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Chlamydial infections in feral pigeons in Europe: review of data and focus on public health implications

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Abstract

Feral pigeons (*Columba livia domestica*), which thrive in most European towns and cities, are commonly infected with the zoonotic bacterium *Chlamydophila psittaci*, the agent of psittacosis (also known as ornithosis) in humans. A number of surveys carried out over the last thirty years across Europe have detected high seropositivity values and high percentages of infection in feral pigeon populations. Overall, when considering data from 11 European countries, seropositivity values to *C. psittaci* in the sampled populations ranged from 19.4 to 95.6 %. In most surveys, the complement fixation test was used, and antibodies were detected in 19.4 to 66.3 % of the samples, with a median of 46.1 %. Indirect immunofluorescence and ELISA tests were employed less frequently, but led to detection of higher percentages of seropositivity (23.7 – 67.7 % and 35.9 – 95.6 %, respectively). Attempts to grow *C. psittaci* in cell culture or embryonated chicken eggs were successful in 2 – 42.3 % and 0 – 57.1 % of samples, respectively, antigen detection methods were positive in 2.3 – 40% of samples, while conventional PCR and real-time PCR using different

genomic targets detected the organism in 3.4 – 50 % of samples. Twenty-five *C. psittaci* isolates from pigeons were typed as *ompA* genotype B (n=14), E (n=10) and E/B (n=1).

The huge increase of feral pigeon populations in Europe is a major cause of concern for the detrimental effect of pigeon droppings on environmental hygiene, in addition to the extensive damage due to the fouling of buildings and monuments. The most important pathogenic organism transmissible from feral pigeons to humans is *C. psittaci*, with 101 cases of disease reported in the literature. Exposure to *C. psittaci*-contaminated dust, direct contact with pigeons through handling and, to a lesser extent, through pigeon feeding have been identified as hazardous exposures in more than half of the human cases, while loose or transient contacts with feral pigeons have been mentioned in about 40 % of the cases.

Education initiatives as to the communication of a health risk resulting from contact with pigeons and pigeon excreta should primarily be targeted at individuals who may be exposed to *C. psittaci*-contaminated dust, such as demolition/construction workers. Recommendations to this category of workers include wearing protective clothes with hoods, boots, gloves and air filter face masks when removing pigeon faeces from roofs, garrets and buildings, especially if working indoors.

Monitoring for *C. psittaci* infections in these workers over time should also be considered. Children should be warned not to handle sick or dead pigeons, and immunocompromised individuals should be advised to carefully limit their contact to feral pigeons.

Culling of pigeons by shooting or poisoning is both unethical and ineffective as the place of the killed birds in the population is quickly filled by new juveniles or immigrating birds from neighbouring areas. Pigeon-deterring systems, such as nets and plastic or metal spikes applied to buildings and monuments will prevent their fouling, and the administration of contraceptive drugs may allow size regulation of the pigeon populations. Nevertheless, the measure that will ultimately lead to permanent reduction and will establish healthy sustainable populations is the restriction of indiscriminate feeding by pigeon lovers. The erection of dovecotes and artificial breeding facilities

should be considered for providing shelter and a balanced diet to the birds, as well as a chance of interaction for pigeon lovers in a hygienically-controlled environment.

Keywords: *Chlamydophila psittaci,* diagnosis, epidemiology, feral pigeons, health hazard, zoonosis

1. Introduction

Feral pigeons (*Columba livia domestica*), also known as "urban", "street" or "city" pigeons, are descendants of the domesticated form of the free-living Rock Dove, or Rock Pigeon (*Columba livia* Gmelin, 1789). During their domestication of more than five thousand years, hundreds of pigeon breeds were produced according to the desires and wishes of man (Haag-Wackernagel, 1998; 1999). Domestication in pigeons is characterized amongst others by a high annual reproduction success, tameness and selection against aggressiveness in males. These features may partly be responsible for the enormous thriving success of the feral pigeon in our cities around the world. In regions where no rock pigeons live, feral pigeons are derived from escaped domestic pigeons, such as the semi-domesticated dovecote pigeon, and from lost homing and fancy pigeons (Haag-Wackernagel, 2003).

After World War II, feral pigeon populations hugely increased worldwide in most larger cities (Simms, 1979) to a level of concern for city administrators and communal health officers. Besides being responsible for the massive fouling of buildings and monuments, feral pigeons were in fact often shown to be naturally infected with a number of viruses, bacteria, fungi and protozoa that are pathogenic to humans (Haag-Wackernagel and Moch, 2004). *Chlamydophila psittaci* (Everett et al., 1999; Garrity et al., 2004), an obligate intracellular bacterium which is the agent of avian chlamydiosis in birds and psittacosis in humans, is the most prevalent organism in feral pigeons worldwide. As a consequence, feral pigeon populations have been repeatedly blamed as vectors for the transmission of *C. psittaci* infections to humans.

104 COST Action 855 (http://www.vetpathology.unizh.ch/forschung/CostAction855.html), a Europe-
105 wide research network on animal chlamydioses and their zoonotic implications, has recently
106 provided a forum for researchers from several countries to discuss the public health risks associated
107 with chlamydiosis in feral pigeons.
108 The purpose of this communication is to (i) review the ecology of feral pigeons and the measures
109 that can be adopted to obtain healthy sustainable feral pigeon populations, (ii) review the methods
110 for detection of chlamydiae and chlamydial antibodies in feral pigeons and the present data on the
111 prevalence of chlamydiosis in avian populations established in several European countries, and (iii)
112 discuss the zoonotic relevance of chlamydial infections in feral pigeons.
113

114 **2. The ecology of feral pigeons in the urban environment**

115

116 Feral pigeons are a valuable enrichment of the urban environment and are one of the few animal
117 species able to survive in our noisy and hectic cities. They also represent a tourist attraction and
118 may have a cleaning up function by eating discarded food. In addition, the feeding and care of feral
119 pigeons are rewarding spare-time activities for many people who enjoy the company of animals,
120 and bring pleasure to a fraction of dedicated citizens, especially to children. In addition, feral
121 pigeons are an interesting study subject with a high scientific and educational value for hobby
122 ornithologists, as well as for biology scientists.
123 Today, the feeding of pigeons by “pigeon lovers” is mainly responsible for the establishment of
124 large pigeon populations in our cities and a supplemental input for their increase is provided by
125 rubbish and seasonally occurring natural food, such as grass and tree seeds in parks and gardens
126 (Haag, 1984). The extensive food supply indeed provides the ecological basis for the large
127 populations that occur in most cities of the world (Haag-Wackernagel, 1993; 1995; 2002; Kösters et
128 al., 1994). Pigeons in fact do not need to commute on risky flights to look for more natural food
129 supplies in the countryside and are minimally threatened by predatory birds, whose populations

