Isolation and Identification of Mycoplasma Species in Dogs

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***Abstract*—** *Mycoplasmas can be associated with several canine health issues, mainly when dogs do not respond to antimicrobial treatment usually aimed at bacterial infections. Different mycoplasma species can be found in both healthy and sick animals; however, the following subjects have yet to be fully understood: The role played by mycoplasmas in canine habitats and the various diseases caused by them. The aim of the present study is to assess the presence of mycoplasma in dog samples at NUDMIC/UFF, RJ, Brazil, over a timeframe of ten years. Out of all assessed dogs, 9.67% (15/155) had respiratory symptoms, whereas the rest of them were asymptomatic. Moreover, 29.96% of the cultured samples (77/257) were positive for mycoplasmas. Typical colonies of said samples were divided into 42.86% (33/77) of oropharynx samples, 51.95% (40/77) of urogenital samples and 5.19% (4/77) of samples from other sources. Species Mycoplasma canis, Mycoplasma edwardii and Mycoplasma cynos were identified by PCR and/or immunoperoxidase. The most common species was M. canis. M. cynos was found in a dog with signs of respiratory disease. Despite the recent improvement in early identification and the biomolecular knowledge surrounding canine mycoplasma, the etiopathogenesis of canine mycoplasmosis remains uncertain..*

Keywords*— *Diseases, dogs, isolation, Mycoplasma, PCR.

I. INTRODUCTION

It has been more than 80 years since Shoetensack first reported mycoplasma species in dogs. The initial studies were very slow and fruitless due to the challenge of growing mycoplasmas in samples contaminated by other bacteria. In addition, there were few techniques to isolate the mycoplasma mixtures available (Rosendal 1979). It is worth remembering that Watson and Crick (1953) published their studies on DNA structure in 1953, and that Mullis et al. (1986) only developed the Polymerase Chain Reaction (PCR) technique in the 1980s. Nevertheless, mycoplasmas in dogs were little studied until the 2000s, when several reports started to arise more often in many countries. However, many mycoplasma, acholeplasma and ureaplasma species had already been described, namely: *Mycoplasma cynos, M. canis, M. edwardii, M. bovigenitalium, M. gateae, M. spumans, M. feliminutum, Acholeplasma laidlawii* and *Ureaplasma* sp. The first three species were the most commonly reported in dogs. A new species, *Mycoplasma mucosicanis* SP. Nov., was isolated from both the mucosa and the urogenital tract of asymptomatic dogs (Spergser et al. 2011).

M. Canis and *M. edwardii* may often appear alone or combined with other mycoplasma species in the upper respiratory tract. However, these species do not seem to be associated with respiratory disease as a primary pathogen *(*Chalker et al. 2004; Johnson et al. 2013). On the other hand, *M. cynos* affects the lower respiratory tract, causing pneumonia alone or often combined with Canine Infectious Respiratory Disease Complex (CIRD) (Chalker et al. 2004; Johnson et al. 2013). The most common symptoms of this disease are dry cough, anorexia and apathy. Lesions caused by *M. cynos* similar to those caused by *M. pulmonis* infection and found in laboratory rodents (Barreto et al. 2003; Souza et al. 2016) — are pathognomonic for mycoplasma pneumonia (Hong and Kim 2012).

M. canis is the species most associated with infertility, mucopurulent discharges and cystitis in the urogenital system (L'Abee-Lund et al. 2003; Ulgen et al. 2006). However, other species, such as *M. edwardii* and *M. spumans*, may also be isolated in healthy animals (Maksimović et al. 2018).

Although the respiratory and urogenital tracts are affected by most isolated species in dogs, *M. edwardii* was isolated from a 12-year-old female dog that has presented acute polyarthritis followed by septicemia (Stenske et al. 2005). Moreover, a picture of purulent meningoencephalitis indicated a brain tissue condition in a six-week-old female dog (Ilha et al. 2010). *Mycoplasma* spp. was isolated from the ear canal of healthy dogs with external otitis for the first time in Paraíba State, Brazil (Santos et al. 2016).

