A preliminary study of *Chlamydophila felis* prevalence among domestic cats in the City of Zagreb and Zagreb County in Croatia

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Introduction

Feline chlamydiosis is a disease in domestic cats caused by *Chlamydophila felis* (*Cp. felis*), which is primarily a pathogen of the conjunctiva and nasal mucosa rather than a pulmonary pathogen. It is capable of causing acute to chronic conjunctivitis, with blepharospasm, chemosis and congestion, a serous to mucopurulent ocular discharge, and rhinitis (Hoover et al., 1978; Sykes, 2005). *C. psittaci*[1] infection in kittens produces fever, lethargy, lameness, and reduction in weight gain (Terwee at al., 1998). According to the literature, chlamydiosis in cats can be treated successfully by administering potentiated amoxicillin for 30 days, which can result in a complete clinical recovery with no evidence of a recurrence for six months (Sturgess et al., 2001).

Rampazzo et al. (2003) investigated the prevalence of *Cp. felis* and feline herpesvirus in cats with conjunctivitis by using a conventional polymerase chain reaction (PCR), and discovered that 14 out of 70 (20%) cats with conjunctivitis were positive only on *Cp. felis* and mixed infections with herpesvirus were present in 5 of 70 (7%) cats.

Helps et al. (2005) took oropharyngeal and conjunctival swabs from 1101 cats and by using a PCR determined *Cp. felis* in 10% of the 558 swab samples of cats with URDT and in 3% of the 558 swab samples of cats without URDT.

Low et al. (2007) investigated 55 cats with conjunctivitis, 39 healthy cats and 32 cats with a history of conjunctivitis that been resolved for at least 3 months. By using conventional PCR assays to determine the prevalence of *Cp. felis* in cats with and without conjunctivitis, they

[1] *Chlamydophila felis* (formerly *Chlamydia psittaci*)

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found that the overall prevalence rate of *Cp. felis* was 3.2%.

In Sibitz et al. (2011), the presence of *Chlamydophila felis* was found using a real-time (RT) PCR in two cats with conjunctivitis out of the 49 which were being investigated, while *Chlamydiae* related to uncultured members of Chlamydiales were found in three more cats with conjunctivitis.

Of 200 cats from 19 cantons in Switzerland that were suspected of having calicivirus (FCV)-related symptoms, 8% were positive for *Cp. felis*, and this microorganism was also present in 1% of 100 healthy cats from the same region. The samples were tested for *Cp. felis* using an RT quantitative PCR (qPCR) (Berger et al., 2015). Fernandez et al. (2017) discovered, by using a real-time polymerase chain reaction (PCR), that 20.5% of 127 cats with URTD were positive for *Cp. felis*, as well as 19.5% of 149 with conjunctivitis and 9.1% of 154 with gingivostomatitis (GS).

However, there could be a possible limitation to the molecular evidence for chlamydophila infection in naturally infected cats because of histological alterations in conjunctiva caused by chronic conjunctivitis (Kiełbowicz et al., 2014).

Based on serum analyses for the presence of antibodies against *Cp. felis* in 214 Swedish cats with no signs of infectious disease, Ström Holst et al. (2006) found that 11% of the cat serums were positive but had low antibody titres. The aim of this preliminary study was to investigate the prevalence of *Chlamydophila felis (Cp. felis)* among domestic cats in City of Zagreb and Zagreb County, Croatia.

Materials and methods

48 cats were investigated. All of them came as patients to clinics of the Faculty of Veterinary Medicine in Zagreb. The cats were both pure and mixed breeds, different sexes, and aged from two months to 18 years old. Each cat was clinically examined.

The cats had not been vaccinated against *Cp. felis,* and before swabs were taken they were not treated with any medication. 48 swabs were taken from the cats' eyes and 48 from their nostrils. Rapid EIA tests were carried out immediately after collecting the samples. For further analyses, the swabs were placed into a 2 mL sucrose phosphate transport medium (Spencer and Johnson, 1983) and stored at – 80 °C until analysis. Conjunctival scrapings were also taken from both eyes for direct immunofluorescence (DIF) tests.

1. Detection of chlamydial antigens

Rapid (immunoenzyme assay) EIA

The 192 ocular and nasal swabs, were examined using a rapid EIA (Clearview Chlamydia MF, Unipath Limited®, Bedford, United Kingdom) according to the manufacturer's instructions. This test is a rapid immunoassay for the direct genus specific qualitative detection of the *Chlamydia trachomatis* antigen, but it can also be used for *Cp. felis* detection (Trávnicek et al., 2002; Pavlin et al., 2005).