130 have been drastically decimated over the years by hunting and by deliberate or accidental
131 poisoning. Regular feeding of pigeons by their feeders throughout the year allows pigeons extra
132 time for breeding, so that some individuals are able to breed throughout the year. Furthermore,
133 several behavioural changes have increased the chances of survival of feral pigeons in urban
134 environments. These birds are in fact extremely adaptable, which also enables them to accept
135 breeding places that are unnatural to them, e.g. on trees or over running ventilation systems.
136 As the density of nesting and roosting pigeons increases, the quality of life in the feral pigeon
137 population deteriorates. In fact, excessive population density activates and stimulates regulation
138 mechanisms that decimate nestlings and juvenile pigeons with infectious and parasitic diseases.
139 Crowded breeding places make pigeons behave more aggressively, which again mostly affects
140 nestlings and juveniles that are the weakest members of the population, leading to a progressive
141 spoiling of their physical condition.
142
143 **3. The impact of oversized feral pigeon populations in the urban environment**
144
145 Feral pigeons are gregarious birds that gather in swarms in streets, squares and parks, and along
146 rivers and lakes. Being often huge, these swarms are of increasing concern for owners of buildings,
147 city administrators and communal health officers (Haag-Wackernagel and Moch, 2004). Concerns
148 have been raised for the detrimental effect of pigeon droppings on environmental hygiene and for
149 the fouling of buildings and monuments. In fact, breeding sites for feral pigeons are usually man-
150 made structures such as holes in the façades of private and community buildings, churches and city
151 towers and structures under bridges (Kösters et al., 1991). Pigeon faeces are thus continuously shed
152 over monuments, statues, roofs, streets and sidewalks, leading to extensive fouling and progressive
153 damage due to the corrosive nature of the acidic contents. A pigeon produces around 12 kg of
154 faeces per year that are mainly deposited at the roosting, breeding and feeding sites (Haag, 1984;
155 Kösters et al., 1991). The progressive damage to marble and limestone is mainly due to the action of

organic acids other than uric acid, which does not seem to be able by itself to deteriorate calcareous stone (Del Monte and Sabbioni, 1986; Dell'Omo, 1996). Pigeon droppings have also proved to be an excellent substrate for the growth of microorganisms such as fungi and bacteria. In particular, the mycelial growth of some fungi (e.g., *Aspergillus* spp.) may by itself cause alteration in marble surfaces through the mechanical action exerted by the fungal hyphae. In addition, some fungal species that grow on pigeon excrement secrete acidic products (especially low-molecular weight organic acids) that contribute to the chemical erosion of calcareous material such as marble. Feral pigeons can be a real problem for historical monuments, and in the case of the cathedral of Milan they have probably contributed to the deterioration of many statues and pinnacles (Bassi and Chiatante, 1976; Mendez-Tovar et al., 1995).

On the other hand, the issues of the contamination of the urban environment caused by feral pigeons and the resultant health risks for humans have been known for a long time given the frequent opportunities of direct and indirect contact with these birds. Close contact with humans commonly occurs in squares, public gardens, parks, markets, and railway stations. In addition, the behavioural habit of pigeons in assembling and resting on roofs, balconies, window sills and shutters brings them even closer to humans. It should be noted that contact is sometimes also actively promoted by enthusiastic pigeon feeders who directly provide the birds with food at their daily gathering.

4. Overview of management strategies of feral pigeon populations

Various attempts have been made in towns and cities worldwide to reduce the size of the feral pigeon populations. A detailed presentation and discussion of all methods applied through the years can be found in several publications, including the ones by Barbieri et al. (1997) and Haag-Wackernagel (1998).

At the beginning of the 20th century, the reduction of large populations was attempted in Washington, London and Dresden by hunting and shooting (Haag-Wackernagel, 1998). Nowadays,

control programmes in some towns and cities still aim to reduce the number of feral pigeons by killing as many individuals as possible, e.g. by trapping, shooting or poisoning. However, it should be noted that several scientific studies have demonstrated that killing alone does not have an effect on the population size because the place of the killed birds is quickly filled by new juveniles, or by birds immigrating from neighbouring areas. Due to the high reproduction rate of feral pigeons of up to 12 fledglings per pair per year, coupled with a low adult mortality rate of 10 % (Haag, 1984), a lasting reduction of their populations simply cannot be achieved by killing.

Attempts for decreasing the birth rate of feral pigeons have been also made through the years in several European towns and cities, with the aim of reducing the size of the populations. Physical measures such as the destruction of pigeon eggs by eggshell puncture or by replacement of fertile eggs with plastic ones, and pharmacological treatments with several drugs have been applied. Cytostatic agents that inhibit the gametogenesis (e.g. busulfan), as well as drugs that interfere with the birds' metabolic activities (azacholesterol, nicarbazine), and natural or synthetic progestinic and estrogenic drugs (progesterone, mestranol, levonorgestrel, ethinylestradiol) have been administered on different occasions (for references, see Ballarini et al., 1989; Bursi et al., 2001). It should be noted that control measures for feral pigeons based on the administration of any such drugs are very controversial. Some results in terms of a reduction of the population size and improvement in the health status of the birds have been reported in the past, and recently also in the city of Ljubljana, Slovenia (Dovč et al., 2003; Dovč et al., pers. comm. 2006). However, in order to remain effective this measure should be supported by other actions such as a feeding ban (Dobeic, 2003). Overall, there is in fact no clear evidence of a significant long-term effect of the administration of drugs on the reduction of the size of feral pigeon populations.

Other measures that have been applied in several European towns and cities for protecting buildings from fouling include pigeon deterring systems such as net-like barriers in front of possible nesting places on the façades of buildings, metal or plastic spikes on preferred resting sites, and electrorepulsive systems. Such measures have been also applied, among others, also in the cities of

Zagreb and Paris (Prukner-Radovčić et al., 2005; Laroucau et al., 2005). Although these devices, if properly installed, can effectively prevent most damages to buildings and monuments, they just displace the problem of fouling to other urban areas and have little or no effect at all on the size of the pigeon populations.

