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Detection of *Mycoplasma* sp using next-generation DNA sequencing is common on nasal swabs from both healthy and unhealthy pet rabbits (*Oryctolagus cuniculus*)

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OBJECTIVE
Upper respiratory infections are a frequent problem in pet rabbits and rodents, and *Mycoplasma pulmonis* is 1 of the most common causes of respiratory infections in pet rats. *M pulmonis* was detected in 1967 in laboratory rabbits via culture of the nares and oropharynx, but overall, *Mycoplasma* is not commonly identified in the upper airway of rabbits. The objective of this study was to compare the prevalence of *Mycoplasma* sp detection via next-generation DNA sequencing on nasal swabs obtained from healthy and unhealthy rabbits.

METHODS
The results of nasal swabs from both healthy and unhealthy rabbits submitted for next-generation DNA sequencing from January 2022 to February 2023 were reviewed. Data gathered included signalment, whether or not *Mycoplasma* sp was detected, and the cell count and relative predominance of *Mycoplasma* sp compared to other organisms.

RESULTS
91 rabbits met the inclusion criteria, of which 49 were healthy and 42 were unhealthy. Overall, 52 of 91 (57.1%) rabbits were positive and 39 of 91 (42.8%) were negative for *Mycoplasma* sp. *Mycoplasma* positivity was significantly ($P < .001$) more common in healthy rabbits (37/49 [75.5%]) compared to unhealthy rabbits (15/42 [35.7%]).

CLINICAL RELEVANCE
The fact that *Mycoplasma* positivity was common in both groups of rabbits, and particularly common in rabbits without upper respiratory signs, suggests that *Mycoplasma* may be normal nasal flora in rabbits. Further research is needed to determine whether *Mycoplasma* could function as an opportunistic pathogen in rabbits.

Keywords: bacterial infections, *Mycoplasma*, rabbits, upper respiratory infections, Lagomorpha

Upper respiratory infections are a frequent problem in pet rabbits (*Oryctolagus cuniculus*)[1] and rodents.[2–4] *Mycoplasma pulmonis* is 1 of the most common causes of upper and lower respiratory infections in pet rats.[4,5] In contrast, *Mycoplasma* is not routinely considered as a cause of respiratory disease in rabbits. *Mycoplasma pulmonis* was first detected in rabbits in a laboratory setting in 1967.[6–8] The source of the *Mycoplasma* was unknown, but possible exposure to rats or mice was speculated.[6] Rabbits from commercial rabbitries were later evaluated for *Mycoplasma* via cultures of the nasopharynx and lungs by the same researcher, but it was not detected.[8,9] Several studies evaluating the microbiota of the respiratory system of rabbits have been published, but identification of *Mycoplasma* is underreported. *Mycoplasma* was not detected in a study evaluating bacterial cultures from nasolacrimal duct flushes in healthy and sick rabbits; however, this finding is unsurprising given that specialized media for *Mycoplasma* were not employed in that study[10] and given the fact that *Mycoplasma* is difficult to culture due to its fastidious nature.[11,12] Another report used *Mycoplasma*-specific culture to screen for

Received March 15, 2024
Accepted August 24, 2024
Published online September 11, 2024

doi.org/10.2460/ajvr.24.03.0075

Mycoplasma in the lungs of rabbits with respiratory signs, but none was detected.[13] In rats, *Mycoplasma* culture has a lower sensitivity than PCR,[12] indicating that culture alone is insufficient to adequately determine the *Mycoplasma* status of animals. Next-generation sequencing (NGS) has the potential to further improve our ability to detect respiratory pathogens in rabbits and rodents.