Mycoplasma canis and *Mycoplasma spumans* were isolated in a cat (Walker et al. 1995) after a dog bite; likewise, *M. canis* was isolated in a 62-year-old woman (Klein, Klotz, and Eigenbrod 2018). These findings suggest the existence of a new mode of mycoplasma transmission.

Isolation had originally prevailed as the diagnostic method, however, PCR has been the method of choice for detecting and typifying mycoplasma in dogs since 2000 (Chalker 2005; Janowski et al. 2008; L'Abee-Lund et al. 2003). This method has contributed to raise the number of reports. The difficulty in obtaining specific antisera for mycoplasma in dogs is an obstacle to serological typing (Chalker 2005). Alternatively, methods such as immunoperoxidase and immunofluorescence are extremely useful in typing mycoplasma isolates, mainly the canine ones (Nascimento et al. 2010).

Despite the onset of enzymatic and biomolecular techniques, isolation remains the gold standard for the diagnosis of animal mycoplasmosis such as the canine one. Therefore, isolation is essential for the validation of PCR methods (Chalker 2005) and to enable the application of immunoenzymatic techniques such as immunofluorescence and immunoperoxidase (Santos et al. 2010; Zeugswetter et al. 2007).

Laboratory challenges in the diagnosis of canine mycoplasmosis lead to underreported cases and to poorly informed veterinarians and breeders, who often use antibiotics that do not work for mycoplasma (Chalker 2005). This scenario results from factors inherent to microorganisms and hosts (Berčič et al. 2012; Maksimović et al. 2018; Mannering et al. 2009) involved in sample preparation and cultivation methods (Chalker 2005; L'Abee-Lund et al. 2003).

In Brazil, Oliveira, Costa, and Silva (1998) assessed the vaginal microbiota diversity of healthy female dogs, but did not isolate mycoplasma. However, Costa et al. (2004) and Nascimento et al. (2010) isolated *Mycoplasma* spp. from the respiratory and urogenital tracts of asymptomatic dogs. These mycoplasma species were then identified as *M. canis* and *M. edwardii* through Indirect Immunoperoxidase, thus confirming the presence of mixed infections in dogs (Nascimento et al. 2010).

Due to the difficulty of diagnosis and of contributing to the knowledge regarding the occurrence of canine mycoplasma. The aim of the present study was to assess the presence of *Mycoplasma* spp. in dogs with and without mycoplasmosis over a timeframe of ten years.

II. MATERIALS AND METHODS

2.1 Study design

Samples were collected from dogs (n=155) in academic or private veterinary clinics in Niterói and Rio de Janeiro Cities. Some of the samples were collected from pet dogs belonging to veterinary students, under their request. The samples were processed at the Diagnostic Mycoplasma Laboratory (NUDMIC) of UFF Veterinary School.

Samples were subjected to cultivation and the isolates were identified by indirect immunoperoxidase. These samples were concurrently assessed by generic PCR, if positive; they were subjected to specific PCR identification.

2.2 Sample collection

Main samples were collected from the oropharynx and urogenital tracts. However, ear and conjunctival swab samples were eventually collected. All the samples were placed in sterile tubes filled with appropriate transport medium (modified Frey's agar and glycerol – 1:1).

2.3 Cultivation and isolation

Aliquots of 0.1 ml were removed from the transport medium. Then, the aliquots were inoculated into 0.9 ml of modified Frey's broth enriched with 50% horse serum and 50% swine serum, and into 0.1 ml of modified Frey's agar plate at 37 ºC under microaerophilic conditions, usually used in the NUDMIC. The cultures were followed up for 21 days by optical screening in a Novex RZ series stereomicroscope at 6.5-45x magnification range.

