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Genetically diagnosis of mycoplasma isolated from respiratory and conjunctival infections in household dogs

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Abstract

Genetic depiction of *Mycoplasma* isolates was done by a PCR approach from the upper respiratory tract and conjunctival infections in domestic dogs in Mosul City. This was accomplished by amplifying the 16S rRNA gene, which is specific to the *Mycoplasma* genus. 100 domestic dogs of various ages, sexes, and breeds participated in the study from 1/2/2022 to 1/1/2023. There were 300 swabs total, including conjunctival, nasal, and oropharyngeal swabs. The swabs were subjected to mycoplasmal culturing in suitable conditions. The growing colonies were examined by light and a dissecting microscope. The DNA of growing colonies was extracted and amplified then migrated in agarose gel to observe the bands. The study's findings revealed the isolation of 58 *Mycoplasma* isolates, with an overall swab isolation rate of around 58% and a high rate of 68% from nasal swabs, then from oropharyngeal swabs and conjunctival swabs 58 and 45% sequentially. PCR technique showed that 34 isolates from the total of 58 isolates were assured for the 16S rRNA gene and yielded a band at 270bp, these findings are regarded as distinctive for the genus *Mycoplasma*. DNA Sequencing results of the 16S rRNA gene revealed that twenty-three 67.65% out of thirty-four *Mycoplasma* isolates were indicated as *Mycoplasma cynos* strain SM-MY-M23 which was dumped in the GenBank nucleotide sequence database under accession number OQ446513, so current results are considered as the first record of *Mycoplasma cynos* in dogs in Iraq.

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Introduction

Mycoplasmas are members of the class *Mollicutes* (soft skin) insinuating the absence of the robust bacterial cell wall and are the minutest and most modest self-replicating prokaryotes (1,2). they are fastidious and demand sterol, or urea for growing. They require enriched media for growth supplemented with serum and yeast extract which delivers amino acids with vitamins, furthermore, penicillin and thallium acetate are added to block the contaminant bacteria and fungi (3), Certain varieties of *Mycoplasma*, which are facultative anaerobes, thrive best in an environment with 5-10% CO_2 and forming characteristically umbonate microcolonies when illuminated obliquely and a (fried egg appearance) in transmitted light (4). Domestic dogs are one of the two (the second one is the cat) most prevalent and familiar domestic animals worldwide. It has cohabited with humans for more than thousands of years as a hunted animal, a source of protection, a favorite, and a buddy (5-8). Notwithstanding the fact that not all *Mycoplasma* infections result in clinical illness, dogs were susceptible to a variety of *Mycoplasma* species (9,10). It is thought that *Mycoplasma* is one of the microbiomes of the upper airways in dogs (11,12), yet, a recent analysis of the normobiota of apparently healthy dogs revealed that *Mycoplasma* regularly moved through the nasal passages to the lower airways. This may propose that Mycoplasma habitation of the upper airways led to the dominance of the lower respiratory tract (13-18). Furthermore, *Mycoplasma* is a significant player in complex respiratory disease in canine (19-24). Many mycoplasmal

species are significant veterinary pathogens that settle on the red blood cells or respiratory, and genital mucosal membranes and cause respiratory infections, mastitis, conjunctivitis, arthritis, and occasionally miscarriage (1,2,25).

The current work sought to identify and classify *Mycoplasma* isolated from respiratory infections and conjunctivitis in domestic dogs through molecular techniques with an observation of DNA sequencing.

Materials and methods

Ethical approval

The endorsement certificate with the number UM.VET.2021.071 was granted by the Commission of scientific morals, which also provided the moral consent to carry out this methodical activity in the College of Veterinary Medicine.

Samples and cultivation

The collected samples underwent standard methodological processing according to Hussein and Hamad (26). The growing colonies were microscopically examined with light and a dissecting microscope. The growing bacteria were examined for modified Diene's stain, and morphological and biochemical tests (25-29). Then the bacterial colonies underwent molecular processing.

