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Laboratory Diagnosis of *Mycoplasma* spp. from the Upper Respiratory Tract and Conjunctival Infections in Shelter Cats

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Abstract

Mycoplasma is a significant microorganism of shelter cats, which can cause respiratory infections and conjunctival inflammation, bringing about huge financial and health misfortunes to pet cats worldwide. This study was undertaken to investigate the prevalence and anatomical distribution of mycoplasmas in a population of shelter cats and establish the association of their presence with ocular or respiratory infections in shelter cats in Baghdad province. A total of 450 swabs were collected, including nasal, oropharyngeal, and conjunctival swabs 150 each from shelter cats of different ages, sex, and breed. The swabs were cultured in PPLO agar, and incubated at 37°C for 2 weeks, and the growing colonies with dissecting microscope. The colonies were also stained with Dienes stain. The prevalence of *Mycoplasma* in all examined cats was 40.7%, as determined by culture. The findings revealed that the prevalence of *Mycoplasma* in upper respiratory tract infections in female cats older than one year was between 46.9% and 48.7%. Conversely, the infections exhibited greater prevalence and a higher rate of isolation in males under one year of age 28.8-30.8%. The present investigation highlighted a significant prevalence of *Mycoplasma* in respiratory swabs obtained from Persian and Himalayan cats, but Scottish and British cats exhibited a comparatively lower rate of positive *Mycoplasmal* culture. To conclude, *Mycoplasmal* infections were more prevalent in upper respiratory diseases among shelter cats.

Keywords: Shelter cats, *Mycoplasma felis*, conjunctival swabs, nasal swabs.

Introduction

Mycoplasmas belong to the class *Mollicutes* and are small prokaryotic cells capable of self-replication. *Mycoplasmas* demand nutrient-rich media for their growth. While almost all of *Mycoplasmas* are capable of surviving in both aerobic and anaerobic conditions, some strains exhibit optimal growth in an environment containing 5-10% carbon dioxide [1, 2]. Several types of microorganisms are significant veterinary pathogens that colonize the mucous membranes of the respiratory and vaginal tracts, as well as red blood cells. This colonization leads to respiratory infections, mastitis, conjunctivitis, arthritis, and occasionally pregnancy loss [3, 4]. The cat is one of the two most prevalent and much favored domestic animals globally (the dog being the other) [5]. For almost 10,000 years, it has coexisted with humans in many roles, such as a hunting companion, guardian, subject of ridicule or admiration, and companion [6]. The incidence of *Mycoplasma* in cats has been reported in several studies [7-9]. Cats exhibited sensitivity to many *Mycoplasma* species, however not all Mycoplasma infections resulted in clinical illness [10, 11]. *Mycoplasmas* are commonly obtained from several animal species. *Mycoplasmas* can be found in the respiratory and ocular mucosa of domestic cats, although they may also colonize other areas on occasion *Mycoplasma* is thought to be a part of the normal bacteria found in the upper airways of cats. However, a recent study examining the

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microbiome of healthy cats revealed that the presence of *Mycoplasma* decreases as it moves from the nasal cavity to the lower airways. These findings suggest that *Mycoplasma* may colonize the lower respiratory tract as a result of infection in the upper airways [12, 13].

The persistence of conjunctivitis, which continues for months or even years, appears to be a significant concern in our veterinary clinics as well as in other nations. The involvement of *Mycoplasma felis* in feline eye diseases is currently uncertain and subject to debate. *Mycoplasma felis* can be cultured from cats that are clinically healthy, leading some researchers to classify them as a component of the normal microflora of the conjunctival sacs and not implicated in the development of conjunctivitis. Alternatively, *Mycoplasma felis* is characterized as a pathogenic species that has been found in cases of feline conjunctivitis [14, 15]. Thus far, there has been no confirmation of *Mycoplasma felis* in cats with ophthalmological issues in Iraq. Cats infected with *Mycoplasma felis* have been documented to experience keratoconjunctivitis and upper respiratory tract disorders. *Mycoplasma* is sometimes isolated from the respiratory tract in connection with clinical symptoms. Furthermore, *Mycoplasma* has been identified as a significant underlying factor in feline infectious respiratory disease complex (FIRDC). Recently, *Mycoplasma felis* and *Mycoplasma gateae*, along with other strains, have been isolated from cats with FIRDC [16]. The primary objective of the present investigation was to employ conventional cultural techniques to isolate and identify *Mycoplasma* from the upper respiratory tract and conjunctival infections in shelter cats, with the additional objective of determining its rate of isolation.