Unlike PCR testing, NGS uses an untargeted sequencing approach. Thus, NGS-based diagnostics are not limited to certain pathogens or specific species of interest.[14,15] Instead, the microbial DNA of all present bacteria and fungi is amplified simultaneously, including Gram positives and negatives and aerobic and anaerobic bacteria. Using this approach, the microbiome of a sample can be analyzed as a whole, allowing for novel pathogen discovery, a complete picture of commensals, and antibiotic resistance tracking. This approach can also be quantitative, allowing the generation of cell counts for each individual member of the microbiome. Having such a cell count is of clinical relevance in determining the clinical importance of potential pathogens detected. In a study[14] comparing paired culture and NGS samples in exotic, zoo, and wildlife species, the probability of a negative result on a sample was higher with traditional culture methods compared to NGS. In addition, slow-growing pathogens, such as *Mycoplasma* sp, could be detected by NGS but not culture.[14]

Due to the unexpected detection of *Mycoplasma* via NGS in several clinical cases of rabbits being managed by the authors for upper respiratory signs, we set out to investigate the prevalence of *Mycoplasma* on nasal swabs originating from pet rabbits. The authors' hypothesis was that *Mycoplasma* detection would be common and would be found in both healthy rabbits and rabbits with signs of upper respiratory disease.

Methods

Sampling and data collection

Cases of rabbits with nasal samples submitted for NGS from January 2022 through February 2023 were reviewed. All samples were from the US, including the states of California, Alabama, New York, Washington, Texas, Utah, Oklahoma, Indiana, Virginia, Ohio, and Maryland. The healthy rabbits consisted of 49 rabbits sampled to establish normal nasal microflora via NGS in healthy domestic rabbits in the US.[16] This dataset included a group of 24 rabbits from Alabama and 25 rabbits from Northern California and included owned pet rabbits and rabbits from rescue organizations.[16] The samples from unhealthy rabbits consisted of samples submitted by various veterinarians in the US as part of the clinical management of these patients. Rabbit breeds included in the study are listed in **Table 1**. The inclusion criteria were rabbits with a known health status (either "healthy," indicating no clinical signs of upper respiratory disease or any known health conditions, or "unhealthy," indicating that swabs were collected to work-up clinical disease) with nasal swabs submitted for NGS to the testing laboratory of MiDOG Animal Diagnostics LLC. Rabbits without a listed health status or sampling site were excluded. Samples obtained via nasolacrimal duct flush or aspiration of nasal abscesses or masses were excluded. Data gathered included signalment (age, breed, and sex), whether or not *Mycoplasma* was detected, and the cell count, percentage, and relative predominance of *Mycoplasma* compared to other organisms. Samples were shipped to the NGS diagnostic center, and sequencing was performed targeting the V1 through V3 region of the 16 S rRNA gene as previously described.[17,18]

Table 1—List of breeds of pet rabbits evaluated for *Mycoplasma* sp via next-generation DNA sequencing of nasal swabs.

Breed	Number of rabbits
Lop	14
Holland Lop	4
Mini Lop	4
Lop (unspecified)	4
Holland Lop X Lionhead	1
Lop X Dutch	1
Mixed breed (unspecified)	11
Dwarf	9
Dwarf (unspecified)	8
Dwarf X Lionhead	1
Lionhead	8
New Zealand	7
New Zealand (unspecified)	5
New Zealand White	1
New Zealand White mix	1
English Spot	6
Flemish Giant	5
Angora	2
Giant chinchilla	2
Rex	2
Agouti	1
American	1
Californian	1
Continental Giant	1
Czech Spotted rabbit	1
Dutch	1
Harlequin	1
Hotot X English	1
TAMUK Composite rabbit	1
Unknown	16

Sample processing

Following sample collection, samples were promptly transferred into vials containing a sterile DNA preservative (DNA/RNA Shield; Zymo Research Corp; catalog [cat.] No. R1108) before processing at the MiDOG LLC testing facility. Genomic DNA extraction utilized the ZymoBIOMICSTM-96 DNA kit (Zymo Research Corp; cat. No. 79 D4304). MiDOG Animal Diagnostics LLC handled sample library preparation and bacterial profiling data analysis using the Quick-16S NGS Library Prep Kit (Zymo Research Corp; cat. No. D6400) with slight adaptations.