2.4 Immunoperoxidase

The indirect immunoperoxidase was performed according to Imada, Uchida, and Hashimoto (1987). The antisera were *M.canis, M. edwardii, M. gateae. M. molare* and *M. arginini*, in the 1/20 dilution. *Mycoplasma agalactiae* was used as negative control at the same dilution; as conjugated to goat anti-rabbit IgG conjugated with peroxidase at 1/80 dilution. The wash buffer was made up of TBBS, horse serum and Tween 20, and the developing solution was made with cold methanol, 4-chloro-1-naphtol, TBS and 30% hydrogen peroxide. Plates were screened for stained colonies in stereomicroscope.

2.5 PCR

DNA was extracted from the samples and/or isolates based on the phenol-chloroform method for the PCR assay according Sambrook (2001). A set of generic primers (GPO3 5'GGG AGC AAA CAG GAT TAG ATA CCC T 3' and MGSO 5'TGC ACC ATC TGT CAC TCT GTT AAC CTC 3") was used to amplify a 270-bp fragment of *Mycoplasma* spp (Van Kuppeveld et al. 1994). Subsequently, 5 µL of target DNA was used for a final 100-µL reaction mixture of 1x PCR buffer, 2 mM MgCl2, 0.5 mM dNTP mix, 2 µL (100 pmol) of both primers and 1.5U of Taq DNA polymerase.

Species-specific PCR was performed as described by Chalker et al. (2004): Clinical specimens were isolated and identified in the reference strains of *M. canis*, *M. edwardii*, *M. cynos*, *M. molare*, *M. gateae* and *M. felis*. Then, primers and processes were described (Table 1). PCR reactions (50 µl) included the following reagents: 5.0 µl of 10x magnesium-free buffer (0.1 M Tris-HCl, 0.5 M KCl, pH 8.3), 1.5 mM MgCl2, 0.5 mL (0.5 units) of Taq DNA polymerase, 0.2 mM of PCR nucleotide mix, 0.025 mg of forward primer (Myc1; 59-CACCGCCCGTCACACCA-39), 0.025 mg of reverse primer for each mycoplasma (Table 1); and 1 µg of DNA from either the sample, the positive control or the negative control (1 µl of water).

TABLE 1
PCR PRIMERS, CYCLES AND AMPLICON SIZE IN DOG SAMPLES

Species	Primer sequence	Cycle conditions (x30)	Product size (bp)
Forward primer Myc1	5 ’CACCGCCCGTCACACCA3’	according to reverse primer	according to reverse primer
M. canis	5’CTGTCGGGGTTATCTCGAC3’	95 °C 1min, 55 °C 30s, 72 °C 1min	247
M. cynos	5’GATACATAAACACAACATTATAATATTG3’	95 °C 45s, 55 °C 30s, 72 °C 20s	227
M. edwardii	5’CTGTCGGGTTATCATGCGAC3’	95 °C 45s, 55 °C 30s, 72 °C 20s	250
M. molare	5’AGCCTATTGTTTTTGATTTG3’	95 °C 1min, 55 °C 30s, 72 °C 1min	397
M. gateae	5’GTTGTATGACCTATTGTTGTC3’	95 °C 1min, 55 °C 30s, 72 °C 1min	312
M. felis	5’GGACTATTATCAAAAGCACATAAC3’	95°C 45 s, 51°C 30 s, 72°C 20 s	238

2.6 Data analysis

Data were compiled in Microsoft Excel® spreadsheets and were calculated the frequences of micoplasma canine. Pearson's chi-square test or Fisher's exact test was used, with a 95% confidence interval to compare the association between the techniques for diagnostic used and collection site. The analyzes were performed using the BioEstat® 5.0 software. (Ayres et al., 2007)

III. RESULTS

3.1 Sample collection

The assessment included 257 samples of 155 dogs from Niterói and Rio de Janeiro Cities. The samples included 55 males, 87 females and 13 dogs of non-specified sex. There was significant difference between the number of males and females ($p<0.05$). Ages ranged from 5 to 180 months (47 months, on average). The two main collection sites were the oropharynx and the urogenital tracts: 41.63% (107/257) and 40.07% (103/257), respectively. Samples from other body parts, such as the ear and the conjunctiva, totaled 18.30% (47/257).