Material and Methods

Study design and animals

Samples

A total of 450 swabs were collected from the nasal (150), oropharyngeal (150), and conjunctival (150) areas of both diseased cats displaying respiratory infection symptoms (Fig.1), as well as healthy shelter cats of various breeds, ages, and genders. The samples were obtained from multiple veterinary clinics in Baghdad City between 01/01/2023 and 01/01/2024. The swabs promptly transferred to the laboratory of the *Mycoplasma* Unit, Department of Microbiology, College of Veterinary Medicine, University of Baghdad, in a sterile approach and placed in a PPLO broth medium.

PPLO broth (CM0403) medium preparation

PPLO broth base (CM0403) (3.7 gm) was suspended in 80 ml of distilled water, then boiled and autoclaved at 121ºC for 15min. The medium was cooled at 45ºC and added the *Mycoplasma* selective supplement – G (SR0059C) that contains horse serum 20 ml, yeast extract (25% w/v) 10 ml, thallous acetate 25 mg, penicillin 20.000 unit (which was prepared previously by adding 20 ml of sterile distilled water then mixed well) to the medium as mentioned in the instruction manual.

PPLO agar (CM0401) medium preparation

PPLO agar base (CM0401) (4.9 gm) was suspended in 80 ml of distilled water, boiled and autoclaved at 121ºC for 15 min. Then cooled at 45ºC and added the *Mycoplasma* selective supplement – G (SR0059C) that contain horse serum 20 ml, yeast extract (25% w/v) 10 ml, thallous acetate 25 mg, penicillin 20.000 unit (which was prepared previously by adding 20 ml of sterile distilled water then mixed well) to the medium as mentioned in instruction manual.

Culture

The swabs placed in a PPLO broth medium and quickly transported to the laboratory of the *Mycoplasma* unit. They were then cultured at a temperature of 37ºC with a 5% concentration of carbon dioxide for a period of 4-14 days inside a container with a lit candle. Upon finding signs of turbidity, a sample from each broth was cultured on PPLO agar medium and placed in a candle jar, where it was incubated at 37ºC with 5% CO_2 for a period of 7-14 days. The inspection of plates for the presence of *Mycoplasma* species colonies initially conducted on days 2, 4, 7, and thereafter on a weekly basis afterward, using a dissecting microscope. The bacterial culture with swabs yielded colonies on PPLO agar that exhibited a distinctive fried egg appearance. These colonies became visible after an incubation period of 4-7 days and had a diameter ranging from 110 to 200 µm. The morphological traits of the grown microorganisms led to a suspicion of Mycoplasma spp. [17].

Usage of (MYCOTRIM®GU), a modified dienes stain, for staining colonies

The stock solution is made by combining 2.5 gr. of Methylene blue, 1.25 gr. of Azure II, 10 gr. of Maltose, 0.25 gr. of Na2CO3, and a hundred ml of distilled water. The working solution is then prepared by diluting the stock solution with distilled water, using a ratio of three volumes of water to one volume of stock solution. To stain the colonies, immerse the agar dish surface containing the *Mycoplasma* colonies in a 1ml solution of Dienes stain. Rinse with distilled water, then treat with 1ml of 95% ethyl alcohol for 1 minute to remove excess stain. Rinse again with distilled water and observe the color of the *Mycoplasma* colonies [18].

Results

Morphology of colonies

The colonies exhibited a convex shape and appeared as small circular shapes with a central region surrounded by a white to colorless halo, resembling the appearance of a fried egg when examined under the microscope. Based on the visual characteristics, the colonies on the agar plate exhibited two distinct types of morphology. In the first type, the colonies were transparent, while in the second type, the colonies looked opaque color (Fig. 2).

Dienes stain

The modified Dienes stain was used to stain *Mycoplasma* colonies grown on PPLO agar. The staining revealed a light blue center surrounded by a dark blue halo, as seen in Fig. 3.

