A Real-Time PCR Assay for the Detection of Atypical Strains of *Chlamydiaceae* from Pigeons

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Abstract

Recent evidence of the occurrence of atypical *Chlamydiaceae* strains in pigeons, different from the established *Chlamydiaceae*, requires the development of a specific and rapid detection tool to investigate their prevalence and significance. Here is described a new real-time PCR assay that allows specific detection of atypical *Chlamydiaceae* from pigeons. This assay has been used to assess the dissemination of these strains in field samples collected from Parisian pigeon populations in 2009. The results suggest a limited dissemination compared to the usually higher prevalence of *Chlamydya psittaci* that is the main species associated with avian chlamydiosis.

Introduction

Avian chlamydiosis is a zoonotic disease caused by *Chlamydia psittaci*, an obligatory intracellular bacterium and member of the family *Chlamydiaceae* [1]. 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 birds as well as the causative strain [2]. Transmission of *C. psittaci* from birds to humans is reported regularly, particularly in high-risk individuals such as breeders or slaughterhouse workers [3]. Although the importance of *C. psittaci* as the causative agent of avian chlamydiosis in birds has been known for decades [4], several studies have recently provided evidence of the occurrence of other chlamydial species in birds such as *C. abortus* [5,6], *C. suis* and *C. muridarum* [7] as well as *C. pecorum* and *C. trachomatis* [8]. Additionally, atypical strains of *Chlamydiaceae* were recently detected in chickens [9,10,11] and pigeons [8,12,13,14].

A better characterisation of these atypical *Chlamydiaceae* and the availability of fast, sensitive and specific detection tools are needed in order to better understand their epidemiological importance. A specific 16S rDNA-based real-time PCR was developed recently for the specific detection of atypical *Chlamydiaceae* from chickens (hereafter named ACC) [14].

The work reported in this paper deals with the development and validation for routine diagnostic purposes of a real-time PCR based on the enolase A (enA) gene target for the specific detection of atypical *Chlamydiaceae* from pigeons (hereafter named ACP). This assay has been used to investigate the dissemination of this agent in Parisian pigeon populations.

Methods

1. Microorganisms

Chlamydial strains and isolates used for determination of specificity, sensitivity and detection limit of the new ACP-specific real-time PCR assay are listed in Tables 1 and 2. *Chlamydiaceae* were propagated in the yolk sac of 7 day-old embryonated chicken eggs and stored at −80°C. Non-chlamydial bacterial and fungal strains obtained from the University of Tours (France) and the Anses strain collection (Maisons-Alfort, France) were used for specificity testing. These included: *Escherichia coli*, *Proteus mirabilis*, *Proteus vulgaris*, *Klebsiella pneumoniae*, *Salmonella enteritidis*, *Salmonella enteritidis* serovar Typhimurium, *Escherichia coli* serovar Typhimurium, *Morganella morganii*, *Proteus mirabilis*, *Serratia marcescens*, *Acinetobacter baumannii*, *Lactococcus lactis*, *Lactobacillus plantarum*, *Yersinia pseudotuberculosis*, *Yersinia enterocolitica*, *Aspergillus fumigatus*, *Aspergillus niger* and *Serratia marcescens*.

2. Preparation of genomic DNA

Chlamydial strains cultivated onto vitellus membranes as well as biological samples were subjected to DNA extraction using the QIAamp DNA Mini Kit (Qagen, Courbevoie, France). DNA extracts were stored at −20°C before analysis.

DNA of non-chlamydial samples was extracted using the Instagene Matrix DNA Kit (BioRad, Marnes-la-Coquette, France) according to the manufacturer’s instructions. DNA concentration and purity of non-chlamydial extracts were spectrophotometrically assessed by reading A260 and A280 and confirmed by visualization on 0.5% (w/v) agarose gels. Finally, DNAs with a mean
Table 1. Characteristics of ACP isolates included in this study.

| ID            | Country   | Year of Isolation | Initial sample         |
|---------------|-----------|-------------------|------------------------|
| 10–743/SC13   | France    | 2010              | Cloacal swab           |
| 10–881/SC42   | France    | 2010              | Cloacal swab           |
| DC96          | Germany   | 2011              | Spleen                 |
| DC97          | Germany   | 2012              | Faeces                 |
| PV_2806/51    | Italy     | 1996              | Intestinal content     |
| PV_2863/2     | Italy     | 1996              | Intestinal content     |
| PV_3515/3     | Italy     | 1996              | Intestinal content     |
| PV_3954/22    | Italy     | 1996              | Intestinal content     |
| PV_7341/13    | Italy     | 1997              | Intestine              |
| PV_7344/2     | Italy     | 1997              | Intestine              |
| PV_155757/2010| Italy     | 2010              | Pool of organs         |
| PV_48558/2010 | Italy     | 2010              | Pool of organs         |
| PV_58394/2012 | Italy     | 2012              | Pool of organs         |