3.2 Cultivation, isolation, immunoperoxidase and PCR

Among the cultures, 29.96% (77/257) were positive for mycoplasma species (Table 2), out of which, 42.86% (33/77) of typical isolated colonies were found in the oropharynx; 51.95% (40/77), in the urogenital tract; and 5.19% (4/77), in other sites (Table 3). These positive samples fermented glucose and were categorized as Mollicutes based on the Dienes staining method. Finally, 31.13% (80/257) of the samples subjected to PCR were positive for *Mycoplasma* spp (Table 2).

TABLE 2
MYCOPLASMA-POSITIVE DOG SAMPLES BASED ON THE DIAGNOSTIC METHOD

Results/Technique	Culture	PCR
Positive	77 (29.96%)[a]	80 (31.13%)[a]
Negative	180 (70.04%)	177 (68.88%)
Total	257 (100.00%)	257 (100.00%)

Same lowercase letters on the rows indicate no significant difference (p> 0.05).

TABLE 3
DOG SAMPLES SUBJECTED TO IMMUNOPEROXIDASE ASSAY ACCORDING TO COLLECTION SITE

Collection site	Oropharynx tract	Urogenital tract	Other sites	Total
Positive	16 (48.48%)[a]	19 (47.50%) [a]	2 (50.00%) [a]	37
Negative	17 (51.52%)	21 (52.50%)	2 (50.00%)	40
Total	33 (100.00%)	40 (100.00%)	4 (100.00%)	77

Same lowercase letters on the rows indicate no significant difference (p> 0.05).

There was no significant difference (p> 0.05) between the diagnostic methods of canine mycoplasma. There was no significant difference (p> 0.05) among the isolates of oropharynx and urogenital tract samples and of other sites.

The specific PCR technique was used to identify 23.75% (19/80) of *M. canis* and 21.25% (17/80) of *M. edwardii*. There was a *M. cynos* sample among *M. spp*. The indirect immunoperoxidase technique was used to identify the isolates, out of which 67.57% (25/37) were *M. canis* and 43.24% (16/37) were *M. edwardii*. There were 4 positive samples for both species (table 4). There was no significant difference between *M. canis* and *M. edwardii* in PCR in comparison to the significant difference between *Mycoplasma.spp* and other species (p <0.05).

TABLE 4
DETECTION OF MYCOPLASMA SPECIES THROUGH PCR AND IMMUNOPEROXIDASE

Technique	PCR			Immunoperoxidase**	
Results	***M canis***	***M edwardii***	***M spp****	***M canis***	***M edwardii***
Positive	19 (23.75%)[A]	17 (21.25%)[A]	44 (55.00%)[B]	25 (67.57%)[a]	16 (43.24%)[b]
Negative	61 (76.25%)	63 (78.25%)	36 (45.00%)	12 (32.44%)	21 (56.76%)
otal	80 (100.00%)	80 (100.00%)	80 (100.00%)	37 (100.00%)	37 (100.00%)

Same lowercase and capital letters on the rows indicate no significant difference (p> 0.05).
**** One of the samples was typified as M. cynos.***
*****The total of 4 samples were positive for both species.***

Results in Table 4 suggest that both methods are efficient to typify mycoplasma species in dog samples, although the indirect immunoperoxidase technique showed better results than those obtained through the PCR technique.

IV. DISCUSSION

The present study investigated the presence of Mycoplasma spp in dogs for over a timeframe of ten years and identified three canine Mycoplasma species. *M. canis* and *M. edwardii* were the most prevalent species, whereas *M. cynos* was found in only one animal among the 15 dogs that have presented respiratory symptoms.