The percentage of *Mycoplasma* isolation from all swabs collected was 40.7%. The highest isolation rate was seen from nasal swabs at 45%, followed by oropharyngeal swabs at 35.3% and conjunctival swabs at 41.3% (Table 1). The findings of the study revealed that respiratory infections were more prevalent in shelter cats as opposed to conjunctival infections observed in all cats that were tested. Table 2 shows that females had a higher occurrence of upper respiratory and conjunctival infections, with percentages of 46.9% and 20.8% respectively, based on their sex. The study results demonstrated that older individuals were more prone to respiratory and conjunctival infections, with rates of 48.7% and 30.8% respectively (Table 3).

The prevalence of conjunctival infections was 43.3% in Persian cats and 58.3% in Himalayan cats, as shown in Table 4. The findings of the study revealed a significant prevalence of *Mycoplasma* in upper respiratory tract infections in cats over one-year-old, with a 100% isolation rate.

Conversely, the situation was different for conjunctival infections, where the prevalence varied according to age. However, depending on gender, the respiratory swabs taken from female cats with the condition were shown to be more suitable for isolating *Mycoplasma* compared to those taken from males. This is because all of the female swabs tested positive for *Mycoplasma* culture, but the male swabs had a greater rate of conjunctival infections and isolation. The study found that the Sphynx breed had a 100% isolation rate of *Mycoplasma* in respiratory swabs, while the Himalayan breed had a rate of 58.3%. In Scottish cats, 42% of conjunctival swabs tested positive for *Mycoplasma* culture, while in British cats, the rate was 25% (Table 4).

Discussion

Mycoplasma species are tiny bacteria characterized by the absence of a cell's peptidoglycan wall. These bacteria are part of the typical microbial flora found in the conjunctiva and upper airways (pharynx, larynx, oral cavity, nasal cavity) of cats. They are widely known to cause conjunctivitis and upper respiratory infections in cats [2, 12]. Nevertheless, the extent to which they are the main factor contributing to lower respiratory disease has been the subject of ongoing discussion for a number of years. No instances of *Mycoplasma spp.* have been found in the trachea, bronchi, or lungs of healthy cats [11]. Most cases of feline infection with *Mycoplasma spp*. in the lower airways were found to have a co-infection with other bacteria or an underlying illness that caused the aspiration of gastric material, impaired local defense systems, or systemic immunosuppression [1, 4, 7]. Therefore, the occurrence and rapid growth of Mycoplasma were seen as a subsequent occurrence.

Cats can be susceptible to infection by many *Mycoplasma species*, which can be detected in the respiratory tract of both healthy and sick cats. However, not all *Mycoplasma* infections result in clinical illness [19, 20]. The colonies in the study were indistinguishable from Mycoplasma when cultured on *Mycoplasma* agar. They exhibited the characteristic appearance of a fried egg, as described in scientific literature [21]. This confirms that in vitro culture is the established technique for isolating and identifying *Mycoplasma*. However, the slow growth rate of these bacteria makes this method time-consuming, even for specialized laboratories [12, 20].

The rate of *Mycoplasma* isolation from the total swabs in our investigation was 40.7%, which significantly differed from the findings reported previously that recorded a rate of 17% [22], and 21.96% [23]. The variations can be ascribed to the methodology of sample collection, the conditions employed for isolation, the case history, and the duration of sample collection [24]. Furthermore, there are other risk factors associated with cats, such as living in a household that has several cats and having a desire to train shelter cats [25]. The *Mycoplasma* isolation rate was highest in nasal swabs at 45%, followed by conjunctival swabs at 41.3% and oropharyngeal swabs at 35.3%. Previous studies by Sandmeyer *et al.* [26] and Brown *et al.* [27] reported different results, with the highest isolation rate observed in conjunctival swabs at 52.86%. They also found the lowest isolation rate in oropharyngeal swabs at 18.30%, which aligns with their findings. The significant rate of isolation from conjunctival swabs provides evidence that *Mycoplasma* naturally inhabits the upper respiratory tract as commensals. However, under stressful conditions, these *Mycoplasma* organisms can transform into pathogens, leading to the development of clinical respiratory cases [28, 29]. This is further supported by the low isolation rates observed from the same swab types in normal healthy cats [30]. The