All experiences up to now have led ultimately to the conclusion that a permanent reduction of feral pigeon populations can only be achieved by reducing their food supply, and the most effective way of achieving this, is through education of the community to not feeding pigeons (Haag-Wackernagel, 1993). Since food supply and availability are in fact the main ecological factors that influence the population size of feral pigeons, the single most important control measure is the enforcement of a feeding ban, which opposes the zoophilic behaviour of pigeon feeders.

In the city of Basel, Switzerland, the importance of the restriction of the feeding of feral pigeons has been brought to the attention of the community through an interdisciplinary project of the University of Basel, the government and the Society for the Protection of Animals of Basel (Haag-Wackernagel, 1995). Large information and education campaigns with leaflets, posters and advertisements in media through radio, television and newspapers conveyed the message that feeding pigeons was in fact harmful to the health of the population, as it leads to over-population and ultimately to poor living conditions for many birds. As a special education initiative, supervised pigeon dovecotes were set up, where birds could stay healthy and find shelter, and could be visited and fed by citizens and pigeon fanciers. This intervention proved crucial for illustrating the beneficial effects of the resizing of the population on the health of the individual pigeons.

5. Microorganisms harboured by feral pigeons, with an emphasis on chlamydiae

Communal health officers, operators in hospitals, schools, railway stations and even prisons, see in feral pigeons a significant hazard for human health and well being (Kösters et al., 1991). A wealth of publications provides firm evidence that feral pigeons are indeed the source of a large number of

zoonotic agents. Epidemiological studies in feral pigeon populations detected at least 110 organisms that are pathogenic to humans (supplemented data according to Haag-Wackernagel and Moch, 2004). Eight of them were viruses, 41 bacteria, 55 fungi and 6 protozoa. However, of these human pathogens harboured by feral pigeons, only seven (namely *Salmonella enterica* serovar *Kiambu*, *Chlamydophila psittaci*, *Aspergillus* spp., *Candida parapsilosis*, *Cryptococcus neoformans*, *Histoplasma capsulatum* and *Toxoplasma gondii*) caused a total of 230 human infections, of which 13 had a fatal outcome (supplemented data according to Haag-Wackernagel, 2006a; 2006b).

Natural infections by *C. psittaci* widely occur in many avian species. In a recent review, Kaleta and Taday (2003) have listed 467 species belonging to 30 orders of birds where *C. psittaci* has been identified. The associated disease may cause significant morbidity and mortality in companion birds and in poultry, and the infection can be transmitted to humans, where clinical signs may also be severe.

C. psittaci commonly infects feral pigeons worldwide. Almost all investigations carried out worldwide in a representative sample size of feral pigeon populations identified some birds seropositive to *C. psittaci*. In 51 investigations of feral pigeon populations carried out from 1966 to 2006, a mean seroprevalence rate of 42.3 % was found with a minimum detection rate of 10 % and a maximum of 95.6 %. Chlamydiae-excreting feral pigeons are often detected as well. In 14 investigations, detection of chlamydial antigen was successful in 13.2 % of feral pigeon specimens with a range of values from 0 to 33.3 % (supplemented data according to Haag-Wackernagel, 2005).

Most infected feral pigeons are asymptomatic and latent carriers of *C. psittaci*. Shedding of the organism occurs in faeces as well as in respiratory and conjunctival secretions, often intermittently and without clinical signs, which makes it difficult to assess the risk of transmission of *C. psittaci* to other animals, including humans. Increased shedding of chlamydiae may be triggered by stress factors such as other concurrent infections or infestations, lack of food, breeding and overcrowding (Andersen and Vanrompay, 2003; NASPHV, 2006). The elementary body (EB), which is the

infectious form of *C. psittaci* shed from the birds, can retain its infectivity for months under suitable environmental conditions (Albrecht et al., 2003) and may travel long distances, carried by the wind (Kukowka et al., 1960). Overt disease with clinical signs of depression, serous conjunctivitis, blepharitis, rhinitis and diarrhea has been reported in pigeons (Andersen and Vanrompay, 2003). Lesions observed at necropsy may include conjunctivitis, hyperemia and enlargement of spleen, hyperemia and degeneration of liver, enteritis and airsacculitis (Pavlak et al., 2000).

6. Direct diagnosis of chlamydial infections in feral pigeons

Chlamydiae can be mainly detected in pigeon faeces, cloacal swabs or smears from the surface of viscera such as liver, spleen and lung by simple laboratory methods, i.e. by staining with several techniques including Giménez, Machiavello, or modified Ziehl-Neelsen (Machiavello, 1937; Stamp et al., 1950; Giménez, 1964; Quinn et al., 1994) or with a direct immunofluorescence assay (DIF). The isolation of *C. psittaci* in tissue culture or in embryonated chicken eggs is still referred to as the gold standard for the direct diagnosis and is the preferred diagnostic method according to OIE (Andersen, 2004), but it requires specialized laboratories and expertise and is time-consuming and expensive. In addition, the test requires that the viability of chlamydiae has been preserved with a suitable transport media when samples are collected and forwarded to the laboratory (Andersen, 1998). The use of the Buffalo Green Monkey (BGM) cell line is recommended for the isolation of avian chlamydial strains (Vanrompay et al., 1992). Chlamydial inclusions in the cytoplasm of the infected cells are visualized by specific staining procedures such as Giemsa (Giemsa, 1902; 1904) or Giménez (1964), or by immunofluorescence. Immunochromatographic (ICT) and ELISA tests for the detection of chlamydial antigens can be used as well and are quick and easy to perform (Fudge, 1991). These two methods can be used also for the detection of non-viable chlamydiae. However, most of the commercially available kits based on these methods were originally developed for detecting chlamydial species other than *C. psittaci* in human samples. Thus, their