Given the importance of stringent quality control in clinical NGS diagnostics, various positive and negative controls were concurrently processed. Negative controls, such as an "extraction negative

control" (using storage buffer DNA/RNA Shield; Zymo Research Corp; cat. No. R1100-50), were included to monitor for potential contamination. Additionally, automated workflow management via a Hamilton Star liquid handling robot (Hamilton Company) minimized human error.

For assurance against contamination and to validate procedures, both cellular and DNA mock communities served as positive controls (ZymoBIOMICS Microbial Community Standard; Zymo Research Corp; cat. Nos. D6300 and D6305). These controls helped address any biases in the DNA extraction process. The ZymoBIOMICS Microbial Community Standard (Zymo Research Corporation) was utilized for performance monitoring across all NGS workflow stages, including bioinformatic analysis.

Sequencing targeted the 16S rRNA V1 through V3 region for bacteriome analysis following a previously described protocol.[17] An Illumina HiSeq 1500 sequencer was employed to achieve a sequencing depth of 7 to 8 million reads, ensuring a minimum of 10,000 reads per sample.

Statistical analysis

Histograms and Shapiro-Wilk *W* tests were used to test for normality. A Pearson Chi-squared test was used to assess for significant differences between healthy and unhealthy rabbits for categorical variables, and a Wilcoxon rank sum test was used to assess significant differences between healthy and unhealthy rabbits for continuous variables. Values of $P < .05$ were considered statistically significant. Commercially available software (R, version 4.3.1, 2023; https://www.R-project.org/; R packages rcompanion and doBy; R Foundation for Statistical Computing) was used for statistical analysis.

Mycoplasma sp PCR

To verify the findings of the study, *Mycoplasma* sp PCR was performed on a portion of the samples. This included 14 samples that had been included in the present study and were archived at the NGS testing laboratory and 1 set of newly collected nasal swab samples. The 14 archived samples were randomly selected using a random number generator (https://www.random.org). The archived samples had been stored at −20 °C and kept in a preservation buffer. These samples were submitted for *Mycoplasma* sp PCR. The newly collected samples were collected in 2024 from a rabbit that was included as part of this study and was positive for *Mycoplasma* sp via NGS in 2022. This sample was frozen at −80 °C prior to shipping. The 2024 samples were submitted for repeat NGS for this individual and for *Mycoplasma* sp PCR. *Mycoplasma* sp PCR was performed by the Department of Infectious Diseases and Immunology at the University of Florida College of Veterinary Medicine.

Results

Based on the inclusion criteria, a total of 116 cases were identified. Twenty-five cases were excluded, with 15 cases excluded due to issues with the sample site and 10 cases excluded due to an unknown or unclear health status **(Figure 1)**. Nine samples were excluded as the source was a nasolacrimal duct, 1 of which was listed as having a nasolacrimal duct abscess. One sample site was listed as a "nasal flush" and was excluded as it was unclear if the source was a nasolacrimal duct flush or a flush of the nares. Three samples were excluded as these appeared to originate from a nasal mass ("nose abscess" in 1, "nasal nodule" in 1, and "nasal FNA" in 1). One sample was excluded as it was listed as a nasal/oral swab. One did not have a sample site listed. Three rabbits were excluded due to an unknown health status. Finally, 7 rabbits were excluded as they were marked as "healthy" but were not part of the group of rabbits specifically sampled to evaluate normal nasal flora in rabbits (see below). The remaining 91 cases were used for analysis.

Forty-nine (53.8%) rabbits were considered healthy, and 42 rabbits (46.2%) had an abnormal health status. As stated above, rabbits not part of the aforementioned study[16] that had their health status listed as "healthy" were excluded as their status as healthy rabbits could not be confirmed.

The age was available for 87 of 91 rabbits. The median age was 3 years (range, 0.33 to 12.5 years) overall, with a median age of 1.33 years (range, 0.33 to 9.5 years) for the healthy rabbits and 6.5 years (range, 0.41 to 12.5 years) for the unhealthy rabbits. Regarding sex, 23 rabbits were castrated males, 24 were spayed females, and 2 were intact males; sex was not listed for 42 rabbits.