Direct immunofluorescence (DIF)

DIF was performed on thirty ocular swabs with a commercially FITC-labelled monoclonal antibody specific for the major outer membrane protein (MOMP) of *Chlamydia trachomatis*

(CHLAMYDIA DIRECT IF IDENTIFICATION, Biomerieux, France) according to the manufacturer's instructions. It can also be used for *Cp. felis* detection.

DNA extraction, conventional polymerase chain reaction (PCR) and sequencing

PCR was performed on six samples (three nostril samples, three eye samples).

Before DNA extraction ocular and nasal swabs were placed in 2 mL of Chlamydia transport medium and homogenised by vortexing. Total DNA was extracted from 200 µL of the sample using QIAamp®DNA Mini Kit (Qiagen, Hilden, Germany) according to manufacturer`s protocols for Blood and body fluid spin protocol. The presence of Chlamydiaceae was confirmed using specific primers 16SF2 (5` CCGCCCGTCACATCATGG 3`) and 23R (5` TACTAAGATGTTTCAGTTC 3`) for amplifying approximately 600 bp long segment containing partial 16S RNA gene, intergenic spacer region and partial 23S RNA gene (Everett et al., 1999). The PCR was preformed using HotStartTaq®Plus PCR (Qiagen, Hilden, Germany) in 25 µL reaction according to manufacturer`s instruction. The PCR program consisted 5 minute at 95 °C for PCR initial activation step followed with 33 cycles of 30 second at 94 °C, 1 minute at 50 °C and 1 minute at 72 °C, with a final extension of 7 minute at 72 °C. Amplicons were visualized by gel electrophoresis on a 1,8% bromide stained agarose gel. PCR products were excised from the gel, purified with Wizard PCR Preps DNA Purification System (Promega Corp., Madison WI, USA) following manufacturer's instructions and sent for sequencing to Macrogen laboratory (Macrogen Inc, Seoul, Korea). Nucleotide sequence data were analysed by BLAST (Altschul et al., 1990) for finding similarity with sequences from NCBI sequence database.

Results

Most of the examined cats were in good health. Their coats were shiny, and not brittle or coarse. Based on the cats' owners' statements, all of the cats had a good appetite and normal behaviour. Their body temperatures were normal, as were their pulses and breathing. However, examination of their conjunctival mucosa showed that 54% had unilateral or bilateral mild conjunctivitis.

One cat, whose biological samples were PCR positive, was underfed and run-down, and its fur was unkempt and in an unhealthy condition. This cat also showed bilateral conjunctivitis with marked mucopurulent discharge from the left eye and nasal discharge from both

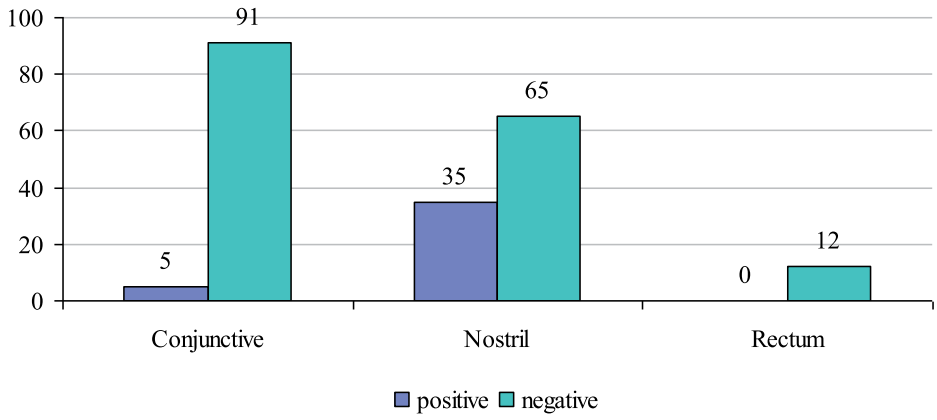

Fig. 1. The results (%) of rapid immunoenzymme assay (EIA) performed on conjunctival, nostril and rectal swab samples

Table 1. Results of direct immunofluorescence performed on conjunctival smears from cats

DIF	Left eye number (%)	Right eye number (%)
Positive result	3 (20.0)	1 (6.7)
Negative result	11 (73.3)	13 (86.6)
Inconclusive result	1 (6.7)	1 (6.7)
Total	15 (100)	15 (100)

nostrils. According to the cat's owner, the mucopurulent ocular discharge had appeared the day before she came to the clinic and the cat had not been treated with any medication. A thoracic radiograph was performed on this cat because of a suspicion of pneumonia and it was examined for FeLV and FIV using FASTest FeLV and FASTest FIV tests (Mega Cor Diagnostic).