Table 2. Chlamydia strains and isolates used for determination of specificity of the ACP-specific real-time PCR assay.

| Species                  | Strains/isolates                                      |
|--------------------------|-------------------------------------------------------|
| Atypical Chlamydia from chicken (ACC) | 08–1274/32, 08–1274/130, 08–1274/190, 08–1274/210, 08–1274/220, 08–1274/230 |
| C. psittaci              | Loth9, VS1, CP3, GR9, TT3, NJ1, Cal 10, VS2255, 06–889A |
| C. abortus               | AB71, 188, LLG4, 526/3 |
| C. pecorum               | 824c, AB10c, IB3c, IB3c, LW679c, SBEc |
| C. felis                 | Dohycat* |
| C. caviae                | GPICc, 98–31961A |

Sources: * Anses Maisons-Alfort, France; † CHU Amiens, France; ‡ INRA Tours, France; § Faculty of Veterinary Medicine – Aristotle University of Thessaloniki, Greece; ¶ Vaccine strain.

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evaluate the dissemination of the ACP. These samples, which contained an internal positive control (Exogenous internal positive control, TaqMan, Life technologies), were previously tested positive in the Chlamydiaceae 23S-rtPCR [17].

Results and Discussion

Recent evidence of the occurrence of atypical Chlamydiaceae strains in chickens and pigeons highlighted the necessity of specific and fast detection tools for the investigation of their epidemiological and clinical importance. Thirteen ACP strains have been isolated up to now in France, Germany and Italy (Table 1). A similar approach to the one used for ACC [14] has been adopted in this study, except that the enolase A (enoA) gene has been selected as target. This gene locus had been previously included in a Multi-Locus Sequence Typing scheme for Chlamydia spp. [15,16]. All enoA sequences of ACP strains in Table 1 proved identical. The consensus sequence was compared in silico to those of the nine established Chlamydia species [15,16] and also to ACC sequences [14]. Alignment (Figure S1) and phylogenetic analysis (Figure 1) revealed that this sequence was specific to ACP and could be a convenient target for their unambiguous identification by real-time PCR.

The specificity of the newly developed enoA-based real-time PCR assay (enoA-rtpCR) was tested with genomic DNA extracts of several bacterial and fungal species. Neither the DNA samples from non-chlamydial species (n = 22), nor the DNA samples from C. psittaci (n = 9), C. abortus (n = 4), C. pecorum (n = 6), C. felis (n = 1), C. caviae (n = 2) and newly described atypical chicken (ACC) isolates included in this study (n = 6) gave rise to a measurable signal. As expected, all DNA extracts from French (n = 2), German (n = 2) and Italian (n = 9) ACP isolates (Table 1) were successfully amplified (data not shown).

In order to determine the detection limit of the new real-time PCR, a decimal dilution series of the titrated 10–743/SC13 DNA was tested. The results showed that the assay was able to detect the DNA from 3 IFUs of the respective pathogen per PCR reaction with an efficiency of 95% (Figure 2). The intra-assay and inter-assay CVs were 2.5% and 1.9% respectively. Therefore, the real-time PCR assay presented here has proved to be sensitive, specific and reproducible and offers the possibility of assessing the epidemiological importance of ACP infections in pigeon populations.

To evaluate the suitability of the assay for this purpose, a retrospective examination of previously collected field samples in Parisian feral pigeon populations [13] has been carried out. Out of the 125 Chlamydiaceae-positive samples, 10 were positive (8%) with the new ACP-specific real-time PCR (Table 3), including samples 09–489/LP23 and 09–589/S46 that had previously been identified as ACP by partial ompA and 16S rRNA sequencing [13]. These findings suggest a low dissemination of ACP in comparison with C. psittaci, which prevalence of 68% had been determined (Table 3) using the incA-based C. psittaci-specific real-time PCR (incA-rtpCR C.psittaci) according to Menard and colleagues [18]. A mixed infection involving an ACP strain and C. psittaci was also found in two samples from Genevilliers (Table 3).

Given the low number of samples tested and the limited geographical range covered, the results of the present study can only be regarded as preliminary. Atypical pigeon strains have been isolated in the laboratories of the authors from the intestinal content or organs of necropsied urban pigeons. Their pathogenicity for pigeons is unknown. Another open issue is the host range. A recently isolated atypical parrot strain from Germany (designated 10DC88) was found to possess an enoA sequence identical to those of ACC, so that it was positive with the ACP-specific real-time PCR assay. This indicates that ACP seem not to be restricted to pigeons and could possibly be circulating in other avian hosts.

All in all, as a rapid and specific diagnostic tool is now available, it seems straightforward to include ACP investigation to analyses of diagnostic samples from birds and to undertake systematic studies to characterize the epidemiology and etiology of ACP infections in avian populations.