study found that upper respiratory tract infections were more prevalent in young cats (less than one-year-old) at a rate of 30.8%. These infections were less common in cats older than one year, but more frequent in diseased females (46.9%) compared to diseased males (28.8%). The infections were mainly observed in Persian and Himalayan cats. However, the isolation rate of *Mycoplasma* was higher in swabs taken from older female cats. The findings were consistent with Hartmann *et al.* [31], who confirmed a significant association between *Mycoplasma* infection and young age (≤1 year). However, the results differed from Wong *et al.* [32], who reported a high rate of *Mycoplasma* isolation from upper respiratory tract infections in children around 1.5 years old. Wong et al. also found a higher rate of *Mycoplasma* isolation in females compared to males, and in Toy cats breed compared to other breeds. Generally, cats that are ≤1 year old, immunocompromised, or sensitive to other respiratory tract infections are more likely to experience upper respiratory tract infections. The inconsistencies may have arisen due to variations in diagnostic processes, sample populations, or inclusion criteria [33]. A 100% prevalence of *Mycoplasma* was observed in conjunctival infections in diseased Sphynx cats, whereas other studies reported a lower prevalence of *Mycoplasma* in such infections [34-36]. This difference may be attributed to the increased breeding of these species for various purposes in recent years.

Conclusions

The findings of our study revealed a significant prevalence of *Mycoplasma* in shelter cats suffering from upper respiratory tract and conjunctival infections, as determined through cultural analysis. Additionally, we observed a high incidence of *Mycoplasma* in Persian and Himalayan cats with upper respiratory tract infections, as well as in sphynx, Scottish, and British cats of various breeds with other types of infections.

Acknowledgment

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Conflicts of interest

The author confirms that there's no evidence of any conflict of interest in relation to the publishing of this article.

Ethical considerations

The commission of Scientific Morality awarded the endorsement certificate with the number P.G./ 2386, together with the moral approval, to conduct this systematic activity in the College of Veterinary Medicine, University of Baghdad.

Fig. 1. Conjunctival swab sampling from diseased sphynx cat

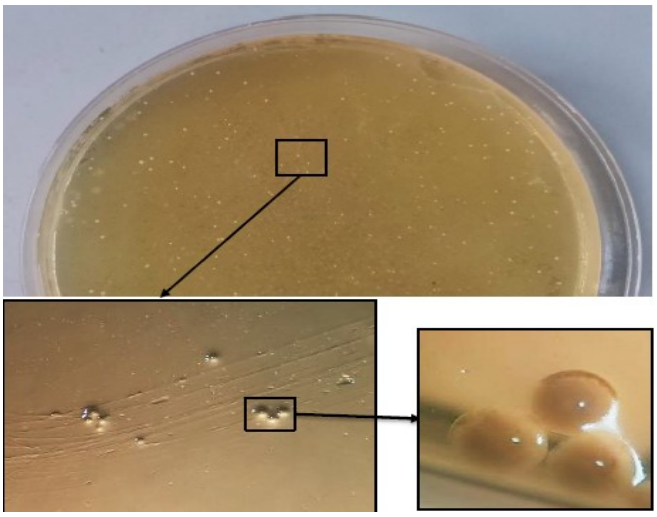

Fig. 2. Types of *Mycoplasma* colony morphology (fried egg) appearance on PPLO agar media

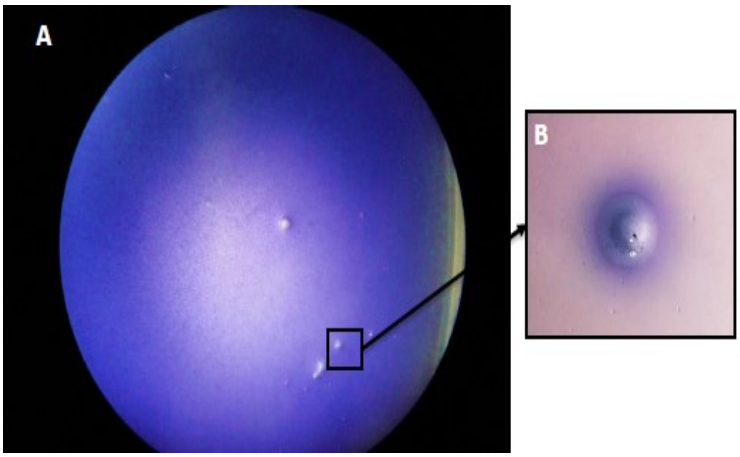

Fig. 3. *Mycoplasma* staining, staining petri dish (A) and staining colony with dienes stain (B)