Mycoplasma canis and *M. edwardii* were found in respiratory and urogenital tract samples from male and female dogs by PCR and immunoperoxidase. These findings support the evidence that these species inhabit both the upper respiratory (Chalker 2005) and the urogenital tracts (Maksimović et al. 2018).

Findings regarding *M. cynos* were similar to those described by Hong and Kim (2012) and Canonne et al. (2018). Hong and Kim (2012) identified *M. cynos* by PCR in lung tissue samples from 5.0% (1/20) of Beagle dogs with respiratory disease. Canonne et al. (2018) studied dogs diagnosed with eosinophilic bronchopneumopathy and chronic bronchitis, as well as healthy dogs, by using the qPCR technique. They detected *M. cynos* in 6.67% (4/60) of sick dogs and in 3.33% (2/60) of healthy dogs.

The detection of *M. cynos* in only one case of respiratory disease suggests that the likelihood of *M. cynos* infection is low and facilitated by the following complications: Serious respiratory diseases such as CIRD, and association with other microorganisms (Chalker et al. 2004; Mannering et al. 2009); moreover, *M. cynos* is more easily detected through PCR (Mitchell et al. 2017). Such species has been defined as an emerging pathogen (Priestnall et al. 2014) associated with other CIRD microorganisms; nevertheless, its ability to cause disease on its own has not yet been proven, since it can be detected both in asymptomatic dogs (Lavan and Knesl 2015) and in dogs vaccinated against CIRD (Mitchell et al. 2017).

The urogenital tract is the common isolation site of several mycoplasma species. However, the association of the urogenital and respiratory tracts to mycoplasmosis remains uncertain due to the incidence of isolation in both healthy animals and in animals with urogenital disorders (L'Abee-Lund et al. 2003; Maksimović et al. 2018; Ulgen et al. 2006).

L'Abee-Lund et al. (2003) isolated *M. canis* in dogs with clinical signs of urogenital disease and suggested that this microorganism could be the causative agent of it. However, the present study showed that *M. canis* was more common in healthy dogs than in the sick ones, as reported by Janowski et al. (2008) and Maksimović et al. (2018). Janowski et al. (2008) suggested that *M. canis* was a part of the vaginal microbiota of healthy female dogs. Maksimović et al (2018) isolated *M. canis, M. spumans, M. edwardii* and *Mycoplasma* spp in vaginal samples from domestic and street female dogs who were either healthy, intact or subjected to total hysterectomy. The present study also evidenced *M. edwardii* and *Mycoplasma* spp in the urogenital tract of healthy animals, but *M. cynos* was not found in it, a fact to be unusual in this site; however, it has been reported in dogs with urogenital disease, along with *M. canis* and *M. spumans* (Jang et al. 1984).

The diagnostic results of the comparison between isolation and PCR have supported the findings by Costa et al. (2004), since there was no significant difference between the aforementioned techniques; thus, both of them can be recommended for diagnosis.

The detection of *M. canis* and *M. edwardii* alone and/or in mixed infections by indirect immunoperoxidase reaction in both the respiratory and urogenital tracts by the present study had already been previously reported (Nascimento et al. 2010). Based on this finding, these species can be part of the microbiota found in both the respiratory and urogenital tracts. Indirect immunoperoxidase can be used to identify mycoplasma species in dogs (Nascimento et al. 2010) and in other animals alike (Santos et al. 2010).

V. CONCLUSION

- Canine mycoplasmas have been found in sick animals, but they are more likely to be found in seemingly healthy animals.
- *M. canis* and *M. edwardii* were the most prevalent species in the assessed sites and through the diagnostic method.
- *M. cynos* was detected in a single animal who presented a respiratory disease.
- The highly consistent detection of mycoplasma species in dogs encourages instructive studies on their etiopathogenesis.

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