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Detection of *Chlamydia psittaci* genotypes in fecal samples of homing pigeons in Croatia

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ABSTRACT

In this investigation fecal samples from a total of 232 homing pigeon lofts, belonging to both racing and breeding pigeons, were examined by specific real-time PCR to reveal the presence of *Chlamydiaceae,* and randomly collected *Chlamydia* positive samples were genotyped by two different molecular methods; *C. psittaci*-specific restriction fragment length polymorphism (RFLP) and Multiple-Locus Variable number tandem repeat Analysis (MLVA), in order to obtain relevant information about the prevalence of different genotypes, and to reveal the potential threat to humans that come into close contact with homing pigeons. Chlamydiosis in birds manifests as an acute, unapparent, sub-clinical, and chronic disease, but frequently also as an asymptomatic infection, which represents an additional danger for public health. Currently, nine different genotypes of *C. psittaci* have been generally accepted, based on PCR amplification, as well as an additional six provisional genotypes, based on DNA microarray. The predominant serotype/genotype in pigeons is B, but also other genotypes were discovered, such as A, C and D, as well as mixed infection. Out of 232 examined samples, 30 (12.9%) of them were PCR positive. DNA from six random positive fecal samples was further examined and the presence of C. *psittaci* was confirmed in all of them. According to RFLP genotyping, from 6 *C. psittaci* positive samples, 4 of them belonged to genotype B, and 2 strains remained untyped, due to the low concentration of DNA isolated. Regarding the MLVA typing, the pattern assigned as 22146334 could be described as a classical "pigeon" MLVA pattern. The presence of 12.9% homing pigeons positive to *Chlamydiaceae* by PCR clearly demonstrates the presence of zoonotic microorganisms and a possible risk for pigeon fanciers that come in close contact with the birds.

Key words: genotyping, *Chlamydia psittaci*, pigeons

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Introduction

Chlamydia (formerly *Chlamydophila*) *psittaci* (*C. psittaci*) infects a wide range of birds all around the world and has been detected in altogether 465 avian species (KALETA and TADAY, 2003). Avian chlamydiosis manifests as an acute, unapparent, sub-clinical, and chronic disease, but frequently also as an asymptomatic infection (ANDERSEN and VANROMPAY, 2003; SUDLER et al., 2004). On the basis of the immunofluorescent reaction of the major outer membrane protein (MOMP) with a panel of specific monoclonal antibodies, *C. psittaci* have been classified into six serovars: A, B, C, D, E and F (ANDERSEN, 1991a, b; VANROMPAY et al., 1993). Nowadays, based on PCR amplification, each serovar could be assigned to an equivalent genotype, so nine different genotypes have been generally accepted: the six already mentioned that predominantly occur in birds, M56 in rodents, WC in cattle (VANROMPAY et al., 1997), and the most recently described, E/B (GEENS et al., 2005). According to SACHSE et al. (2008), adjustments to the present genotypes scheme should be made by introduction of six provisional genotypes, as detected by DNA microarray.

Urban pigeons are often infected with *C. psittaci* all over the world, and a high percentage of seropositive, but also infected birds can be found, respectively (GAFFURI et al., 2000; DOVČ et al., 2004; MAGNINO et al., 2009). The predominant serotype/genotype in pigeons is B (HEDDEMA et al., 2006a; GASPARINI et al., 2011; GEIGENFEIND et al., 2012), but also other genotypes have been discovered, as A (VANROMPAY et al., 1997; SACHSE et al., 2012), C and D (DICKX et al., 2010), E/B (GEENS et al., 2005), mixed infection (GEIGENFEIND et al., 2012), or provisional genotypes (SACHSE et al., 2012).

