

PCR CONFIRMATION OF *CHLAMYDOPHILA FELIS* FROM NASAL AND CONJUNCTIVAL SWAB SAMPLES OF A DOMESTIC CAT IN CROATIA

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Until now there is no evidence that chlamydiosis does exist among cats in the Republic of Croatia regardless of the fact that feline chlamydiosis is a worldwide disease. This report describes the clinical examination of a one year old cat with bilateral conjunctivitis, as well as the diagnostic methods used to confirm infection with Chlamydomphila (Cp.) felis. We have used rapid enzyme immunoassay test (EIA) for antigen detection, in order to examine the swabs taken from the eyes and nostrils. The positive result was confirmed by direct immunofluorescence (DIF) made on scrapings from cat's conjunctivas. Conventional polymerase chain reaction (PCR) made on swabs taken from cat's conjunctivas and nostrils and sequencing the PCR product was used to confirmed infection of cat precisely with Chlamydomphila felis. No increase of IgG antibodies against chlamydia was noted using indirect immunofluorescence (IFA) method.

Key words: Chlamydomphila felis, cat, DIF, IFA, PCR

INTRODUCTION

Chlamydia was isolated from cats, for the first time, in the United States. The microorganism was called *Chlamydia (C.) psittaci*^{*}, it was considered to be the major cause of feline respiratory disease and it was also called feline pneumonitis agent (Baker, 1942). Nowadays, regardless suspicion (Lipman *et al.*, 1994) only one chlamydial organism had been identified in cats, *Chlamydomphila (Cp.) felis* (Sykes, 2005).

It is primarily a pathogen of the conjunctiva and nasal mucosa rather than a pulmonary pathogen (Hoover, 1978). *Cp. felis* 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). Experimentally *C. psittaci* infection to kittens produces fever, lethargy, lameness, and reduction in weight gain (Terwee *et al.*, 1998). According to literature, chlamydiosis in cats can be treated successfully by administering potentiated

* *Chlamydomphila felis* (previously named *Chlamydia psittaci*)

amoxicilin for 30 days and that could result in a complete clinical recovery with no evidence of recurrence for six months (Sturgess *et al.*, 2001).

The presence of bacteria *Cp. felis* were confirmed among cats in USA (Cello, 1967; Bannasch and Foley, 2005), Great Britain (Darougar *et al.*, 1977; Johnson, 1984; Wills *et al.*, 1984), Canada (Shewen *et al.*, 1978), Australia (Studdert *et al.*, 1981), Japan (Fukushi *et al.*, 1985; CAI *et al.*, 2002), Slovenia (Dovč *et al.*, 1994), New Zealand (Gruffydd-Jones *et al.*, 1995) and Sweden (Holst *et al.*, 2006).

Despite this worldwide spreading, the presence of *Cp. felis* in cats in Croatia has not been reported to date, so this short report presented for the first time shows that the bacteria *Cp. felis* is also inherent among cats in Croatia.

MATERIAL AND METHODS

On May 2008, the one year old female cat (mixed breed) with conjunctivitis was a patient at the Clinic for Internal Diseases, Veterinary Faculty University of Zagreb. The cat had not been vaccinated against *Cp. felis*, and before swabs were taken it was not treated with any medication. No microorganisms were isolated on routine examination of various cell culture techniques of an ocular swab from the affected cat. The fast diagnostic tests FASTest FeLV and FASTest FIV (Mega Cor Diagnostic) were also done. Thoracic radiograph was performed. Swabs were taken from cat's eyes and nostrils respectively. The rapid EIA tests were done immediately after collecting the samples. For further analyses swabs were placed into 2 mL sucrose phosphate transport medium (Spencer and Johnson, 1983) and stored at -80°C until analyses. Conjunctival scrapings were also taken from both eyes for direct immunofluorescence (DIF) tests. The blood sample was taken from the *vena cephalica antebrachii*, it was obtained when the swabs were taken. The haematological examinations were done immediately after blood was taken using haematological counter BAKER System Serrono 9120 CP (Serrono-BAKER Diagnostic, Inc, Allentown, Pennsylvania, USA) and biochemical examinations were done using automatic analyser Olympus AU 600 (Olympus Diagnostica GMBH, Hamburg, Germany).

The rest of the serum was stored at -20°C until tested for chlamydial antibodies using indirect immunofluorescence (IFA).

1. Detection of chlamydial antigen

Rapid EIA

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

Direct immunofluorescence (DIF)

DIF is performed on 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 be used for *Cp. felis* detection.