286 reliability for testing animal samples (and especially faeces) is generally lower than culture-based
287 and molecular methods. In particular, false-positive results may occur due to the cross-reactivity
288 with the lipopolysaccharide (LPS) antigen of other Gram-negative bacteria (Vanrompay et al.,
289 1994; Andersen, 2004).
290 As an alternative to the cultivation of chlamydiae, molecular methods have been adopted in many
291 laboratories in the last few years. In particular, several PCR protocols have been made available.
292 The genomic targets for the PCR assays include the single *ompA* and *ompB* genes (Hewinson et al.,
293 1997; Kaltenböck et al., 1997; Yoshida et al., 1998; Hartley et al., 2001; Sachse and Hotzel, 2003),
294 the 16S rRNA gene (Ossewaarde and Meijer, 1999) or the *pmp* gene family (Laroucau et al., 2001;
295 2007). Recently, real-time PCR protocols have been also recommended, targeting either conserved
296 *Chlamydiaceae* genomic sequence (Ehricht et al., 2006) or a species-specific and genotype-specific
297 *C. psittaci* genomic sequences (Geens et al., 2005b; Heddema et al., 2006a; Pantchev et al., 2008).
298 These tests are very sensitive and their detection limits has been found to be equivalent to a few
299 genomic copies of chlamydiae (Geens et al., 2005b; Ehricht et al., 2006; Heddema et al., 2006a).
300 Very recently, two DNA microarrays for the detection of chlamydiae were developed. One targets
301 the 23S rRNA gene of the *Chlamydiaceae* family and allows species identification (Sachse et al.,
302 2005), while the other has been specifically developed for *ompA*-based *C. psittaci* genotyping
303 (Sachse et al., 2008).
304 Among all chlamydial species, only *C. psittaci* has been detected in pigeons so far. Isolates of this
305 species have been grouped into serovars by a microimmunofluorescence assay employing serovar-
306 specific monoclonal antibodies (MAbs) directed against the major outer membrane protein
307 (MOMP). Six avian serovars (A to F) are currently recognized (Andersen, 1991), and at least three
308 of them infect pigeons. Serovar B is considered to be host specific and the most prevalent pigeon-
309 associated serovar worldwide (Vanrompay et al., 1993; Hoop et al., 2002; Andersen, 2005;
310 Laroucau et al., 2007). Serovar E also commonly infects pigeons. Initially, it was detected less
311 frequently compared to serovar B both in the US (Andersen, 2005) and in Europe (Vanrompay et

al., 1993; 1997; Duan et al., 1999). Serovar A, which is commonly associated with psittacine birds, has also been detected in feral pigeons (Vanrompay et al., 1993).

Besides serotyping, a genotyping procedure consisting of restriction enzyme analysis of the PCR-amplified MOMP gene (*ompA*) of chlamydiae (restriction fragment length polymorphism or RFLP analysis) has been introduced for typing of avian *C. psittaci* strains (Sayada et al., 1995). This technique is highly reproducible and can be directly applied to clinical samples without the need for culturing the organism. However, a major drawback is its limited discriminatory ability compared to other genotyping methods. In fact, PCR-RFLP lacks high sensitivity since the DNA content of the sample may not be high enough to generate large amounts of amplified product and unambiguous restriction cleavage patterns. In addition, this technique fails to recognise the new genotype E/B or any of the atypical *C. psittaci* strains.

Seven genotypes in total, from A to F and an additional E/B recognized by nucleotide sequencing, have been identified in birds. Genotypes A, B, C, D, E and E/B have all been detected in pigeons, and mixed infections with different genotypes have been documented as well (Geens et al., 2005a). Serovars and genotypes are closely related (Table 1). All *C. psittaci* genotypes can be transmitted to humans (Andersen and Vanrompay, 2003; Geens et al., 2005b; Heddema et al., 2006c; Gaede et al., 2008), including the recently described genotype E/B, whose zoonotic transmission from parrots and from turkeys has been just reported (Harkinezhad et al., 2007; Verminnen et al., 2008).

Besides the current *C. psittaci* classification based on the *ompA* gene sequence, a novel approach based on the identification of tandem repeats in DNA (multilocus variable number of tandem repeats analysis or MLVA) has been recently applied to *C. psittaci*. This method targets eight distinct genomic areas dispersed throughout the genome, none of them being localised within the *ompA* gene (Laroucau et al., 2008). So far, isolates from pigeons have yielded four distinct MLVA patterns (number 1, 7, 12 and 19).

For more detailed reading on diagnostic issues, the reader is referred to the review "Recent developments in the laboratory diagnosis of chlamydial infections" in this volume.

7. Serological diagnosis of chlamydial infections in feral pigeons

The most widely used serological test for detection of antibodies to *C. psittaci* in pigeons is the complement fixation test (CFT), which is the standard test for chlamydial antibodies in birds according to the OIE (Andersen, 2004). The test was originally described by Bedson (1935), and it can be used for analysing sera from pigeons, as recommended by Page (1975). The CFT has been generally considered suitable for the analysis of sera from pigeons since it often detects both high percentages of seropositivity in the sampled population and high antibody titers in individual pigeons. However, this test has some important limitations. It only detects antibodies capable of fixing the complement and directed to a group-specific chlamydial antigen, while other serological methods can detect all IgG capable of binding the antigen, as well as species- and type-specific antibodies (Salinas et al., 1993b). As an alternative to the CFT, an indirect immunofluorescence test (IIF) and a microimmunofluorescence test (MIF) have been also employed (Salinas et al., 1993b; Dovč, 1995; Donati et al., 2006). Moreover, the use of an ELISA based on the chlamydial LPS, which is more sensitive than the CFT, easier to standardize and more suitable for large-scale epidemiological studies, has been also recommended (Schmeer, 1983; Fudge, 1991).
For more detailed reading on serological methods, the reader is referred to the review "Recent developments in the laboratory diagnosis of chlamydial infections" in this volume.

8. Data on chlamydial infections in feral pigeon populations across Europe

Tables 2 and 3 illustrate laboratory data from surveys on *C. psittaci* infections carried out in the feral pigeon populations of 11 European countries over the last 30 years.
Overall, when considering data from all countries, seropositivity values to *C. psittaci* in the sampled populations ranged from 19.4 % to 95.6 %. Four different methods were used for the detection of

antibodies, with CFT being the most frequently employed (6 countries) followed by ELISA and IIF (2 countries each) and MIF (one country). In one survey, a pool of sera was examined with three serological methods (CFT, MIF and ELISA) for comparison. With CFT, seropositivities ranged from 19.4 % to 66.3 % with a median of 46.1 %, while similar values were obtained in surveys employing the indirect immunofluorescence test (23.7 – 67.7 %). The highest seropositivities, ranging from 56 % to 95.6 %, were reported with the LPS-based ELISA.

