DNA DETECTION AND EVALUATION OF ANTIBODIES AGAINST *CHLAMYDOPHILA FELIS* IN DOMESTIC CATS FROM THE NORTHEAST OF THE STATE OF SÃO PAULO, BRAZIL.

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Key words: Feline chlamydiosis, indirect immunofluorescence assay, complement fixation test, PCR
ABSTRACT

This is a report of the results of indirect immunofluorescence assay (IFA), complement fixation test (CFT) and direct DNA detection by PCR to determine Chlamyphila felis infection in domestic cats. A total of 145 sera and conjunctival swab samples collected from cats showing clinical signs or clinical history compatible with feline chlamydiosis were collected in catteries, veterinary clinics and public shelters in five cities in northeast of the state of São Paulo, Brazil. Antibodies against Chlamydiaceae were detected in 72.41% and 6.9% of the sampled animals, and titers were over 256 in IFA and CFT. Compared with IFA, CFT was found to be less sensitive in anti-Chlamyphila spp. antibody detection in cats. Bacterial DNA was detected in swab samples of nine cats (6.21%) distributed among all analyzed groups. Results obtained in serological and PCR analyses indicate that C. felis is found in cats from northeast of the state of São Paulo, and veterinary practitioners should consider this bacterial infection as a possible threat for cats in Brazil.

Key words: Feline chlamydiosis, indirect immunofluorescence assay, Complement Fixation test, PCR.

INTRODUCTION

Chlamyphila felis, formerly known as Chlamydia psittaci (Everett 2000), is classified as member in Chlamydiaceae family, an obligate intracellular bacterium, with cell walls resembling those of gram-negative bacteria. It was first isolated from cats with respiratory disease in the United States, in 1942 (Baker et al. 1942).

C. felis is primarily a conjunctival pathogen, capable of causing acute to chronic conjunctivitis, with blepharospasm, chemosis, congestion and serous to mucopurulent ocular discharge in cats (Hoover et al. 1978). Nasal discharge, sneezing and other signs of upper respiratory tract disease (URTD) may occur in some cats (O’Dair et al. 1994). Clinical signs of the disease are influenced by the age of the cat, its immunocompetence, which tissues are affected, and the inoculum (Ramsey 2000). Transmission is thought to occur mainly by direct contact with infected ocular secretion.

Diagnosis of C. felis in domestic cats can be carried out by demonstration of the antigen or serological tests. Many surveys, involving both detection of the organism and serology, have
been conducted to determine the prevalence of *C. felis* in cat populations of many countries. The presence of complement-fixation antibodies in cat sera ranged from 0 to 12.7% (Studdert & Martin 1970, Studdert et al. 1981, Povey & Johnson 1971, Lazarowicz et al. 1982, Fukushi et al. 1985). Indirect immunofluorescence assay (IFA) in English and Swedish cats with clinical signs showed antibody titers ranging from 9.1% to 11%, respectively (Holst et al. 2006; Gunn-moore et al. 1995). In contrast, 45% of healthy cats in England were positive in IFA (Gethings et al. 1987). IFA was more sensitive than the CFT in the detection *Chlamydiaceae* from cat sera (Wills et al. 1987). However, not all animal sera or immunoglobulin isotypes are able to fix complement (Schmeer et al. 1987, Kaltenboeck et al. 1997). Furthermore, CFT is not feasible with haemolytic or anti-complementary sera (Cross and Clafin 1963).


It is important to detect whether a cat is shedding *C. felis* for several reasons: it may aid the choice of appropriate antibiotics for sick animals and direct the proper disinfection program; besides, accurate diagnostic techniques may ensure that cats shedding the organism will not be introduced in *Chlamydophila*-free households (Gaskell 1993).