Using a rapid immunoenzyme assay (EIA) produced 5-35% positive results, depending on which mucosa were examined. The results are shown in Fig. 1.

The eye samples which were positive when using the EIA were examined using DIF, and the results are shown in Table 1.

Of six clinical samples (three nostril samples and three eye samples), only two were found to be positive for *Cp. felis* using a PCR. More precisely, the positive samples were taken from the left nostril and left eye of the same cat. The positive and control samples produced an approximately 600 bp long product in the first round of PCR (Fig. 2). For confirmation of the positive results and determination of *Chlamydia*, species sequencing was performed.

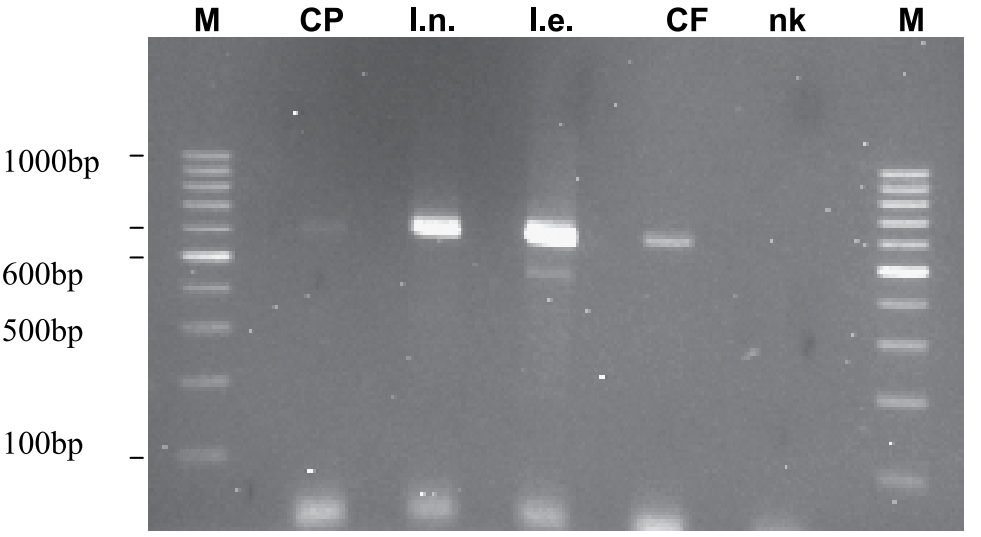

Fig. 2. Agarose gel electrophoresis of PCR products extracted from clinical samples (left nostril and left eye of one cat): Column M = 100 bp long DNA standard: Column CP = positive control *Cp. psittaci*: Column l.n. = epithelial cell swab sample of M47 cat's left nostril: Column l.e. = epithelial cell swab sample of cat's left eye: Column CF = positive control *Cp. felis:* Column nk = negative control

A comparison of obtained 445 bp nucleotide sequences including partial 16S RNA gene, intergenic spacer region and partial 23S RNA gene with sequences from GenBank database demonstrate the 100% identity with different *Cp. felis* strains such as *Cp. felis* strain Fe/C–56 (accesion number AP006861.1), FB baker (VR-120) (accesion number U68457.4), FB Vaccine (accesion number U68459.1) for example. The compared region was from 149200 bp to 149644 bp numbering from start of the genome of *Cp. felis* strain Fe/C–56 (accesion number AP006861.1).

Discussion

This research was a preliminary study of the presence and prevalence of *Cp. felis* among the cat population in Croatia. Our experience showed a lack of knowledge about feline chlamydiosis in small animal veterinary clinics and inadequate investigation of cat patients suffering from conjunctivitis or from upper respiratory tract disease (URDT) related to chlamydiosis.

Since cats suffering from chronic chlamydiosis can have no clinical symptoms (Wills, 1986), this research included healthy cats as well as cats with conjunctivitis, which is believed to be the primary symptom of cat chlamydiosis (Sykes, 2005). Thus, 27 of 48 examined cats had conjunctivitis and 23 of them were seemingly healthy. An EIA test was used only as an orientational diagnostic test to choose the cats whose biological materials would be forwarded for further specific diagnostic procedures. Using an EIA, positive samples were identified. In Trávnicek et al (2002), 76-80% of examined cats were EIA positive, but the authors examined only those cats with conjunctivitis. This research, which included cats both with and without conjunctivitis, established a range of 5-35% of positive samples, depending on which mucosa was examined. 59.3% of cats with conjunctivitis were EIA positive, as opposed to 8.6% of seemingly healthy cats. However, according to Sykes (2005), the incidence of *Cp. felis* among healthy cats is, in general, low.