Overall, 52 of 91 (57.1%) rabbits were positive and 39 of 91 (42.9%) rabbits were negative for *Mycoplasma*. Of the healthy rabbits, 37 of 49 (75.5%) were positive and 12 of 49 (24.5%) were negative for *Mycoplasma*. Of the unhealthy rabbits, 15 of 42 (35.7%) were positive and 27 of 42 (64.3%) were negative for *Mycoplasma. Mycoplasma* detection was significantly different between healthy and unhealthy rabbits ($P < .001$) and was significantly more common in healthy rabbits compared to unhealthy rabbits. The median cell count of *Mycoplasma* in *Mycoplasma*-positive samples was 290,000 cells/sample (range, 1,700 to 12,000,000 cells/sample). For healthy, *Mycoplasma*-positive rabbits, the median cell count was 580,000 cells/sample (range, 2,100 to 12,000,000 cells/sample); for unhealthy, *Mycoplasma*-positive rabbits, it was 110,000 cells/sample (range, 1,700 to 2,500,000 cells/sample). The difference in cell count between healthy and unhealthy rabbits was significant ($P = .01$). Of all the bacterial species identified in each sample with *Mycoplasma*, *Mycoplasma* was the predominant organism in 11 of 52 samples **(Table 2)**. The relative abundance of *Mycoplasma* in *Mycoplasma*-positive samples compared to all other bacteria was not significantly different between healthy and unhealthy rabbits ($P = .89$). The median percentage of *Mycoplasma* in comparison to other bacteria in *Mycoplasma*-positive samples was 4.74% (range, 0.04% to 66.63%), with a median percentage of 4.85%