DNA extraction

The suspected colonies were subcultured in *Mycoplasma* broth medium for 7 days at 37°C. The extraction was accomplished affording to the guidelines of the kit manufacturing company (Geneaid, Presto ™ Mini gDNA Bacteria Kit Quick Protocol, Korea). One ml of bacterial broth was transferred to an eppendrof tube, and centrifuged for 5 min. at 16000 rpm, afterward threw away the floating and added 200µl of GT buffer to centrifuged eppendrof tubes, vortex 2 min., added 20µl of proteinase K which dissolved previously by ddH2O, vortex 1-2min., incubated at 60℃ at 10 min., afterward added 200µl of GB buffer, again vortex 10 sec. and incubated at 70ºC for 10 min., then quickly combined by forcefully shaking the lysate sample after adding 200 µl of utter ethanol. Transferred the mixture to GD column which was spotted in 2ml gathering tube, centrifuged at 14-16000 rpm for 2min., next obsolete the 2ml gathering tube holding the floating material and substitute with a novel 2ml gathering tube, furthermore added 400µl of W1 buffer to GD column, centrifuged at 16000rpm for 30 sec. Neglected the floating and spot the GD column remade in the 2 ml gathering tube, the following adds 600µl of wash buffer (Ethanol mixed) to the GD column and centrifuged at 16000rpm for 30sec. Again, neglected the floating material tube and put the GD column back in the new 2ml gathering tube, next drying the sample by centrifugation again at 14-16000rpm for 3min. Finally inserted 100µl of preheated elution buffer was into the midst of the template column and waited for at 3min., after that centrifuged at 16000rpm for 1min. The *Mycoplasma* DNA was set up in floating which was set into a novel tube and kept in freezing unto applied as a DNA template (30).

Preparing the primer

The primer sequence for the amplification of the 16S rRNA gene for *Mycoplasma* (31) (Table 1), The Macrogen Company (South Korea) produced this primer. in a lyophilized form. As a stock solution, primer was disbanded in nuclease-free water to an eventual concentricity of 100 pmol/l. This primer's working solution was created by mixing 90µl of nuclease-free water with 10µl of primer stock solution, which was kept at -20 Celsius degrees in the freezer (32,33).

PCR detection of 16S rRNA gene of *Mycoplasma*

The reaction mixture for PCR was prepared in a total volume of 20µl for the 16S rRNA gene (33).

PCR amplification condition

The 16S rRNA gene amplification was done in a thermal cycler (An Analytik Jena/Biometra, Germany) and the using program included three stages, the first stage was the initial denaturation at 94ºC for 10 minutes, the second stage included 30 repeated cycles of three steps (Denaturation at 94ºC for 45 seconds, annealing at 55ºC for 45 seconds, and extension at 72ºC for 1 minute), while the last stage was the final extension at 72ºC for 5 minutes.

Table 1: Primer used in the existing study

Primer		Sequence (5′ 3′)	Size (bp)	Reference
16S rRNA gene	F	TGGGGAGCAAACAGGATTAGATACC	270	(31)
	R	TGCACCATCTGTCACTCTGTTAACCTC		

Agarose gel electrophoresis

The fineness of the extricated DNA was examined by employing electrophoresis in 1.5% agarose gel (Biobasic Candia Inc, Canada) mixed with 3 µl of Ethidium Bromide stain (Promega, USA) by adding 1µl of DNA loading dye (Promega, USA) to 5µl of extracted DNA. In order to segregate the amplified products, 1.5% agarose gel electrophoresis was also employed. Each PCR result was placed into the agarose gel's well at a volume of 5 µl. The electrophoresis was performed using a power supply MP

300V at 80 V for one hour, and an electrophoresis tank (BioRad, USA) holding 1X TBE buffer (Promega, USA). A 100 bp DNA ladder, 4 µl (Promega, USA) served as a common molecular weight indicator. Lastly, the band was saw under the UV transilluminator (An Analytik Jena/Biometra, Germany) (34,35), and the photo of the gel was captured using a digital camera.

Sequencing 16S rRNA gene

Sanger dideoxy sequencing was used to assess the sequencing data after PCR product purification and sequencing, and the BLAST algorithm at the NCBI server [available at]. The sequence data were deposited in GenBank.

Results

Bacterial isolation and identification

Results of the culture of *Mycoplasma* isolates showed that these isolates were pure and formed characteristically umbonate microcolonies when illuminated obliquely and had a fried egg appearance (Figure 1).