In the Republic of Croatia, according to state laws, all avian flocks and birds in breeding and transport must be tested for avian chlamydiosis. The investigations implemented in Croatia so far have revealed the frequent presence of *Chlamydia* in urban pigeons (PAVLAK et al., 2000; PRUKNER-RADOVČIĆ et al., 2005). Racing and breeding pigeons, also known as homing pigeons, are both very popular in Croatia. Lofts are mostly kept outdoors, and as such, contact with infected urban pigeons or other birds is possible. Samples collected from different bird species (mostly fecal samples, swabs or organs) are examined by using several diagnostic methods, however, particular classification and identification is completed by conventional PCR and real-time PCR (HEWINSON et al., 1997; EHRICHT et al., 2006; PANTCHEV et al., 2008). Determining of the *C. psittaci* genotypes is especially important from the public health point of view, since it is well known that certain genotypes can cause severe infection in humans (especially A and D), and other (mainly B) just mild respiratory symptoms (DICKX et al., 2010) or no clinical signs at all (SACHSE et al., 2012). Numerous cases of zoonoses caused by *C. psittaci*, linked to pigeons, were described by HAAG-WACKERNAGEL and MOCH (2004).

Genotyping techniques have already been used for the typing of avian *C. psittaci* strains by SAYADA et al. (1995). Recently, a novel approach, based on the finding of tandem repeats in DNA (Multiple-Locus Variable number tandem repeat, MLVA) has been applied for genotyping of *C. psittaci.* This method targets eight distinct genomic areas, dispersed throughout the genome (LAROUCAU et al., 2008). More recently, DNA microarray technology was also used for typing of *C. psittaci* and was shown to discriminate among already known genotypes and identify so far untyped strains (SACHSE et al., 2008; SACHSE et al., 2009).

In this investigation, fecal samples belonging to both racing and breeding pigeon flocks were examined by *Chlamydiaceae*-specific real-time PCR (targeting the 23S rRNA gene) and *C. psittaci*-specific real-time PCR (targeting the *ompA* gene), and randomly collected *Chlamydia* positive samples were genotyped by two different molecular methods; *C. psittaci*-specific RFLP and MLVA scheme, in order to obtain relevant information about the prevalence of all, up to now, known genotypes, and to reveal the potential threat to humans that are in close contact with homing pigeons.

Materials and methods

Fecal samples from a total of 232 homing pigeon lofts, originating from both racing and breeding pigeons of different ages and sizes of population, were taken by the official veterinary authorities in all 21 counties of the Republic of Croatia and in the City of Zagreb for routine diagnosis of *Chlamydiaceae*, according to state law, and send to the Laboratory for Chlamydia at the Department of Poultry Disease and Clinic, of the Faculty of Veterinary Medicine, University of Zagreb. One pooled fecal sample, belonging to approximately 10% of the pigeon population was taken for each loft. The DNA was extracted using a GenElute Mammalian Genomic DNA Miniprep Kit (Sigma-Aldrich, USA), according to the manufacturer's instructions, and examined by *Chlamydiaceae*-specific real-time PCR, as described by EHRICHT et al. (2006).

Further, randomly chosen DNA samples, belonging to 6 different pigeons lofts, were examined at Unité Zoonoses Bactériennes, Anses Maisons-Alfort, France, by *C. psittaci*-specific real-time PCR, as described by PANTCHEV et al. (2008), and genotyped by alternate molecular tools - MLVA and RFLP. Genotyping by RFLP, based on restriction enzymes *Alu*I and *Mbo*II, was performed as described by SAYADA et al. (1995). Fragments were separated on a 2% NuSieve agarose gel and visualized under a UV light. MLVA were conducted as described by LAROUCAU et al. (2008), by using 8 sets of primers: ChlaPsi_280, ChlaPsi_480, ChlaPsi_605, ChlaPsi_810, ChlaPsi_222, ChlaPsi_281, ChlaPsi_929 and ChlaPsi_1788.

Results

Table 1. Genotyping of pigeon's *C. psittaci* strains (n 6).