TABLE 1. Respiratory and conjunctival *Mycoplasma* isolation Results from in-shelter cats

Type of sample	No. of samples	No. and % of positive samples	Total %
Nasal Swab	150	68(45.3%)	15.1%
Oropharyngeal Swab	150	53(35.3%)	11.7%
Conjunctival Swab	150	62(41.3%)	13.7%
Total Count	450	183(40.7%)	40.7%

TABLE 2. Prevalence of *Mycoplasma* infection based on sex in shelter cats

Type of sample (Sex)	No. of samples	No. and % of positive samples	Total % from 150 sample
Male	52	15(28.8%)	61(40.6%)
Female	98	46(46.9%)	

TABLE 3. Prevalence of *Mycoplasma* infection based on age in shelter cats

Type of sample (Age group)	No. of samples	No. and % of positive samples	Total % from 150 sample
≤ 12 Months	68	21(30.8%)	61(40.6%)
> 12 Months	82	40(48.7%)	

TABLE 4. Prevalence of isolation rate of *Mycoplasma* between different breeds of shelter cats

Type of Breed	No. of samples	No. and % of positive samples	Total %
Persian	30	13(43.3%)	8.66%
Himalayan	12	7(58.3%)	4.66%
Scottish	7	3(42.8%)	2%
British	8	2(25%)	1.3%
Sphynx	2	2(100%)	1.3%
Other types	91	34(37.36%)	22.66%
Total Count	150	61(40.6%)	40.6%

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التشخيص المختبري لأنواع المفطورات من اصابات التهاب الجهاز التنفسي العلوي والملتحمة في القطط المنزلية

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المفطورات هي كائنات دقيقة تتواجد بشكل طبيعي في الجهاز التنفسي للقطط ، والتي يمكن أن تسبب التهابات في الجهاز التنفسي والملتحمة، مما يؤدي الى حصول خسائر مالية وصحية كبيرة للقطط المنزلية في جميع أنحاء العالم. أجريت هذه الدراسة لمعرفة مدى انتشار المفطورات وتوزيعها التشريحي بين القطط المنزلية وإثبات ارتباط وجودها مع التهابات العين أو الجهاز التنفسي للقطط المصابة في محافظة بغداد. تم جمع 450 مسحة من مناطق مختلفة من جسم القطط المصابة، بما في ذلك الأنف والفم والبلعوم والملتحمة، 150 مسحة لكل منها على التوالي من القطط المصابة من مختلف الأعمار والاجناس والسلالات. تم زرع المسحات في الوسط الخاص بالمفطورات، وحضنت عند درجة حرارة 37 درجة مئوية لمدة أسبوعين، وتم الكشف عن المستعمرات النامية باستخدام المجهر التشريحي. وصبغت المستعمرات أيضًا بصبغة دينيس. بلغت نسبة انتشار المفطورات في جميع القطط المفحوصة 40.7%. كشفت النتائج أن نسبة انتشار المفطورات في التهابات الجهاز التنفسي العلوي لدى إناث القطط الأكبر من سنة تتراوح بين 46.9% إلى 48.7%. وعلى العكس من ذلك، أظهرت العدوى انتشارًا أكبر ومعدل عزل أعلى لدى الذكور الذين تقل أعمارهم عن سنة واحدة بنسبة 28.8-30.8%. سلط البحث الحالي الضوء على انتشار كبير للمفطورات في مسحات الجهاز التنفسي التي تم الحصول عليها من القطط الفارسية والهيمالايا، ولكن القطط الأسكتلندية والبريطانية أظهرت معدل أقل نسبيا من الاصابة بالمفطورات. في الختام، كانت العدوى بالمفطورات أكثر انتشارا في أمراض الجهاز التنفسي العلوي عن اصابات ملتحمة العين بين القطط المنزلية.

الكلمات المفتاحية: وسط المايكوبلازما، المفطورات، القطط الاليفة، التهاب الرئة، التهاب ملتحمة العين.