Conventional polymerase chain reaction (PCR)

Total DNA was extracted from 1 mL of the sample using QIAamp®DNA Mini Kit (Qiagen, Hilden, Germany) according to manufacturer's protocols for bacteria. Before DNA extraction ocular and nasal swabs were placed in 2 mL of Chlamydia transport medium and homogenised by vortexing. The presence of *Chlamydiaceae* was confirmed using specific primers 16SF2 and 23R targeting 16S–23S rRNA gene intergenic spacer region (Everett and Andersen, 1999) The PCR was performed using HotStartTaq®Plus PCR (Qiagen, Hilden, Germany) in 25 µL reaction according to manufacturer's instruction. The PCR program consisted of 5 minutes at 95 °C for PCR initial activation step followed with 33 cycles of 30 seconds at 94 °C, 1 minute at 50 °C and 1 minute at 72 °C, with a final extension of 7 minutes at 72 °C. Amplicons were visualized by gel electrophoresis on a 1.5% bromide stained agarose gel. The size of the amplicons were approximately 600 bp. Amplicons were excised from the gel and PCR products were purified with Wizard PCR Preps DNA Purification System (Promega Corp., Madison WI, USA) following manufacturer's instructions.

DNA sequencing and nucleotide analysis

The sequencing of PCR product was performed in MacroGen Inc. (Seoul, Korea). Sequence data were assembled and edited using Chromas v.1.45 (<http://www.technelysium.com.au/chromas14x.html>) Analysis of nucleotide sequences were conducted using NCBI Blast program (<http://blast.ncbi.nlm.nih.gov>).

2. Detection of antibodies against *Chlamydophila felis*

Indirect immunofluorescence (IFA)

Specific antibodies against *Cp. felis* were determined in cat's serum using indirect immunofluorescence test (FELINE CHLAMYDIA IG-g ANTIBODY KIT (FULLER LABORATORIES, California, USA) according to manufacturer's recommendations. This test could provide detection and quantitative determination of IgG class antibodies against chlamydia. As the species specific conjugate, we were using Anti-cat IgG (whole molecule) FITC conjugate (SIGMA, USA), developed in goat using purified cat IgG. Goat anti-cat IgG is conjugated to Fluorescein Isothiocyanate (FITC). Titer 1:40 could be considered as a positive titer.

RESULTS

Clinical examination showed that the cat was underfed, depressed, the fur was sluttish and defected. Body temperature was normal, as well as its pulse and breathing. The cat showed bilateral conjunctivitis with marked mucopurulent discharge from the left eye and nasal discharge from both 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 medications. Thoracic radiograph was negative. The fast diagnostic tests FASTest FeLV and FASTest FIV (Mega Cor Diagnostic) were negative, as well as the results of microbiological examinations.

The results of rapid EIA, DIF, PCR and IFA are given in Table 1, and the positive result of DIF are shown in Fig. 1.

Table 1. The results of rapid EIA, DIF, PCR swabs of eyes and nostrils) and results of IFA cat's sera) in the cat which showed bilateral conjunctivitis

Test \ Swab	Left eye	Right eye	Left nostril	Right nostril	Antibodies titer IgG
Rapid EIA	positive	negative	positive	positive	–
DIF	positive	negative	–	–	–
PCR	positive	negative	positive	dubious	–
IFA	–	–	–	–	negative

"–" test was not done

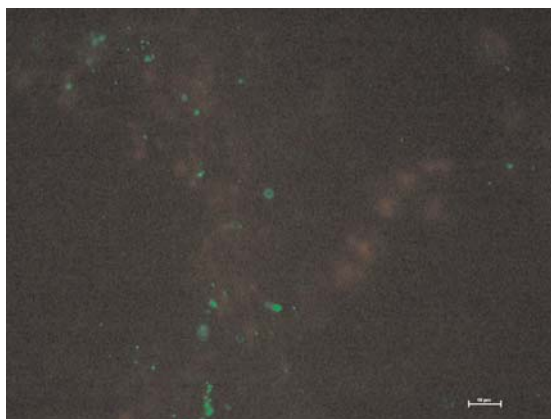


Figure 1: The positive result of direct immunofluorescence test performed on left eye (1:400 AXIOSKOP, OPTON, Germany)

The results of observed haematological, as well as biochemical values of examined blood samples were within normal.

With PCR approximately 600 bp long product was obtained from swabs of eye and nostril. For conformation of positive results and determination of Chlamydia species sequencing was preformed. Comparison of obtained 445 bp nucleotide sequences with sequences from GenBank database demonstrate the 100% identity with different *Cp. felis* strains such as *Cp. felis* strain Fe/C-56 (accession number AP006861.1), FB baker (VR-120) (accession number U68457.4), FB Vaccine (accession 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 (accession number AP006861.1).