Sample ID	23S-rtPCR *Chlamydiaceae*	*omp*A rtPCR *C. psittaci*	*C. psittaci* genotyping	
			PCR-RFLP pattern	MLVA
1	+	+	genotype B	22146334
2	+	+	genotype B	22146334
3	+	+	nec*	nec
4	+	+	genotype B	22146334
5	+	+	genotype B	22146334
6	+	+	nec	nec

*nec - not enough concentrated DNA

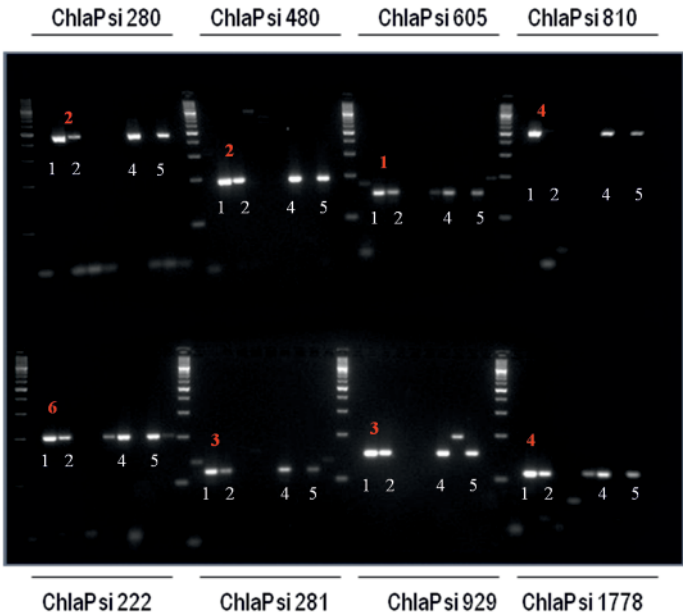

Fig. 1. MLVA of 4 pigeon's C. *psittaci* strains. Numbers in white corresponds with the sample ID number (1, 2, 4 and 5) and numbers in red with the MLVA pattern (22146334). A 100 bp ladder is run on each side of a group of samples. Samples numbered as 3 and 6 were not included since there was not enough DNA. Primers used are signed as ChlaPsi 280, 480, 605, 810, 222, 281, 929 and 1778.

Altogether 232 fecal samples, from racing and breeding pigeon lofts were examined by real-time PCR, to reveal the presence of *Chlamydiaceae*, according to Croatian laws (ANONYM., 2011). Of these, 30 (12.9%) fecal samples were shown to be PCR positive. DNA from six random positive fecal samples were further examined and the presence of C. *psittaci* was confirmed in all of them (Table 1).

According to RFLP genotyping, from six *C. psittaci* positive samples, four of them belonged to genotype B, and two strains remained untyped, due to the low concentration of extracted DNA. Regarding the MLVA typing, the pattern assigned as 22146334 (Fig. 1), described as a classical "pigeon" MLVA pattern, was found in all four examined samples.

Discussion

The detection rate of *Chlamydia* found in the fecal samples of homing pigeons in Croatia is in concordance with the findings of other authors (DICKX et al., 2010; SACHSE et al., 2012). Recently it was also described that the detection rate of *Chlamydia* in pigeon fecal samples by PCR could be even better than in swabs, due to the combining of feces from possibly high shedders in one representative sample belonging to an entire loft (SACHSE et al., 2012). This could confirm the results of this study, as from only two samples was the DNA yield too low and genotyping was not possible.

Genotyping, performed by two different methods, revealed the presence of genotype B in all examined samples. The results of RFLP typing were consistent with MLVA schemes, but with one difference - MLVA did not provide the distinction between B and E genotypes. This apparent discrepancy probably reflects the fact that none of the markers chosen in this study target *omp*A (LAROUCAU et al., 2008), as were in RFLP. The results of genotyping are in conclusion with the majority of other authors, who also reported that pigeons predominantly carry genotype B (VANROMPAY et al., 1997; HEDDEMA et al., 2006b; PANNEKOEK et al., 2010; GASPARINI et al., 2011; GEIGENFEIND et al., 2012), but other genotypes were also discovered (VANROMPAY et al., 1997; DICKX et al., 2010; GEIGENFEIND et al., 2012). The genotype B, originating from nonpsittacine birds, has always been described as having low or medium virulence, but there are also some reports that it may be the cause of human disease (HEDDEMA et al., 2006b)