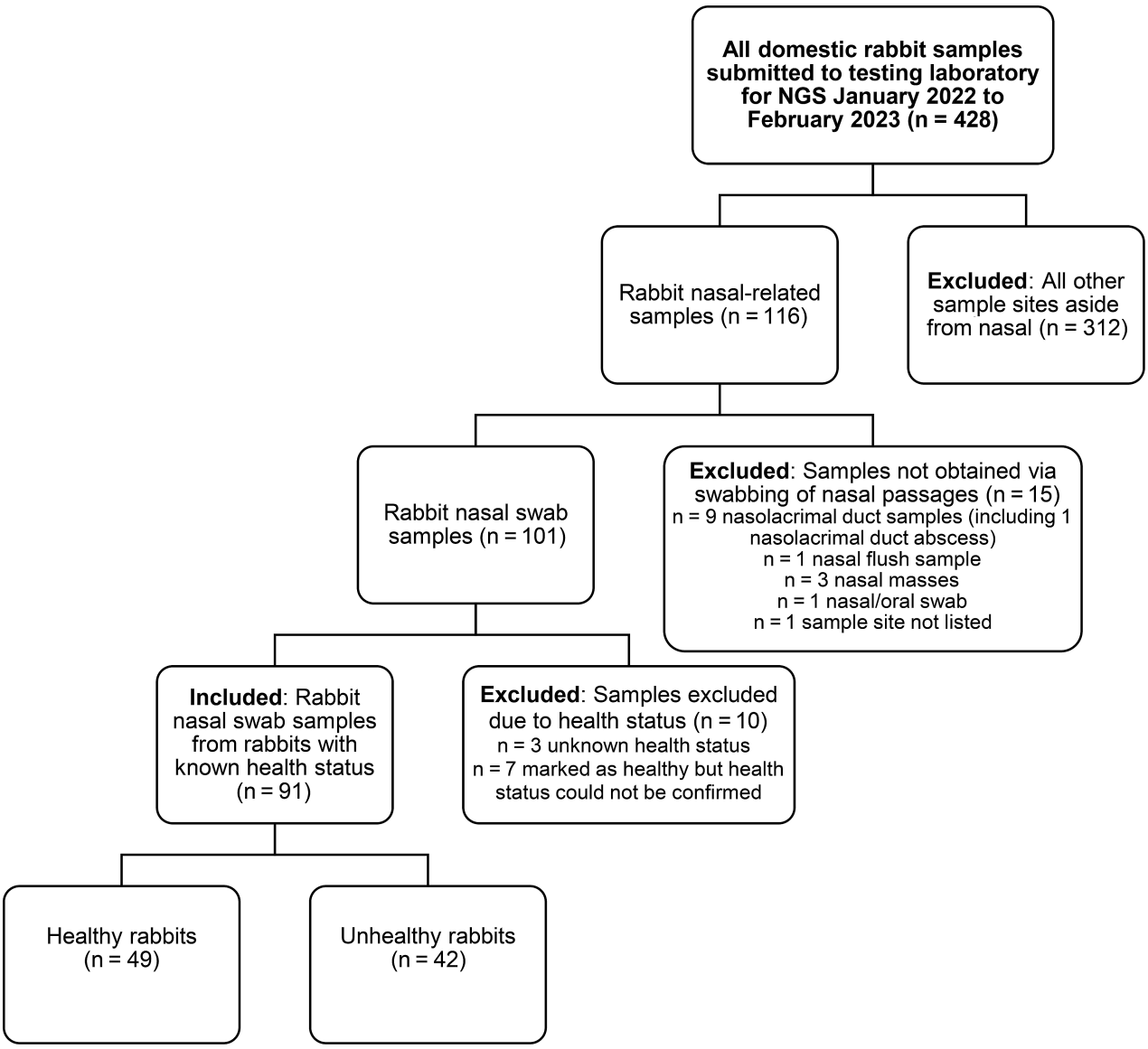

Figure 1—Flow chart describing the inclusion and exclusion criteria for samples from domestic rabbits (*Oryctolagus cuniculus*) that underwent next-generation DNA sequencing (NGS) from January 2022 through February 2023.

Table 2—Relative abundance of *Mycoplasma* sp compared to other bacterial species identified in nasal swab samples from *Mycoplasma*-positive rabbits.

Relative predominance of *Mycoplasma*	Number of samples out of total number of *Mycoplasma*-positive samples	Range of *Mycoplasma* cell counts
1	11/52	15,000–12,000,000
2	3/52	700,000–6,700,000
3	5/52	110,000–6,600,000
4	8/52	100,000–6,900,000
5	5/52	32,000–2,400,000
6+	20/52	1,700–2,100,000

A value of "1" in the left column indicates that *Mycoplasma* sp was the most abundant bacterial species based on cell count in comparison to other bacterial species identified in the sample; a value of "2" indicates it was the second most abundant, etc.

(range, 0.04% to 66.63%) in healthy rabbits and 4.65% (range, 0.05% to 63.05%) in unhealthy rabbits. This was not significantly different between healthy and unhealthy rabbits (P = .84).

For rabbits with a known sex, *Mycoplasma*-negative rabbits included six castrated males and six spayed females, and *Mycoplasma*-positive rabbits included 18 spayed females, 17 castrated males, and

2 intact males. There was no significant difference in *Mycoplasma* detection by sex (P = .71), breed (P = .22), or age (P = .09).

Of the 14 archived samples submitted for *Mycoplasma* sp PCR, 10 samples were positive for *Mycoplasma* sp on NGS, and 4 were negative for *Mycoplasma* sp on NGS. Of the 10 NGS-positive samples, 5 of 10 were PCR-positive for *Mycoplasma* sp. Of the 4 NGS-negative samples, 3 of 4 were PCR-negative for *Mycoplasma* sp.

The rabbit in which repeat sampling was performed was positive for *Mycoplasma* sp via NGS in both 2022 (7,200 cells/sample) and 2024 (480,000 cells/sample) but was negative on *Mycoplasma* sp PCR.