Although a vaccine for feline chlamydiosis is available in Brazil, there are no studies that demonstrate the occurrence of the disease in this country. The study reported here was a preliminary analysis to determine the occurrence of *Chlamydophila* spp infections in cats from the northeast of the state of São Paulo, Brazil, using serological analyses and direct detection of Chlamydial DNA. We also compared the results of Complement Fixation Test (CFT) and Indirect Immunofluorescence Assay (IFA) in the detection of antibodies against *Chlamydophila* spp in cats.

**MATERIALS AND METHODS**

**Samples and experimental design.**

A total of 145 serum and conjunctival swab samples collected from cats with or without clinical signs or clinical history compatible with feline chlamydiosis, vaccinated or unvaccinated against the disease, were assayed. Samples were obtained from catteries, veterinary clinical
clinics and public shelters in five cities (Jaboticabal, São José do Rio Preto, Sertãozinho, Ribeirão Preto and Descalvado) in the northeast of the state of São Paulo, Brazil. All procedures were performed according to the Ethical Principles in Animal Research adopted by the Brazilian College of Animal Experimentation and to the 2000 Report of the AVMA Panel on Euthanasia (AVMA 2001).

Animals were bled from the jugular vein using disposable syringes. Serum was stored at -20°C until analysis. Conjunctival swabs were obtained by rolling a dry sterile cotton-tipped swab firmly over the conjunctival sacs of both eyes. Swabs were placed in a tube containing 1mL of ethanol (100%), and tubes were frozen at -20°C until testing.

Experimental groups were divided according to clinical history: G1 - unvaccinated cats without clinical signs/history compatible with feline chlamydiosis (n = 51); G2 – unvaccinated cats with clinical signs/history compatible with feline chlamydiosis (n = 45); G3 - vaccinated cats without clinical signs/history compatible with feline chlamydiosis (n = 28), G4 –vaccinated cats with clinical signs/history compatible with feline chlamydiosis (n = 21).

From the 145 cats analyzed, 49 (33.79%) had been vaccinated against *C. felis* (Fel-O-Vax LVK-IV®, FortDodge, EUA) and 96 (66.21%) had not been vaccinated. At the time of sample collection, 45.52% (66/145) of the sampled cats showed clinical signs and/or history compatible with feline chlamydiosis.

**Serum assay.**

Sera were assayed using Indirect Immunofluorescence Assay (IFA) and Complement Fixation Test (CFT). IFA titers for antibodies to *C. felis* were determined using a commercial Kit [IFA(Bion® Chlamydia-G Antibody Test System, Bion Enterprises, USA)] with goat-anti-cat IgG conjugated with fluorescein isothiocyanate (FITC - Sigma, USA). Briefly, sera were initially screened at 1:16 dilutions in phosphate buffered saline (pH 7.2), and samples were considered negative in the absence of fluorescent staining at the starting dilution. Positive samples at 1:16 were diluted from 1:16 until 1:1024, and endpoint dilution was determined by faint but definite/inclusion fluorescence.

Complement Fixation Test (CFT) was performed according to the method described by BIER et al. (1968), modified for microplates (Raso et al. 2006). Briefly, 25 µL of test sera and 25 µL of optimal amount of chlamydial antigen, followed by 50 µL of complement (50 per cent hemolytic end point-two of complement) were added to each well and incubated overnight at 4°C. Sensitized sheep-red blood cells were added to each well in 25 µL volumes and plates were
then incubated for 30 min at 37°C. Controls for serum, antigen, complement and hemolytic systems were included in every run. Finally, plates were centrifuged at 800 x g for 5 min and the degree of lysis was visually assessed. Samples showing more than 50% lysis at serum dilutions 1:16 or higher, in the presence of 2 units of complement, were considered positive (titer ≥ 16).

**Chlamydial DNA detection.**

**Genomic DNA extraction.**

Swab samples were vortexed for 2 min and then centrifuged at 20,000 x g for 30 min at 4ºC. The pellet was resuspended into 40 µL of buffer (0.1 M NaCl, 10mM TRIS, 1 mM EDTA; 5% Triton x100) and 9 U proteinase K (Invitrogen, USA), incubated at 56ºC for 90 min, and then centrifuged at 2,000 x g for 2 min. DNA extraction was performed in the supernatant using GFX Genomic Blood DNA Purification Kit (GE Healthcare Life Sciences, UK), according to manufacturer’s instructions.