In most surveys, *C. psittaci* was also detected using direct methods in a percentage of up to 50 % of the examined samples. Direct detection was carried out either from cloacal or combined conjunctival/choanal/cloacal swabs of live birds, or from the intestinal content or viscera collected at necropsy. Pigeon droppings were examined in one survey only. The isolation of *C. psittaci* in cell culture or embryonated chicken eggs was attempted in 8 countries, and yielded positive results in 2 - 42.3 % and 0 - 57.1 % of samples, respectively. Non-culturable antigen detection methods were employed in 4 countries and were positive in 2.3 – 40 % of samples. As to molecular methods, several PCR protocols were applied, with genomic targets as diverse as the *ompA*, the 16S rRNA and the 23S rRNA genes, which allowed the detection of *C. psittaci* in 3.4 - 50 % of the samples. Genotyping was carried out on 25 *C. psittaci* strains detected in pigeons sampled in 3 countries. Fourteen of them were assigned to genotype B, 10 to genotype E and one to genotype E/B.

9. Comments on the different methods employed for diagnosing chlamydial infections in feral pigeons

Overall, chlamydial infections are widespread in the feral pigeon populations of several European towns and cities. Indeed, in most of them, moderate to high percentages of seropositivity to *C. psittaci* have been detected for several years. The results of the serological investigations in some countries may not be directly comparable due, among others, to the different cut-offs used in the analysis, yet they clearly indicate that European feral pigeons are frequently exposed to *C. psittaci*.

This finding is not unexpected, since all investigations performed worldwide in a representative number of birds have demonstrated the detection of chlamydial antibodies in feral pigeons. In most surveys mentioned in this communication, serological investigations have been carried out using CFT, which has for a long time proven to be a suitable method, since pigeon sera are able to fix guinea pig complement, unlike sera from other birds such as turkeys and some parrot species. ELISA, IFI and MIF have been also used, albeit less frequently. Limited data have been published concerning the comparison of the sensitivity and specificity of serological methods for the detection of anti-*C. psittaci* antibodies in pigeon sera. Milon et al. (1983) and Trap et al. (1986) compared IIF and CFT, and concluded that IIF was more sensitive than CFT for the analysis of pigeon sera, but also suggested that the cut-off of 1:40 for the IIF was too high. Ceglie et al. (2007) recently reported good agreement between the CFT and the MIF and again detected more positive samples with the latter test. Eidebenz (1990) tested a blocking antibody ELISA against the CFT and found that the former was specific and more sensitive than CFT. Salinas et al. (1993b) compared the performance of five serological methods (CFT, indirect CFT, IIF, MIF and ELISA) for the detection of chlamydial antibodies in pigeon sera. Taking the IIF as a reference method, they found that the ELISA and MIF were more sensitive and allowed the detection of more positive samples than the CFT, but the ELISA was found to be less specific than the other methods. Overall, serological methods other than the CFT have shown a higher sensitivity, but still needs to be fully evaluated as to their specificity (Andersen, 2004).

In the surveys considered in this communication, infection with *C. psittaci* has been frequently demonstrated directly with both non-cultural and molecular methods. In addition, the carriage of viable organisms by apparently healthy birds has been ascertained in some cases by isolation of *C. psittaci* in cell culture or embryonated eggs. The cultural method is highly sensitive provided that the viability of chlamydiae in the sample has been preserved, while the use of non-culturable antigen detection methods has been questioned in the last few years due to the possible occurrence of false-positive results arising from cross-reactivity with other bacterial antigens. On the other

hand, some of the recently developed molecular methods, i.e. PCR-RFLP, real-time PCR and DNA microarrays, look particularly attractive for their specificity, sensitivity and flexibility, since they allow more rapid detection and typing of *C. psittaci* without the need for culturing the organism, which also makes them safer. There is no consensus at the moment for recommending a single PCR assay for the diagnosis of avian (including pigeon) chlamydiosis. However, in a comparison of different conventional PCR protocols, the PCR assay targeting the *pmp* gene family has been recently found highly specific and more sensitive, up to 10 times, than assays targeting other chlamydial genes (Laroucau et al., 2007). Real-time PCR protocols are characterized by high sensitivity and also allow quantification of genome copy numbers in the samples. A validation study has recently compared conventional PCR, real-time PCR, immunohistochemistry, cell culture and a DNA microarray assay. Sensitivies of microarray testing and real-time PCR have been found to be equivalent (Borel et al., 2008).

10. The zoonotic relevance of chlamydiae acquired from feral pigeons

The most important pathogenic organism transmissible from feral pigeons to humans is *C. psittaci*, the agent of avian chlamydiosis in birds and psittacosis (also known as ornithosis) in humans. The clinical presentations of the disease in humans range from a mild influenza-like illness to a severe atypical pneumonia and systemic disease with extra-pulmonary involvement. Humans get infected by inhalation of aerosols contaminated with faecal dust, feather particles or dried excreta from infected birds (Leopold, 1965).

In 1941, Meyer described the first case of transmission of *C. psittaci* from feral pigeons to humans. A mother and her daughter had picked up a sick feral pigeon in the street in New York City. The pigeon died after 4 days and, two weeks later, both mother and daughter developed ornithosis with fever and pneumonia. Two thirds of the feral pigeons examined in their environment were positive for *C. psittaci* (Meyer, 1941). Since the first description, a number of case reports have

442 demonstrated the successful transmission of chlamydiae from pigeons to humans (for references,
443 see Süss et al., 1996). A recent extensive search of the literature identified 101 case reports of
444 ornithosis in humans where the route of transmission could be traced to a contact with feral pigeons
445 (Haag-Wackernagel and Moch, 2004).
446 In most cases (95 %), detailed information has been provided as to the circumstances of interaction
447 between humans and pigeons (supplemented data according to Haag-Wackernagel, 2006a; 2006b).
448 Overall, 53 % of all reported cases of disease in humans could be referred to a close contact with
449 feral pigeons or their excreta as detailed hereafter. About one fourth (27 %) of cases was related to
450 occupational exposure to contaminated dust, while fewer cases (15 %) followed the handling of sick
451 or dead pigeons. Fatalities – one case each - following both exposures were reported. Few cases (11
452 %) were linked to the habit of feeding pigeons. On the other hand, loose or transient contact with
453 feral pigeons were mentioned in 43 cases (42 %) of human disease, of which 11 were children and 6
454 were immunosuppressed patients. In this case, the activities leading to infection included, e.g.,
455 eating lunch in a park frequented by pigeons, walking through a pigeon flock, and living in a
456 neighbourhood frequented by pigeons. Only in a limited percentage of cases (5 %) no information
457 was provided as to the nature and circumstances of the contact with pigeons.
458 Demolition/construction labourers may get professionally exposed to *C. psittaci*-contaminated dust
459 when they work in parts of buildings such as over roofs, in garrets and close to gutters where pigeon
460 faeces have accumulated. Inside such buildings, the beating of wings of pigeons may further
461 contribute to build up and spread contaminated aerosols that are especially hazardous. Children may
462 be particularly exposed to the risk of infection when they handle sick birds, as they may be prone to
463 give them some shelter and assistance. Feeding pigeons may lead to exposure to chlamydiae when
464 birds congregate and spread contaminated dust with the beating of their wings, or when pigeon
465 feeders indulge in intimate contacts with the birds.
466 Loose or transient contact with feral pigeons leading to disease in humans is difficult to identify and
467 document, but may indeed be relevant, as shown by the analysis of the occurrence of the disease in

people only temporarily exposed to infected birds or contaminated aerosols, e.g. customs officers transiently exposed to imported parrots (De Schrijver, 1995) and veterinarians visiting a duck processing plant on a single occasion (Palmer et al., 1981; Kaleta, pers. comm. 2008).