Conclusions

A sensitive and specific real-time PCR assay for the detection of atypical Chlamydiaceae from pigeons has been developed and preliminary validated. The use of this new tool could contribute to a better understanding of the importance of these bacterial agents.
Supporting Information

Figure S1  Alignment of partial enoA sequence for ACC (08–1274/3 reference), ACP (10–743/SC13 reference) and various established Chlamydiaceae strains (one representative of each enoA sequence type; [15,16]). Nucleotide homologies are represented by dots. The numbers refer to alignment positions. ACP-specific primer sequences are boxed and the probe sequence is underlined. (TIF)
References

1. Greub G (2010) International Committee on Systematics of Prokaryotes. Subcommittee on the taxonomy of the Chlamydiae minutes of the closed meeting, 21 June 2010, Hof bei Salzburg, Austria. Int J Syst Evol Microbiol 60: 2694.

2. Andersen AA (1997) Two new serovars of Chlamydia psittaci from North American birds. J Vet Diagn Invest 9: 159–164.

3. Deschuyfleer TP, Tyberghien LF, Dickx VL, Geens T, Saclen JM, et al. (2012) Risk assessment and management of Chlamydia psittaci in poultry processing plants. Ann Occup Hyg 56(5): 380–9. Review.

4. Vanrompay D, Ducatelle R, Haesebrouck F (1997) Chlamydia psittaci infections: a review with emphasis on avian chlamydiosis. Vet Microbiol 43: 93–119.

5. Herrmann B, Rahman R, Bergestrom S, Bournedahl J, Olsen B (2000) Chlamydyphila abortus in a Brown skua (Catharacta antarctica lonnbergi) from a subantarctic island. Appl Environ Microbiol 66: 3654–3656.

6. Pantchev A, Sting R, Bauerfeind R, Tyczka J, Sachse K (2009) New real-time PCR tests for species-specific detection of Chlamydyphila psittaci and Chlamydyphila abortus from tissue samples. Vet J 181: 145–150.

7. Lemus JA, Fargallo JA, Vergara P, Parejo D, Banda E (2010) Natural cross chlamydial infection between livestock and free-living bird species. PLoS One 5e13512.

8. Sachse K, Kuechlewind S, Ruegger A, Schubert E, Rohde G (2012) More than classical Chlamydia psittaci in urban pigeons. Vet Microbiol 157: 476–480.

9. Gaede W, Reckling KF, Dresenkamp B, Kenklies S, Schubert E, et al. (2008) Chlamydyphila psittaci infections in humans during an outbreak of psittacosis from poultry in Germany. Zoonoses Public Health 55: 184–188.

10. Laroucau K, Vorimore F, Berndt A, Berndt A, Schubert E, et al. (2009) Isolation of a new chlamydial agent from infected domestic poultry coincided with cases of atypical pneumonia among slaughterhouse workers in France. Infect Genet Evol 9: 1240–1247.

11. Robertson T, Bibby S, O’Rourke D, Belfiore T, Lambie H, et al. (2009) Characterization of Chlamydiaceae species using PCR and high resolution melt curve analysis of the 16S rRNA gene. J Applied Microbiol 107: 2017–2020.

12. Vicari N, Laroucau K, Vorimore F, Barbieri I, Sachse K, et al. (2009) Molecular analysis of four chlamydiid isolates from the intestine and cloacal swabs of feral pigeons sampled in Milan and Ferrara, Italy. (Oral communication). 1st European Meeting on Animal Chlamydioses and Zoonotic Aspects. Murcia, Spain. 14–16/06/09.

13. Gasparini J, Erin N, Bertin C, Jacquin L, Vorimore F, et al. (2011) Impact of urban environment and host phenotype on the epidemiology of Chlamydiaceae in feral pigeons (Columbia livia). Environ Microbiol 13: 3186–3193.

14. Zecovic A, Vorimore F, Marhold C, Horvatek D, Wang D, et al. (2012) Molecular characterization of atypical Chlamydia and evidence of their dissemination in different European and Asian chicken flocks by specific real-time PCR. Environ Microbiol 14(8): 2212–2222.

15. Pannekoek Y, Morelli G, Kunecek B, Morre SA, Ossewaarde JM, et al. (2008) Multi locus sequence typing of Chlamydiaceae: clonal groupings within the obligate intracellular bacteria Chlamydia trachomatis. BMC Microbiol 8: 42.

16. Pannekoek Y, Dickx V, Beekman DS, Jolley KA, Keijzers WC, et al. (2010) Multi locus sequence typing of Chlamydia reveals an association between Chlamydia psittaci genotypes and host species. PLoS ONE 5: e14179.

17. Ehricht R, Slickers P, Goedner S, Hothel H, Sachse K (2006) Optimized DNA microarray assay allows detection and genotyping of single PCR-amplifiable target copies. Mol Cell Probes 20(1): 60–63.

18. Ménard A, Clerc M, Subtil A, Mégraud F, Bébétier C, et al. (2006) Development of a real-time PCR for the detection of Chlamydia psittaci. J Med Microbiol 55: 471–473.