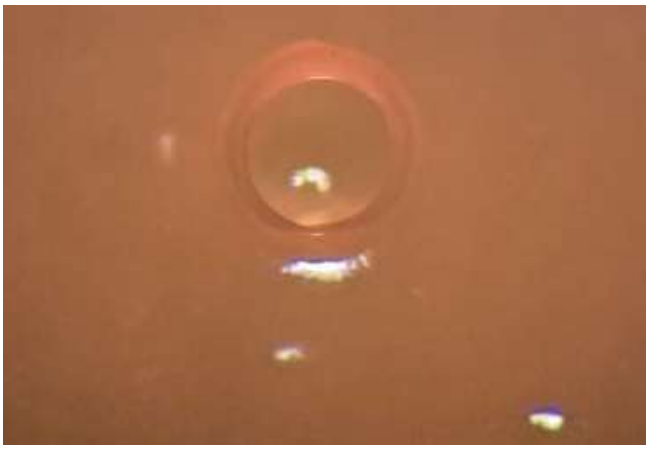

Figure 1: Fried egg appearance characteristics for *Mycoplasma* colony.

Isolation rates

The existing investigation found that 58% of samples were affirmative for *Mycoplasma* isolation. Oropharyngeal swabs and conjunctival swabs came in second and third, respectively, to nose swabs in terms of isolation 68, 58, and 45%.

DNA extraction

The confirmation of *Mycoplasma* isolates was done by detecting the 16S rRNA gene using PCR and agarose gel. The findings exhibited that 34 isolates (from a total of 58 isolates) had the Mycoplasmal 16S rRNA gene which has a 270bp product size, (Figure 2). These results confirmed that 34 isolates were *Mycoplasma* and pure.

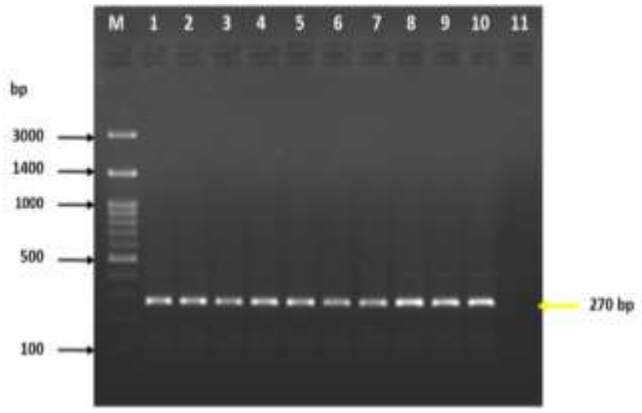

Figure 2: Amplification of *Mycoplasma* 16S rRNA gene. Lane M: 100 bp DNA ladder. Lanes 1-10 positive samples. Lane 11 negative control.

Nucleotide sequence accession number

The genome sequence of the 16S rRNA gene revealed that twenty-three 67.65% out of thirty-four *Mycoplasma* isolates were indicated as *Mycoplasma cynos* strain SM-MY-M23 from the nasal swabs in the household dogs suffering from respiratory signs have been submitted to the GenBank database of nucleotide sequences with entry number OQ446513.

Discussion

Respiratory diseases are frequent in dogs (10), and dogs are more sensitive to numerous types of respiratory infectious diseases (12), including *Mycoplasma* which was a pathogenic agent of respiratory disease in dogs (35,36). The flourishing bacteria in the contemporary investigation were matching to *Mycoplasma* when grownup on the medium, and bare the fried egg exterior as recounted in former reference books (3,25), which showed that in vitro culturing was the ideal style for identifying and isolating *Mycoplasma*, yet, the leisurely growth of *Mycoplasma* renders this technique time-wasting unto precise laboratories (3,13).

The existing study's 58% *Mycoplasma* isolation rate deviates significantly from the data stated by Sakmanoglu *et al.* (37) who documented 27% and Randolph *et al.* (38) and Barreto *et al.* (39) who chronicled 29.96%. The variances are perhaps imputed to the methods of sample gathering, isolation circumstances, case history, and specimen gathering time (3). The greater Mycoplasmal isolation ratio was recorded in nasal swabs 68%, then in the other two types of swabs (oropharyngeal swabs 58%, and conjunctival swabs 44%, however, various findings were established by former articles Randolph *et al.* (38) and Mitchall *et al.* (40) that showed oropharyngeal swabs 42.86% arranged at the peak of isolation and approved to them that conjunctival swabs 18.30% were the lowermost of isolation. The high rate of isolation from oropharyngeal swabs confirms that