Although the prevalence of chlamydial infections in feral pigeons is consistently high across Europe, the actual risk for humans of acquiring psittacosis from these birds is difficult to quantify. In general, the relevance of feral pigeons as a source of zoonotic chlamydiae is poorly understood. It is somehow puzzling to note that in spite of the exceptionally wide distribution of *C. psittaci* in feral pigeon populations and the variety of possible contacts with humans, only very few cases of transmission of *C. psittaci* from feral pigeons to humans have been reported worldwide. One possible underlying reason is that many pigeon-derived *C. psittaci* strains may not be highly pathogenic in humans, or at least not as pathogenic as the strains commonly encountered in other birds, e.g. parrots, ducks and turkeys. In this scenario, pigeon-borne psittacosis in humans would often be undetected or misdiagnosed, due to the associated poor clinical or non-specific influenza-like signs. Actually, feral pigeons are known to harbour a variety of genotypes of *C. psittaci*. In the surveys of feral pigeons whose results are summarized in this communication, only genotypes B, E, and E/B have been detected. Other genotypes that may occur in pigeons, namely A, C and D, which are often present in parrots, ducks and turkeys, respectively, and have been associated with more severe disease in humans, have not been identified. The recently described occurrence of a mild form of psittacosis in humans infected with *C. psittaci* genotype E/B provides evidence that mild disease induced by *C. psittaci* in humans may be overlooked (Harkinezhad et al., 2007). The genotyping of additional strains of *C. psittaci* recovered from feral pigeons is needed in order to assess the relative prevalence of each genotype in these avian populations and ultimately to trace human cases of psittacosis to infections in this animal reservoir.

On the other hand, it may be difficult to unequivocally trace a human case of ornithosis to contact with feral pigeons, since contact with other *C. psittaci*-infected free-living birds that dwell close to humans may have simultaneously occurred. For example, free-living tits (*Parus major* and other

494 *Paridae*) are frequently infected with chlamydiae (Holzinger-Umlauf et al., 1997) and they too
495 might be a source of infection for humans.
496 Assessing the risk of acquiring psittacosis from feral pigeons is also difficult because there is a lack
497 of information and understanding about the mechanism of infection of humans through loose and
498 transient contact with these birds. Research is ongoing to clarify this issue, as well as investigations
499 on indirect ways of transmission of *C. psittaci* from feral pigeons. For example, the relevance of
500 additional transmission routes of *C. psittaci* to humans in the urban environment, such as the
501 inhalation of contaminated water droplets from public fountains where feral pigeons regularly
502 bathe, is currently being investigated at the University of Basel (Geigenfeind and Haag-
503 Wackernagel, pers. comm. 2007).
504
505 **11. Recommendations aimed at preventing pigeon-related psittacosis in humans**
506
507 The degree of exposure to feral pigeons and their excreta, as well as the susceptibility to *C. psittaci*
508 is not homogeneous in the human population. Thus, specific measures for the prevention of feral
509 pigeon-related cases of psittacosis in humans should be adopted at different levels.
510 Education initiatives to communicate the health risks and recommendations for minimizing this risk
511 should be primarily directed at occupationally exposed groups, such as demolition/construction
512 labourers that are exposed to dust contaminated with pigeon excreta. Preventive measures for these
513 categories include wearing protective clothes with hoods, boots, gloves and P2 or P3 air filter face
514 masks when removing pigeon faeces from roofs, garrets and buildings, especially if working
515 indoors. Keeping the pigeon droppings damp while removing them is a simple hygienic measure
516 that helps reduce the risk of inhaling *C. psittaci*-contaminated dust. After work, all clothing should
517 be disposed of, or disinfected in case of future intended use. In Switzerland and Germany, clear
518 guidelines have been published for the prevention of psittacosis when working in areas frequented
519 by feral pigeons and contaminated with their excreta (Tiefbau-Berufsgenossenschaft, 2006).

Monitoring for *C. psittaci* infections over time, by direct detection of the organism and/or by specific antibody testing, should also be considered for this category of workers.

It may be also speculated that other workers in the urban environment, such as street sweepers and traffic wardens might be particularly exposed to *C. psittaci* through inhalation of dust contaminated with pigeon excreta. However, no information is available as to an increased risk of infection in this group compared to the general population. Targeted studies might be helpful to clarify this issue.

Recommendations should also be directed to vulnerable sections of the population that may develop severe clinical manifestations after exposure to *C. psittaci*. Accordingly, children should be warned not to handle sick or dead pigeons and immunocompromised individuals should be educated to carefully limit their contact with feral pigeons and enforce strict hygienic procedures when dealing with the birds.

In many European towns and cities, a reduced and healthier population of feral pigeons should be included among the aims of administrators and health officers, as a general intervention for preserving urban hygiene. The management of feral pigeon populations in the urban environment is a complex issue that requires careful planning. Before any intervention, an evaluation of the local situation as to the number of birds and their aggregation sites is mandatory. Fencing of buildings with pigeon deterring systems such as net-like structures and other mechanical devices represents a first-line intervention measure for preventing fouling. Administration of contraceptive drugs may be useful for reducing the bird population, but this measure is unlikely to lead to a permanent solution and should be coupled with others, in particular with a feeding ban. Pigeon feeders should be encouraged to stop or limit their activity by at least enforcing a feeding ban in defined urban areas that are close to hospitals, railway stations, kindergardens and prisons, where avoidance of pigeon aggregation is considered as a priority. Building dovecotes and artificial breeding facilities may be also considered for providing a balanced diet to the pigeons and a chance of interaction between pigeon lovers and the birds in a hygienically controlled environment. The personnel attending dovecotes should be adequately informed about the health risks arising from contact with pigeons