Homing and urban pigeons often shed chlamydiae intermittently in the absence of clinical signs, which makes diagnosis very cumbersome. On the other hand, shedding of this pathogen through feces, and respiratory and conjunctival secretions, increases the risk of transmission of *C. psittaci* to other animals and to humans (ANDERSEN and VANROMPAY, 2003; HARKINEZHAD et al., 2009; VAN DROOGENBROECK et al., 2009). According to HARKINEZHAD et al. (2009) up to 7% of apparently healthy people, coming in close contact with pigeons, were positive by PCR to *C. psittaci* or had antibodies

against it. In the study conducted by DICKX et al. (2010) the number of positive persons was even higher - 12.5%.

Previous investigations conducted in the City of Zagreb (the Capital of Croatia) revealed approximately 15% of urban pigeons positive to *C. psittaci* (PRUKNER-RADOVČIĆ et al., 2005; HORVATEK et al., 2007). The current investigation revealed the presence of 12.9% homing pigeons positive to *Chlamydiaceae* by PCR, which clearly demonstrates the presence of zoonotic microorganisms and a possible risk for pigeon fanciers that come into close contact with the birds. Homing pigeons positive to *C. psittaci* are treated with antibiotics, but due to the possibility of development of resistant strains, as described by DUGAN et al. (2004), it is obligatory to treat the infected animals under strict veterinary supervision.

In this investigation the identity of the species of all Chlamydiaceae-positive samples was not determined, and according to other authors not only *C. psittaci*, but also *C. abortus*, *C. pecorum*, or *C. trachomatis* can be found in pigeons and other birds (PANTCHEV et al., 2009; SACHSE et al., 2012), so further investigations will be needed to clearly identify positive samples.

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SAŽETAK

Ukupno su bila pretražena 232 uzorka izmeta golubova na prisutnost bakterija porodice *Chlamydiaceae* pomoću za *Chlamydiaceae* specifične lančane reakcije polimerazom u stvarnom vremenu. Nasumično odabrani pozitivni uzorci pretraženi su i na prisutnost bakterije *Chlamydia psittaci* (*C. psittaci*), te su u slučaju pozitivnog nalaza i genotipizirani pomoću dvije različite molekularne metode - MLVA i RFLP, kako bi dobili svrsishodne informacije o učestalosti različitih genotipova, te otkrili potencijalnu prijetnju za zdravlje ljudi koji dolaze u bliži kontakt s golubovima. Klamidioza u ptica očituje se kao akutna, inaparentna, supklinička ili kronična bolest, ali često i kao asimptomatska zaraza, što predstavlja dodatnu opasnost za javno zdravstvo. Novijim istraživanjima, temeljenim na lančanoj reakciji polimerazom (PCR) nađeno je devet različitih genotipova *C. psittaci*. Prevladavajući serotip/genotip u golubova je B, ali su u njih nađeni i drugi genotipovi, kao što su A, C i D, te mješovite infekcije. Od 232 pregledana uzorka, njih 30 (12,9%) bilo je pozitivno na prisutnost bakterija iz porodice *Chlamydiaceae*. Nasumično izabrana DNK šest pozitivnih uzoraka dodatno je pretražena i na prisutnost *C. psittaci*, te je nalaz bio pozitivan u svih šest uzoraka. Pomoću PCR-RFLP metode, od 6 uzoraka pozitivnih na bakteriju *C. psittaci*, četiri su pripadala genotipu B, dok se dva uzorka nisu mogla pretražiti zbog niske koncentracije izdvojene DNK. Pomoću MLVA metode, četiri pretražena uzorka označena su oznakom 22146334, što se opisuje kao "golublji" genotip. Nalaz 12,9% golubova pozitivnih na prisutnost bakterija porodice *Chlamydiaceae* jasno pokazuje prisutnost uzročnika u jatima golubova u Hrvatskoj, te mogući rizik za uzgajivače koji dolaze u bliski kontakt s pticama.

Ključne riječi: *Chlamydia psittaci*, genotipovi, golub, Hrvatska

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