell culture isolation
					No. of +ve samples	Mean Ct	No. of +ve samples	
Slaughterhouse								
08-1274/2	Slaughterhouse	500	Chicken	4	1/10 (10%)	38.6	0/1	
08-1274/1	breeder	500	Chicken	8	0/10	–	–	
08-1274/5		500	Chicken	12	0/10	–	–	
08-1274/4		500	Chicken	16	4/10 (40%)	33.0	0/4	
08-1274/3		500	Chicken	20	10/10 (100%)	28.8	0/10	+
08-1274/6		100	Guinea fowl	7	0/10	–	–	
08-1274/8		120	Guinea fowl	13	0/10	–	–	
08-1274/7		120	Guinea fowl	16	0/10	–	–	
Customers								
08-1274/9	Breeder 1	320	Chicken	20	3/10 (30%)	34.6	0/3	
08-1274/10		–	Guinea fowl	20	0/10	–	–	
08-1274/17		–	Duck	–	1/5 (20%)	35.1	1/1	+
08-1274/11	Breeder 2	600	Chicken	15	0/10	–	–	
08-1274/12		–	Guinea fowl	15	1/8 (12.5%)	40.1	0/1	
08-1274/13	Breeder 3[a]	600	Chicken	–	9/9 (100%)	26.3	0/9	+
08-1274/23		600	Chicken	12	10/10 (100%)	27.2	0/10	+
08-1274/24		600	Chicken	4	3/10 (30%)	39.0	0/3	
08-1274/14	Breeder 4	–	Chicken	–	0/10	–	–	
08-1274/15		–	Guinea fowl	–	0/8	–	–	
08-1274/16	Breeder 5[a]	600	Chicken	8	1/10 (10%)	38.8	0/1	
08-1274/19		600	Chicken	18	10/10 (100%)	27.0	0/10	+
08-1274/18	Breeder 6[a]	600	Chicken	18	0/10	–	–	
08-1274/20	Breeder 7[a]	600	Chicken	12	1/10 (10%)	38.9	0/1	
08-1274/21		600	Chicken	17	10/10 (100%)	26.6	0/10	+
08-1274/22	Breeder 8[a]	600	Chicken	16	9/10 (90%)	31.8	0/9	+
08-1274/25	Reproducer	407	Chicken	43	0/10	–	–	

[a] Breeders linked to the same supplier of 1-day-old chickens, i.e. 08-1274/25.