Mycoplasma is a habitual symbiont of the upper respiratory tract. Whenever weary events occur, *Mycoplasma* transforms into infectious and the disease emerges. The weakened Mycoplasmal isolation from the analogous swab kinds of apparently healthy dogs also provides evidence for this by Barreto *et al.* (39). The output of the amplicon band at 270 bp., a positive band of isolates of *Mycoplasma*, exposes the importance and vast role of this technique in the detection of *Mycoplasma* species, determination of virulence factors, sequencing and identification of phylogenetic tree of *Mycoplasma* isolated in domestic dogs (41). The genome sequence of the 16S rRNA gene specialized for *Mycoplasma* genus achieved in the existing study was indicated *M. cynos* strainSM-MY-M23 from nasal swabs in the household dogs suffering from respiratory signs and was recorded in accession number OQ446513 in GenBank, so these results have differed from the data recorded by Walker *et al.* (42) who indicated *M. cynos* strain (C142) from tracheal wash in dogs, in accession number HF559394 in the United States, this confirms the part of *M. cynos* in the occurrence of respiratory diseases in household dogs, with its multiple virulence factors that will be detected by our subsequent study. Thus, the current study is one of the first studies in recording *Mycoplasma cynos* strainSM-MY-M23 isolated from upper respiratory and conjunctival infections in household dogs in Iraq, especially in Mosul city.

Conclusion

The output of the existing investigation denoted *Mycoplasma cynos* by sequencing of 16S rRNA gene specialized for *Mycoplasma* genus so further studies must be done to know the virulence determinants of *Mycoplasma cynos* in dogs to preclude the dispersal of infective strains of *Mycoplasma cynos* between dogs.

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Conflict of interest

Regarding the publishing of the current investigation, the authors thus state that yonder is no conflict of advantage**.**

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التشخيص الجيني للمفطورات المعزولة من إصابات التنفسية وملتحمة العين في الكلاب المنزلية

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الخلاصة

جري وصف الأنماط الوراثية للمفطورات المعزولة من الإصابات التنفسية وملتحمة العين في الكلاب المنزلية في مدينة الموصل باستخدام تقنية تفاعل البلمرة المتسلسل والتي تم إنجازها عن طريق تضخيم الجين الرايبوزومي الخاص بتحديد جنس المفطورات ومن ثم متابعة التسلسل الجيني. أخذت المسحات من ١٠٠ كلب منزلي من كلا الجنسين وبأعمار وأنواع مختلفة في الدراسة للفترة من٢٠٢٢/٢/١ الى ٢٠٢٣/١/١٥، حيث جمعت ٣٠٠ مسحة شملت على مسحات من الأنف، البلعوم الفمي وملتحمة العين. أخضعت المسحات لزرع المفطورات وبالظروف الملائمة لها. أكدت المستعمرات النامية بفحصها بالمجهر الضوئي والتشريحي. بعد ذلك استخلص الحامض النووي منقوص الأوكسجين للمستعمرات النامية وتم تضخيمه وترحيله على الأكاروز لملاحظة الحزم. كشفت نتائج العزل، عزل ٥٨ عزله من المفطورات وبإجمالي نسبة عزل من المسحات الكلية ٥٨%، وبأعلى نسبة عزل من المسحات الأنفية ٦٨%، تلتها مسحات البلعوم والفم ومسحات ملتحمة العين بنسب ٥٨ و ٤٥% على التوالي. بينت نتائج تفاعل تقنية البلمرة المتسلسل بان ٣٤ عزلة من مجموع ٥٨ عزلة كانت مؤكدة لإنتاج الجين الريبوسومي، والحصول على حزم بحجم ٢٧٠ زوج قاعدي وعدت هذه النتائج مميزة لجنس المفطورات. بينت نتائج تسلسل الحامض النووي الرايبوزومي أن ثلاثة وعشرين عزلة ٦٧,٦٥% من أصل أربعة وثلاثين عزلة من الميكوبلازما كانت من نوع المفطورات الكلبية من عتره SM-MY-M23 وتم ثبيتها في قاعدة بيانات تسلسل النيوكيلوتيدات لبنك الجينات تحت الرقم التصنيفي OQ446513، واعتبرت هذه النتائج كتسجيل أول لهذا النوع من المفطورات في الكلاب في العراق.