**PCR for Chlamydophila spp.**

PCR for detection of *Chlamydophila* spp was performed with primers designed by Buxton et al. (1996) that correspond to the conserved regions of chlamydial major outer membrane protein gene (MOMP). Primer sequences were synthesized as follows: oligo 420 (5’-CAGGATATCTTGCTGCTTTAA-3’) and oligo 422 (5’-GCAAGGATCGCAAGGATC-3’), which amplify a 260-base pair (bp) DNA fragment. Reaction was performed in a 25 µL amplification mix composed of 10 mM Tris-HCl (pH 8.8) (Buffer 10X Biotools, Spain); 0.2mM of dNTPs (Biotools, Spain), 0.2 mM of each primer (Invitrogen, EUA); 1.25 U of Taq DNA polimerase (Biotools, Spain), and 5 µL of the extracted DNA. PCR cycling conditions were 10 min at 94ºC; 34 cycles at 94ºC for 1 min, 54ºC for 1 min and 72ºC for 1 min; and a final extension at 72ºC for 4 min. Both positive (*C. felis* vaccine - Fel-O-Vax LVK-IV®, FortDodge, EUA) and negative (autoclaved ultrapure water) controls were included in each PCR reaction. A 10 µL aliquot of each reaction was submitted to 1.5 % agarose gel electrophoresis. Samples were considered positive when a clearly visible band of the expected size was apparent in an ultraviolet transilluminator, after staining with ethidium bromide. Identification of the MOMP amplicons was confirmed by DNA sequencing of the control sample. A fragment of purified amplified DNA was automatically sequenced for confirmation (ABI Prism 310 Genetic Analyser – Applied Biosystem®/ Life Technologies Corporation, Carlsbad, California 92008, USA) and used for subsequent phylogenetic analysis. Consensus sequences were obtained by analyzing the sense and antisense sequences using the CAP3 software (http://mobyle.pasteur.fr/cgi-
Comparisons with GenBank sequences were carried out using the basic local alignment search tool (BLAST). Nucleotide sequences of positive control (Accession number HQ335353) showed 96% of similarity with *Chlamyphila felis* MOMP gene.

Univariate analysis was carried out using MiniTab® Statistical Software v. 14, 2003. Data entered into a computer database was analyzed by chi-square test. Fisher’s exact test was used if the expected value in a cell of the contingency table was lower than five. A statistically significant association was considered when $P$ value of less than, or equal to, 0.05. Kappa’s statistics was used to quantify the actual degree of agreement between IFA and CFT (Landis & Koch 1977).

**RESULTS**

Table 1 shows the results of Complement fixation tests for antibody titers (%) against *Chlamyphila spp* carried out in parallel with Immunofluorescence (IFA) in a total of 145 serum samples from domestic cats with clinical signs or clinical history compatible with feline chlamydiosis, vaccinated or unvaccinated against the disease. From the 145 serum samples, 105 (72.41%) were reactive in IFA, and 10 (6.9%) were reactive in CFT. Antibodies against *Chlamyphila spp* were detected by IFA in 50.98% (26/51) G1 samples, in 66.66% (30/45) G2 samples, and in all animals of G3 (n=28) and G4 (n=21), with titers ranging from 1:16 to 1:1024.

CFT-reactive samples were detected in only 10 of 145 samples examined (6.9%), with titers ranging from 1:16 to 1:512. No complement-fixation antibody was detected in cat sera from G1 and G2, but these antibodies were detected in G3 (20.69%) and G4 (19.05%). Thus, all positive serum samples came from vaccinated cats, and 4 of 10 of these samples (40%) came from cats with clinical signs and/or history compatible with feline chlamydiosis.