546 and be regularly monitored for *C. psittaci* infections by DNA or antigen detection methods and/or
547 by antibody testing. For the sake of animal protection, overtly sick birds should be captured and
548 taken into veterinary care. In case chlamydiosis is confirmed, the birds should be appropriately
549 treated with effective drugs such as tetracyclines (chlortetracycline, doxycycline), quinolones
550 (enrofloxacin, difloxacin) or macrolides (clarithromycin) (Theis, 2007; Kinndle, 2007). In the case
551 of very poor conditions, the birds should be euthanized in order to adapt the population to the
552 reduced food supply resulting from public restriction of feeding. Education initiatives directed to
553 the general public are strongly encouraged to illustrate the relationship between feeding,
554 overcrowding, and the deterioration of living conditions of pigeons. In this context, reliable and
555 unbiased information concerning the health hazards arising from the uncontrolled increase of feral
556 pigeon populations should also be provided to the citizens through a variety of media. Regular
557 interaction with the associations involved in the protection of animal welfare and health, such as the
558 Society for the Protection of Animals, is recommended in order to illustrate the implementation of
559 regulatory measures which need to be adopted. Education and information are fundamental, since
560 the imposition of feeding bans usually does not prove successful given the solidarity that pigeon
561 feeders tend to get from the general population. In this scenario, the usefulness of sanctions for
562 those who defy the ban is questionable, since they might actually prove ineffective as to their
563 intended scope of controlling the bird populations.
564

565 **12. Conflict of interest statement**
566

567 None of the authors (Magnino, Haag-Wackernagel, Geigenfeind, Helmecke, Dovč, Prukner-
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Table 1 – Geographical distribution of serotypes and genotypes of *C. psittaci* detected in feral pigeons

Country	**Serovars**	**Genotypes**	**References**
United States	B, E	B, E	Andersen, 1997; Vanrompay *et al.*, 1997; Geens *et al.*, 2005a
Belgium	A, B	A, B, D	Vanrompay *et al.*, 1993; 1997
England	n.d.	A, B	Sayada *et al.*, 1995
The FYR of Macedonia	n.d.	B, E	Ilieski *et al.*, 2007
France	B	B, E	Duan *et al.*, 1999; Laroucau *et al.*, 2008
Italy	A, B, E	A, B, D, E, E/B	Geens *et al.*, 2005a; Laroucau *et al.*, 2008
Japan	n.d.	C	Sayada *et al.*, 1995
The Netherlands	n.d.	B	Heddema *et al.*, 2006b
Switzerland	n.d.	B	Hoop *et al.*, 2002

n.d. = not determined

Table 2 - A summary of surveys for detecting antibodies to *C. psittaci* in feral pigeon populations in some European towns and cities

Country	Town or city	Year of sampling	Laboratory test	Results positive/total (% positive)	Notes	Reference
Bosnia and Herzegovina	Sarajevo	2005	IIF	53/176 (30.1 %)	sera from captured pigeons with no clinical signs	Rešidbegović *et al.*, 2006
		2006	IIF	62/234 (26.5 %)	sera from captured pigeons with no clinical signs	Rešidbegović *et al.*, 2007
Bulgaria	Pleven	1993-1994	CFT	7/20 (35%)	cut-off: 1 :8	Martinov *et al*., 1997
Croatia	Zagreb	1992-1997	CFT	18/44 (40.9 %)	cut-off: 1:8	Vlahović *et al.*, 1998
		1988-1993	CFT	410/834 (49.2 %)	higher antibody titers were detected in pigeons with lesions at necropsy cut-off: 1:8	Pavlak *et al.*, 2000
		2000-2003	Ab-ELISA (LPS)	174/182 (95.6 %)	high antibody titers were detected in 57/182 sera (32.8 %)	Prukner-Radovčić *et al.*, 2005
France	Toulouse	1980-1982	CFT, IIF	186/501 (37.1 %)	CFT cut-off: 1:8 IIF cut-off: 1:40	Milon *et al.*, 1983
			CFT, IIF	46/101 (45.5 %)		
	Paris	1984	CFT, IIF	315/475 (66.3 %)	CFT cut-off: 1:8 IIF cut-off: 1:40	Trap *et al.*, 1986
		1990	CFT	176/415 (42 %)	cut-off: 1:8	Laroucau, pers. comm. 2007
		1999	CFT	316/658 (48 %)	pigeons sampled in the 20 districts, in the Bois de Boulogne and in the Bois de Vincennes	Laroucau *et al.*, 2005

					cut-off: 1:8	
		2003 (March)	CFT	38/75 (51 %)	cut-off: 1:8	Laroucau, pers. comm. 2007
		2003 (December)	CFT	21/43 (49 %)	cut-off: 1:8	Laroucau, pers. comm. 2007
	Troyes	2007 (June)	CFT	7/29 (24 %)	cut-off: 1:8	Laroucau, pers. comm. 2007
Germany	Giessen	1979-2004	CFT	46/81 (56.8 %)	sera from diseased and necropsied feral pigeons	Kaleta and Hönicke, 2004; Kaleta, pers. comm. 2007: Helmecke, 2007
			CFT	286/1,474 (19.4 %)	sera from feral pigeons with no clinical history cut-off: 1:8	
Italy	Pisa	1988	CFT	263/495 (53.1 %)	cut-off: 1 :16	Cerri *et al.*, 1989
	Bologna and Ferrara	1997	CFT	39/178 (21.8 %)	sera from feral pigeons with no clinical history cut-off: 1:16	Renzi and Magnino, 1998
	Bolzano	2006	CFT	38/68 (55.9 %)	cut-off: 1:10	Ceglie *et al.*, 2007
	Venice	2006	CFT	111/267 (41.6 %)	cut-off: 1:10	Ceglie *et al.*, 2007
	Padua	2006	CFT	65/100 (65 %)	cut-off: 1:10	Ceglie *et al.*, 2007
	Verona	2006	CFT	78/167 (46.7 %)	cut-off: 1:10	Ceglie *et al.*, 2007
Spain	Murcia	1991	CFT	36/128 (28.6 %)	CFT cut-off: 1:10	Salinas *et al.*, 1993a