The *Mycoplasma* species identified in the present study appeared to be a taxon within the *Mycoplasma* genus with unknown species identity. The DNA sequence was not able to be aligned to a sequence in a database (PubMed, Greengenes, MiDOG LLC database), indicating that no sequence of that specific species has been deposited by any researchers. Based on the DNA sequence, it was determined with 99% confidence that the species identified is a species within the genus *Mycoplasma*, but the specific species could not be determined. In addition, it could not be determined whether all rabbits had the same *Mycoplasma* species or if multiple different *Mycoplasma* species were found.

Discussion

The present study found that *Mycoplasma* sp detection via NGS of nasal swabs is common in both healthy rabbits and rabbits with upper respiratory signs. There was a significant difference in *Mycoplasma* detection between healthy and unhealthy rabbits, with detection being more common in healthy rabbits. In addition, healthy rabbits with *Mycoplasma* had significantly higher cell counts than unhealthy rabbits with *Mycoplasma*. Based on the frequent detection of *Mycoplasma* among the nasal flora of healthy rabbits, it is suspected that *Mycoplasma* may be normal nasal flora in rabbits. However, the potential for *Mycoplasma* to cause disease when a rabbit is immunosuppressed, as occurs in other species,[4,5] is unknown.

Mycoplasma pulmonis was first reported to be detected in rabbits in 1967.[6] In that report, *M pulmonis* was consistently detected in 4 New Zealand white laboratory rabbits evaluated via cultures of the nares and oropharynx over a period of 2 months.[6] As 2 of these rabbits were clinically healthy and 2 had upper respiratory signs, it was unclear if *M pulmonis* was the cause of clinical disease.[6] Aerobic bacterial cultures were not performed in that study to evaluate for other bacterial agents.[9] Interestingly, 1 of the rabbits was used as a control in an *M pneumoniae* immunization study.[6] Rabbits have been used as models for experimentally induced mycoplasmal synovitis and arthritis.[19,20] Both *M arthritidis* and *M pulmonis* injections successfully induced synovitis with prolonged inflammation but rapid clearance of viable organisms,[19] and inflammatory changes were less severe with *M pulmonis* compared to *M arthritidis*.[20]

The relatively high frequency of *Mycoplasma* detection noted in the present study may initially be surprising given the historically limited descriptions of *Mycoplasma* detection in rabbits. However, recent studies[21,22] using rabbits as a model for rhinosinusitis in humans have likewise demonstrated a high relative predominance of *Mycoplasma* in rabbits via 16 S rRNA amplification. In 1 study,[21] farm-sourced New Zealand white rabbits underwent transient sinus occlusion, and swabs were collected from the middle meatus of each nostril before, during, and after occlusion and evaluated for bacterial community makeup using amplification of the V3 to V4 region of the 16S rRNA gene. Baseline samples in the same study showed a predominance of *Helicobacter, Moraxella, Mycoplasma*, and *Neisseria*, but bacterial diversity was significantly increased during unilateral sinus occlusion.[21] In another study[22] using *Pasteurella*-free laboratory rabbits as a model for rhinosinusitis, Proteobacteria and Tenericutes (a phylum that includes the *Mycoplasma* genus) predominated in control samples prior to sinus blockage, and Tenericutes was significantly more prevalent in controls compared to rabbits with sinusitis. These studies, along with the present one, further support that *Mycoplasma* may be normal nasal flora in rabbits.

No significant differences in *Mycoplasma* detection were noted by age, sex, or breed in the present study. In regard to breed, the lack of significant differences may be in part due to the large number of breeds included and the fact that several rabbits were mixed or unknown breeds. Certain disorders are more common in particular breeds of rabbits. For example, lop rabbits are prone to aural[23,24] and dental[23] disease, and lionhead and dwarf lop rabbit are prone to dacryocystitis.[25] If *Mycoplasma* is indeed normally carried in the nasal passages of rabbits, it would make sense that carriage is common in a variety of breeds without a particular breed being predisposed.