Antibody titers obtained in the two different serological methods (IFA and CFT) did not present a positive correlation, demonstrating that CFT is not a sensitive method to search for anti-*Chlamyphila spp* antibodies in domestic cats (kappa=0.05, sensitivity 9.5%, specificity 100%, positive predictive value 100% and negative predictive value 29.6%).

From the 145 conjunctival swab samples, 9 (6.21%) were positive for *Chlamyphila spp* in PCR, as shown in table 1. One from 9 samples of G1 (1.96%), 5 of G2 (11.11%), 2 of G3 (7.14%) and 1 of G4 (4.76%) were positive for *Chlamyphila spp*. DNA (Table 1). From these nine positive samples, three came from cats without clinical signs/history of infection, three came from vaccinated cats, and five (55.5%) were positive in IFA.
Thus, this study gives a clear indication that there is no correlation between results of IFA and CFT. Only ten cats reactive in CFT were reactive in IFA. Among PCR-positive animals, four were negative in IFA and none was positive in CFT.

**Table 1.** Occurrence (%) of *Chlamydophila felis* in cats from Northeast of São Paulo State, Brazil, by indirect immunofluorescence assay (IFA), complement fixation test (CFT) and polymerase chain reaction (PCR).

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<th>IFA +</th>
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<td>105/145 (72.41%)</td>
<td>10/145 (6.9%)</td>
<td>9/145 (6.21%)</td>
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1*G1 n= 51/145 (35.17%)  50.98% (26/51)a  0% (0/51)a  1.96% (1/51)a  
2*G2 n=45/145 (31.03%)  66.66% (30/45)ac  0% (0/45)a  11.11% (5/45)a  
3*G3 n=28/145 (19.31%)  100% (28/28)b  20.69% (6/28)b  7.14% (2/28)a  
4*G4 n=21/145 (14.48%)  100% (21/21)bc  19.05% (4/21)bc  4.76% (1/21)a  
P-Value  0.0000001  0.000125  1

Letters different in the same column indicate statistically significant difference.

1*G1 = *Group* unvaccinated cats without clinical signs/historic compatible with chlamydiosis
2*G2 = *Group* unvaccinated cats with clinical signs/historic compatible with chlamydiosis
3*G3 = *Group* vaccinated cats without clinical signs/historic compatible with chlamydiosis
4*G4 = *Group* vaccinated cats with clinical signs/historic compatible with chlamydiosis

**DISCUSSION**

*Chlamydophila spp* is widely spread in the studied population of vaccinated and unvaccinated animals, as indicated by IFA results (72.41%). Using a similar approach, Wills et al. (1988) found 62% (140/227) of IFA-reactive cats. Although high antibody titers indicate previous exposure, they do not necessarily mean active infection (McDonald et al. 1998). However, IFA results should be carefully interpreted because of cross-reactivity with other bacteria.

It was observed that prevalence was higher in vaccinated than in unvaccinated animals, suggesting efficient seroconversion in animals of group G3 and G4. These data corroborate findings of another study that has observed that 100% vaccinated cats were IFA-positive (Wills et al. 1987). Unvaccinated cats without clinical signs (G1) showed positive results in IFA in 50.98% (26/51) of the cases, similar to the results by Gethings et al. (1987), who found 45% of positive
results in a population of cats with similar clinical history. However, in England and Sweden, only 9.1% and 11% of the animals, respectively, were IFA-positive (Gunn-Moore et al. 1995, Holst et al. 2006). Among unvaccinated cats with clinical signs (G2), 68.89% (31/45) were IFA-positive, as also reported by Wills et al. (1988), who found 60% (69/116) of IFA-positive animals. In unvaccinated cats, paired serology in IFA can confirm diagnosis of *C. felis* infection. Recent studies using ELISA with recombinant antigens show that this technique may distinguish between vaccinated and infected cats (Ohya et al. 2008).