DISCUSSION

Until now, in southeastern Europe, only in Slovenia (Dovč *et al.*, 1994) the presence of *Chlamydophila felis* among domestic cat population was confirmed. Our experiences showed that veterinarians in small animal veterinary practice have little knowledge about clinical symptoms of feline chlamydiosis and they don't use proper diagnostic methods in cat patients suffering from conjunctivitis or upper respiratory tract disease (URDT).

However, feline chlamydiosis agent is just one of the agents which caused URDT, but it rarely caused pneumonia (Wills *et al.*, 1984). So, cats were treated only with topical ophthalmic ointments which led to improved clinical symptoms, but a complete clinical recovery could not be obtained. The examined cat was about one year old which placed it in the group of cats susceptible to sickness (Sykes, 2005).

The fact that the cat had serous ocular discharge from the left eye whereas mucopurulent ocular discharge from the right eye indicated that the process had begun just on one eye. According to literature chlamydiosis in naturally infected cats usually begins on one eye (Masubuchi *et al.*, 2002). Except pathological changes on eyes, lack of weight gain and threadbare fur, there were no other clinical signs of chlamydioses. EIA was used only as an orientational diagnostic method (Travnicek *et al.*, 2002; Pavlin *et al.*, 2005). Positive result of the EIA method was just the direction for further procedures of specific and more sensitive diagnostic methods.

The presence of chlamydial antigen found due to DIF confirmed the diagnoses of chlamydial infection. The results of conventional PCR and sequencing of the product obtained by PCR confirmed that the cat was infected with *Chlamydophila felis*. When the nucleotide sequence of the 16S-23S rRNA gene intergenic spacer region was compared with the same region of the known *Cp felis* strains Fe/C-56 (accession number AP006861.1), deposited in GeneBank database (Benson *et al.*, 2002), we found a 99% similarity.

Tozon *et al.* (2006) establish that cats with antibody titre of 1:320 shows clinical symptoms, but the examined cat was showing typical clinical symptoms without an increase of antibodies. The cat's owner didn't notice any signs of illness during unilateral serous ocular discharge and she required veterinary aid at the moment when the inflammatory process was manifested by mucopurulent ocular discharge. According to literature data (Hoover *et al.*, 1978) it could be presumed that the cat was infected about 15 days before the owner got it to the veterinarian. In fact, the clinical symptoms began at least 5-10 days after infection, primarily as serous conjunctivitis, and after 3-5 days serous conjunctivitis turned into mucopurulent conjunctivitis. Terwee *et al.* (1998) found that chlamydia-specific IgG plasma antibody was evident at day 26 in infected animals which developed conjunctivitis. That means that at the moment when blood samples were taken, considerable upgrowth of the antibodies titer couldn't develop.

The cat has been successfully treated for 30 days using potentiated amoxicilin (Sturgess *et al.*, 2001). This case indicates that feline chlamydiosis does exist in Croatia. The results obtained present a direction according to which further examinations of chlamydiosis prevalence within the cat population in the Republic of Croatia can be undertaken.

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DOKAZ BAKTERIJE *CHLAMYDOPHILA FELIS* PCR-om U UZORCIMA OBRISAKA NOSA I OČIJU U DOMAĆE MAČKE U HRVATSKOJ

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SADRŽAJ

Unatoč rasprostranjenosti klamidioze u mačaka širom sveta, sve do sada nije bilo dokaza da klamidioza domaće mačke postoji u populaciji domaćih mačaka u Republici Hrvatskoj. U ovom radu opisujemo kliničke simptome u jednogodišnje mačke s bilateralnim konjunktivitisom te prikazujemo dijagnostičke metode kojima smo u konačnici dokazali da je bolest uzrokovana upravo bakterijom *Chlamydophila (Cp.) felis*. Koristili smo brzi enzimski imunološki test (EIA) za detekciju antigena kako bismo istražili uzorke obrisaka nosa i očiju. Pozitivan rezultat potvrđen je specifičnijom i osjetljivijom dijagnostičkom metodom direktne imunofluorescencije kojom smo istražili obriske očiju. Konvencionalna reakcija lančane reakcije polimerazom na uzorcima konjunktiva i nosa te sekvenciranje produkata dobivenih PCR-om dokazali su da je bolest uzrokovana upravo bakterijom *Chlamydophila felis*. Metodom indirektno imunofluorescencije nije dokazan porast specifičnih IgG antitela.