Various species of *Mycoplasma* have been described as causes of respiratory infections in several mammal, avian, and reptile species. Commonly encountered mycoplasmal syndromes in zoological companion animals include respiratory infections in rats,[4,5] poultry,[26] and tortoises.[11] Commonalities between these infections in different species include the fact that animals can be subclinical carriers or develop disease of varying severity and the fact that the infection is unlikely to be cleared.[4,27,28] Rats may serve as a reservoir of mycoplasmosis for mice[29]; it is unknown whether rabbits carrying *Mycoplasma* could spread the infection to these rodents if housed in closed proximity. The opposite situation, namely rats spreading *Mycoplasma* to rabbits, which was speculated in the original study describing *Mycoplasma* in rabbits,[6] is thought to be an unlikely reason for the rabbits to be carrying *Mycoplasma* in the present study due to the high numbers of rabbits affected. However, information regarding whether the rabbits lived in households with rats or could have been exposed to rats prior to adoption was not available.

The samples obtained from unhealthy rabbits were obtained by a variety of different veterinarians

during normal clinical practice. Therefore, it is unknown whether the parameters of sampling were consistent (eg, whether the veterinarian wore gloves during sampling, how deep the nasal swab was inserted, whether the swab could have been contaminated by the skin, etc). Another potential weakness of this study is that information regarding clinical signs was not provided for the majority of the unhealthy rabbits, and information regarding any imaging findings was not available. Therefore, it is unclear if *Mycoplasma* is associated with clinical signs or imaging changes to the upper respiratory system in rabbits. However, the results of this study and others do suggest that *Mycoplasma* may be normal nasal flora in rabbits, and it is considered somewhat unlikely that *Mycoplasma* is associated with clinical signs. Further cases demonstrating histopathologic changes associated with *Mycoplasma* on biopsy or necropsy samples of rabbits with respiratory disease would be indicated to determine if *Mycoplasma* is a clinically relevant pathogen in rabbits. Next-generation sequencing may also be useful in the detection of *M pulmonis* in pet rats, a species in which mycoplasmosis is a common and clinically relevant organism.

In this study, half of the 10 submitted samples were positive for *Mycoplasma* sp on NGS but negative on PCR. It is possible that NGS was more sensitive, the PCR was more specific, or that there was a loss of genetic material over time due to sample storage and shipping. The majority of the NGS-negative samples were also negative on PCR; the single case that was negative on NGS but positive on PCR is more difficult to explain. Possible reasons for this include sample contamination.

The single rabbit that underwent repeat sampling was persistently positive for *Mycoplasma* sp. via NGS in both 2022 and 2024. Further research is needed to determine if prolonged *Mycoplasma* sp carriage is the norm in rabbits, or if some rabbits may eventually clear the organism. In other species, animals with *Mycoplasma* spp tend to be infected for life as the organism is extremely difficult to clear.

Future research could also compare serology, *Mycoplasma* culture, *Mycoplasma* PCR, and NGS for the detection of *Mycoplasma* sp in pet rabbits. Unfortunately, *Mycoplasma* PCR was only performed on a portion of the samples in this study due to financial constraints. Therefore, formal comparison between NGS and PCR was not performed.

In summary, *Mycoplasma* was detected on nasal swabs evaluated via NGS in a large proportion of both healthy and unhealthy pet rabbits. This was significantly more common in healthy rabbits, suggesting that rabbits can carry this organism without clinical signs and that it may be normal nasal flora in this species. Further research is needed regarding the potential pathogenicity of this organism in domestic rabbits.

Acknowledgments

The authors wish to thank Alexandra Burne, Dr. Mary Brown, and the University of Florida College of Veterinary Medicine Mycoplasma diagnostic lab for performing the Mycoplasma PCR testing.

Disclosures

Drs. McCready and Siperstein have nothing to disclose. Dr. Krumbeck is an employee of the NGS-based testing laboratory. Dr. Brandao is a member of the *AJVR* Scientific Review Board, but was not involved in the editorial evaluation of or decision to accept this article for publication.

No AI-assisted technologies were used in the generation of this manuscript.

Funding

Funding for the Mycoplasma sp. PCR testing was provided by the Oklahoma State University College of Veterinary Medicine Veterinary Clinical Sciences Department.

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