In the cat population studied, 6.9% of the animals showed complement-fixation antibodies. These results are compatible with those found in Japan and Switzerland, where 2.1 and 5% cats were positive to *C. felis*, respectively (Fukushi et al., 1985, Lazarowicz et al. 1982). All cats reactive in CFT were vaccinated, and 40% had clinical history of feline chlamydiosis. Statistical analysis showed that vaccinated cats presented higher prevalence of complement-fixation antibodies, suggesting that the presence of such antibodies may be credited to active immunization of cats.

Complement fixation test has been used in the past to determine the prevalence of *C. felis* in cat populations (Studdert et al. 1981, Fukushi et al. 1985). However, many studies have been unable to detect complement-fixation antibodies in cats experimentally infected with *C. felis* (Cello 1971, Shewen et al. 1980), suggesting that complement fixation is an unreliable serological test for antibodies against this agent in cats.

In relation to the comparison of the two serological techniques, our data corroborate previous reports (Wills et al. 1988, Griffiths et al. 1996, Markey et al. 1993) that compared IFA with CFT, showing that CFT is specific (100%), but it is not a sensitive (9.5%) method to search for anti-*Chlamydia spp* antibodies in domestic cats, since cats showing IFA titers greater than 1024 may not have detectable complement-fixation antibodies to *Chlamydia*. Predictive values suggest that IFA can be used in routine testing and research procedures.

The great disadvantage of serological methods is that results only indicate previous exposure to the agent, not necessarily the presence of active infection (Wills et al 1988). In fact, detection of active infection is important to prevent the spread of the disease to other cats and to monitor the effectiveness of a treatment (McDonald et al. 1998). The most effective test to determine the status of infected animals is PCR.

From the 145 conjunctival swab samples, *Chlamydiaceae* DNA was detected in 9 (6.21%) animals. From the positive samples, three were from animals without clinical signs and/or history
of feline chlamydiosis. Direct diagnosis of *C. felis* in cats has been reported even in the absence of clinical signs of disease (Gruffydd-Jones et al. 1995, McDonald et al. 1998), and may occur due to subclinical infections, as already noticed by McDonald et al. (1998). In Korea, a total of 78 cats without ocular and upper respiratory tract disease were examined and all specimens were negative for *C. felis* in PCR (Kang & Park 2008). In the present study, from nine positive samples, three were from vaccinated cats, contradicting previous reports (Rampazzo et al. 2003) that showed that none of the vaccinated cats were PCR-positive for this microorganism. Thus, the objective of active immunization against *C. felis* is to reduce the severity of clinical signs of the disease (Wasmoen et al. 1992), but not to prevent shedding of the organism (Wills et al. 1987).

Among the nine PCR-positive animals, antibodies were detected by IFA in five of them (55.5%). The presence of PCR-positive - but serology-negative - animals may be explained by primary immune response to *Chlamydophila* infections. About two weeks after the first clinical signs, there is an increase in IgA and IgM antibodies, which reach their peaks in about five weeks, and decline at about 10 weeks. At the moment of maximum IgA and IgM activity, IgG production begins and reaches its peak around 12 weeks after the first symptoms. This latter immunoglobulin can be detected for years (Matter et al. 2006). In our study, IgG was the class of antibodies analyzed; as a consequence, if the animals studied were in the initial phase of the disease, antibodies against the agent would not have been detected.

**CONCLUSIONS**

Results indicate that exposure to *C. felis* is frequent in the cat populations studied, once *Chlamydophila spp* was present in conjunctival swabs collected from cats with and without clinical signs of chlamydiosis. In unvaccinated cats, paired serology can confirm the diagnosis of *C. felis* infection, and IFA may be an efficient technique to detect anti-*Chlamydophila spp* antibodies because of its simple protocol, quick implementation and good diagnostic sensitivity and specificity. This study also showed that vaccination induces production of IgG antibodies specific to *C. felis* but does not prevent infection by the etiological agent, once vaccinated animals (G3 and G4) were positive for *Chlamydophila spp*. DNA.
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