The next chosen diagnostic procedure was DIF (Dorin et al., 1993; Dovč et al., 1994; Pavlin et al., 2005; Tozon et al., 2006). The samples of three EIA positive cats were not appropriate for further examination using DIF because of poor fixation. Thus, 15 cats out of 18 EIA positive animals, irrespective of whether their mucosa was positive, were involved in further examinations using DIF. There were 13 cats with conjunctivitis and 2 seemingly healthy cats. Right and left eye swab samples were examined, and the results showed that three cats with conjunctivitis whose eye swabs were positive for EIA were also DIF positive.

A conventional PCR was performed on the samples of the three DIF positive cats. Eye as well as nostril swabs were examined. The result of the PCR method was positive for both the left nostril and left eye swab samples in one cat with bilateral conjunctivitis.

Data reported in the literature show about 3.3% of PCR positive cats among 60 examined healthy cats and cats with conjunctivitis (Di Francesco et al., 2004). According to Low et al. (2007), the overall prevalence rates of *Cp. felis* in cats with and without URDT was 3%. These results are very similar to the ones obtained in this study (4%).

In recent literature, among cats with conjunctivitis, a wide range of positive results using different PCR techniques was obtained. Rampazzo et al. (2003) established 20%, Helps et al. (2005) 10%, and Sibitz et al. (2011) 4% positive rates among cats with conjunctivitis, while Berger et al. (2015) established 8%.

At the same time, among cats without symptoms of conjunctivitis, the above researchers established 0% (Rampazzo et al., 2003), 3% (Helps et al., 2005), and 1%

(Berger et al., 2015) of cats being positive for *Cp. felis*.

Despite the fact that these data are very similar to the obtained results in which only one out of 48 (4%) examined cats was PCR positive, they are not completely comparable because not all of the 48 examined cats were examined using a PCR. However, this result can be a starting point for further examination to establish the prevalence of the agent in the Croatian cat population, regardless of whether they have conjunctivitis or not.

Abstract

This research was preliminary study of the presence and prevalence of *Chlamydophila felis* (*Cp. felis*) among domestic cats in the City of Zagreb and Zagreb County in Croatia. 48 pure and mixed breed cats were examined. 192 conjunctival and nasal swab samples were examined using a rapid immunoenzyme assay (EIA). 30 conjunctival scraping samples of 15 cats that were EIA positive were examined using DIF. Four of them were positive, while the results of two samples were inconclusive. These six samples (three nostril samples and three eye samples) were examined using a conventional polymerase chain reaction (PCR), and only two samples from the same cat were found to be positive for *Cp. felis*. The sequencing of the PCR product confirmed that the causative agent of the cat's conjunctivitis was *Cp. felis*. By using direct immunofluorescence as well as a polymerase chain reaction procedure and sequencing, the results of the research established the existence of the bacteria *Cp. felis* in one out of 48 domestic cats.

Key words: *feline chlamydiosis, cat, Chlamydophila felis, immunofluorescence, PCR, Zagreb, Zagreb County*

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Preliminarno istraživanje učestalosti pojave bakterije *Chlamydophila felis* u domaće mačke u Gradu Zagrebu i na području Zagrebačke županije

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Cilj ovog preliminarnog istraživanja bio je ustanoviti učestalost pojave bakterije *Chlamydophila felis* (*Cp. felis*) u domaće mačke u Gradu Zagrebu i na području Zagrebačke županije. Istraživanjem je bilo obuhvaćeno 48 mačaka različitih pasmina. Brzim enzimskim imunološkim dijagnostičkim postupkom (EIA) pretraženo je 192 uzoraka obrisaka konjunktiva i nosnica te 12 uzoraka obriska sluznice rektuma. Trideset obrisaka konjuktiva s 15 mačaka, pozitivnih na EIA, pretraženo je postupkom izravne imunofluorescencije (DIF). Četiri su uzorka bila je pozitivna, a u dva je nalaz bio sumnjiv. Potom je tih šest uzoraka (tri uzoraka obriska nosne sluznice i tri obriska konjunktive) pretraženo postupkom lančane reakcije polimerazom (PCR). Dva su uzorka, podrijetlom s iste mačke, bila pozitivna. Rezultat sekvencioniranja produkta PCR dokazao je da je bolest u te domaće mačke bila uzrokovana upravo bakterijom *Cp. felis*. Korištenjem postupka izravne imunofluorescencije, lančane reacije polimerazom te sekvenciranjem produkta PCR dokazano je postojanje bakterije *Cp. felis* kao uzročnika mačje klamidioze u jedne od 48 mačaka.

Ključne riječi: *klamidioza mačaka, mačka, Chlamydophila felis, imunofluorescencija, PCR*