				(28.6 %)		
			MIF	44/128 (33.5 %)	MIF cut-off: 1:32	
			Ab-ELISA (EB)	45/128 (35.9 %)		
Slovenia	Ljubljana	1991-1992	IIF	10/15 (67.7 %)	cut-off: 1:40	Dovč, 1995
		2000	IIF	33/139 (23.7 %)	cut-off : 1:40	Dovč *et al.*, 2004
		2006	IIF	26/86 (30.2 %)	cut-off: 1:40	Dovč, pers. comm. 2006
Switzerland	Luzern	2001	Ab-ELISA (LPS)	33/59 (56 %)		Haag-Wackernagel, 2006a

Legenda
CFT = complement fixation test; Ab-ELISA (EB) = antibody-detection ELISA based on crude antigen from chlamydial elementary bodies;
Ab-ELISA (LPS) = antibody-detection ELISA based on chlamydial lipopolysaccharide; IIF = indirect immunofluorescence assay;
MIF = microimmunofluorescence assay

Table 3 - A summary of surveys for detecting *C. psittaci* in feral pigeon populations in some European towns and cities

Country	Town or city	Year of sampling	Laboratory test	Results positive/total (% positive)	Notes on the type of sample(s)	Reference
Bosnia and Herzegovina	Sarajevo	2006	PCR (*ompA*)	3/8 (37.5 %)	tissue samples from dead birds	Rešidbegović *et al.*, 2007
Bulgaria	Sofia	2006	EI	2/15 (13.3 %)	pool of spleen, liver and lung from necropsied birds	Martinov, 2006
Croatia	Zagreb	1992-1997	DIF	4/39 (10.2 %)	tissue samples from necropsied birds	Vlahović *et al.*, 1998
		2000-2003	ICT	3/107 (2.8 %)	cloacal swabs	Vlahović *et al.*, 2004
			EI	0/3 (0 %)		
		2000-2003	Ag-ELISA	44/278 (15.8 %)	cloacal swabs	Prukner-Radovčić *et al.*, 2005
		2006	Ag-ELISA	120/787 (15.3 %)	cloacal swabs	Prukner-Radovčić, pers. comm. 2007
France	Toulouse	1980-1982	EI	3/101 (3 %)	pool of spleen and lung	Milon *et al.*, 1983
			TC	3/150 (2 %)	cloacal, intestinal and pharyngeal swabs	
	Paris	1984	EI	4/7 (57.1 %)	tissue samples from necropsied birds	Trap *et al.*, 1986
		2003 (March)	Rt PCR (23S rRNA)	5/33 (15.2 %)	cloacal swabs	Laroucau, pers. comm. 2007
		2003 (December)	Rt PCR (23S rRNA)	4/20 (20 %)	cloacal swabs	Laroucau, pers. comm. 2007

		(December)	(23S rRNA)	(20 %)		2007
	Troyes	2007 (March)	Rt PCR (23S rRNA)	5/33 (15.2 %)	cloacal swabs	Laroucau, pers. comm. 2007
		2007 (June)	Rt PCR (23S rRNA)	1/29 (3.4 %)	cloacal swabs	Laroucau, pers. comm. 2007
Germany	Giessen	1979-2004	TC	10/77 (13 %)	tissue samples from necropsied birds	Kaleta and Hönicke, 2004; Kaleta, pers. comm 2007; Helmecke, 2007
Italy	Pisa	1988	EI	14/35 (40 %)	pool of viscera (lung, liver and spleen) from necropsied birds	Cerri *et al.*, 1989
	Trento	1995	TC	12/35 (34.3 %)	intestinal content from necropsied birds with no clinical history	Manfredi *et al.*, 1997
	Milan	1996-1997	TC	30[1]/163 (18.4 %)	intestinal content from necropsied birds with no clinical history	Rampin *et al.*, 1998
	Bologna and Ferrara	1997	TC	34[2]/178 (19.1 %)	intestinal content from necropsied birds with no clinical history	Renzi and Magnino, 1998
	Bergamo	1998-1999	TC	11/26 (42.3 %)	tissue samples from necropsied birds with no clinical history	Gaffuri *et al.*, 2000
	Venice	2006	PCR (16S rRNA)	7/50 (14 %)	liver and spleen collected from necropsied birds	Ceglie *et al.*, 2007
The FYR of Macedonia	Skopje	2004-2005	Ag-ELISA	10/25 (40 %)	conjunctival, choanal and cloacal swabs	Mitevski *et al.*, 2005
		2006	Rt PCR (23S rRNA)	2[3]/36 (5.6 %)	cloacal swabs	Ilieski *et al.*, 2007
	Prilep	2004-2005	Ag-ELISA	4/16 (25 %)	conjunctival, choanal and cloacal swabs	Mitevski *et al.*, 2005

				(25 %)	swabs	
	Kumanovo	2004-2005	Ag-ELISA	2/10 (20 %)	conjunctival, choanal and cloacal swabs	Mitevski *et al.*, 2005
	Bogdanci	2004-2005	Ag-ELISA	2/12 (16.7 %)	conjunctival, choanal and cloacal swabs	Mitevski *et al.*, 2005
	Vinica	2006	Rt PCR (23S rRNA)	10^4/20 (50 %)	cloacal swabs	Ilieski *et al.*, 2007
	Stip	2006	Rt PCR (23S rRNA)	4^5/60 (6.7 %)	cloacal swabs	Ilieski *et al.*, 2007
The Netherlands	Amsterdam	2005	Rt PCR (*ompA*)	26^6/331 (7.9 %)	fresh faecal droppings	Heddema *et al.*, 2006b
Spain	Murcia	1991	TC	7/39 (18 %)	cloacal swabs	Salinas *et al.*, 1993a
			EI	5/39 (12.8 %)	cloacal swabs	
Slovenia	Ljubljana	2006	DIF	2/86 (2.3 %)	cloacal swabs	Dovč, pers. comm. 2006
			EI	1/86 (1.2 %)	cloacal swabs	
Switzerland	Luzern	2001	Ag-ELISA	2/60 (3.3 %)	cloacal swabs	Haag-Wackernagel, 2006a
			EI	1/60 (1.6 %)	cloacal swabs	

Notes
(1) Three isolates were genotype B
(2) Four isolates were genotype E and one was genotype E/B
(3) One PCR product was genotype E
(4) Four PCR products were genotype E
(5) One PCR product was genotype B, and one was genotype E

(6) Ten PCR products were genotype B

Legenda
TC = tissue culture; EI = egg inoculation; Ag-ELISA = antigen-detection ELISA; DIF = direct immunofluorescence assay; ICT = immunochromatographic test
PCR = polymerase chain reaction; RFLP = restriction fragment length polymorphism; Rt PCR (gene) = real-time PCR (targeted gene)