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Detection of *Mycoplasma* spp. in horses with respiratory disorders

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Abstract

Background: Bacteria belonging to the genus *Mycoplasma* are small-sized, have no cell walls and small genomes. They commonly cause respiratory disorders in their animal hosts. Three species have been found in the respiratory tract of horses worldwide, that is., *Mycoplasma (M.) equirhinis*, *M. pulmonis* and *M. felis*, but their role in clinical cases remains unclear.

Objectives: The aim of this study was to i) develop and validate tools to detect, isolate and identify different *Mycoplasma* spp. strains in clinical equine respiratory-tract specimens and ii) subsequently define the prevalence of the three species in France depending on sample types and horse characteristics (age, breed, sex).

Study design: Validation of a workflow for mycoplasma diagnosis and subsequent prevalence study.

Methods: Mycoplasma-free tracheal wash samples spiked with numerated strains and DNA dilutions were used to validate the culture methods and real-time PCR (rt-PCR) assay. Isolated strains were identified by 16S rRNA gene sequencing. Prevalences were determined on a population of 616 horses with respiratory disorders, sampled in France in 2020.

Results: In total, 104 horses (16.9%) were found to be positive for *Mycoplasma* spp. by at least one method. *M. equirhinis* was the predominant circulating species, accounting for 85% of the rt-PCR-positive samples and 98% of the 40 cultured strains.

Main limitation: The proposed pre-enrichment procedure improves the sensitivity of detection but hinders the quantification of the initial mycoplasma load in the clinical specimens.

Conclusions: Prevalence of mycoplasma varied with age, breed, and type of sample.

KEYWORDS

detection, horse, *M. equirhinis*, *Mycoplasma* spp., respiratory tract

Albertine Léon and Florence Tardy contributed equally.

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1 | INTRODUCTION

Bacteria belonging to the genus *Mycoplasma* (*M.*) are small-sized cells that have no cell walls. Their small genomes and thus narrow metabolic pathways make them the simplest organisms able to self-replicate in cell-free media *in vitro*.¹ They are fastidious to culture, requiring specific complex growth media and controlled CO₂ and humidity conditions.² More than a hundred *Mycoplasma* species have so far been found in different animal hosts, where they are either commensals, opportunists or primary pathogens.³ *Mycoplasma* infections commonly target the respiratory tract. Mycoplasmal respiratory infections are typically caused by *M. pneumoniae* in humans,⁴ *M. bovis* in cattle,⁵ and *M. gallisepticum* in poultry.⁶ Respiratory disorders in horses are a major cause of poor performance^{7,8} and thus of significant economic losses for the equine industry,⁹ and include both equine asthma and bacterial or viral infections.¹⁰ Bacteria responsible for bronchopneumonia, pneumonia or pleuropneumonia of horses are numerous, including non-enteric (e.g., *Pasteurella* or *Actinobacillus* spp.) or enteric (e.g., *Klebsiella* spp.) Gram-negative bacteria or anaerobes (e.g., *Bacteroides* spp.) or more frequently gram-positive aerobes (*Streptococcus equi* subsp. *zooepidemicus* being the commonest while *Rhodococcus equi* is seldom found in immunocompetent adults).^{11,12} Viral respiratory infections are mostly caused by equine influenza virus or equine herpesviruses (EHV-1 and EHV-4) and are associated with various signs such as fever, cough and nasal discharge.¹³ An old study conducted in 1975 reported the isolation of three *Mycoplasma* species, that is., *M. equirhinis*, *M. felis* and *M. pulmonis*, from nasopharyngeal swabs of both healthy horses and horses with severe respiratory disorders.¹⁴ In the early 1980s another *Mycoplasma* species, isolated from the respiratory tract of horses, was described, namely *M. fastidiosum*.¹⁵ From that day on, there have been regular reports of *M. felis* and *M. equirhinis* but not *M. pulmonis*, nor *M. fastidiosum*^{16,17} in the respiratory tract of horses in various countries, but their clinical significance remains unclear.¹⁸ The difficulty in specifying the aetiological role of these bacteria is partly due to the lack of systematic detection during diagnostic investigation and the absence of characteristic clinical signs. For instance, *M. felis*, a recognised cat pathogen, was suspected to be involved in pericarditis and/or pleuritis of horses, as it was isolated from pleural fluid in which no other bacterial species were detected.^{19,20} *M. pulmonis*, a rodent pathogen, has occasionally been isolated from the nasopharynx of horses.²¹ *M. fastidiosum* has never been documented in clinical cases so far. *M. equirhinis*, considered an opportunistic pathogen in horses,³ was recovered from tracheal washes (TW) of horses with or without clinical signs.^{16,17} In France, there are still no data available on how often mycoplasmas circulate in equids and which species are involved. A rough estimate based on routine real-time PCR (rt-PCR) analysis at LABÉO suggested an approximate prevalence of *Mycoplasma* spp. of 15.2% in clinical respiratory specimens. Unfortunately, we cannot reasonably compare this prevalence figure with other datasets from different countries due to differences in experimental designs and detection methods.^{16–18,22} Furthermore, we have no details concerning the *Mycoplasma* species present.

This study was designed to optimise and validate a set of tools to detect *Mycoplasma* spp. in horse respiratory specimens and to identify the strains involved to species level. We then assessed these tools on a large set of clinical specimens collected in 2020. The prevalence of *Mycoplasma* spp. was analysed according to sample type and to the main characteristics of the horses (age, breed, sex) in order to determine whether presence of a mycoplasma is significantly associated with specific horse characteristics.

2 | MATERIALS AND METHODS

2.1 | Study population and samples

A total of 616 samples were collected in 2020 from horses in France and submitted to LABÉO laboratory (Saint-Contest, France) for diagnosis of suspected respiratory disorders. Samples were either tracheal washes (TW, *n* = 473, 76.8%) including tracheal aspirates or bronchoalveolar lavages (BAL, *n* = 143, 23.2%). Horses with known characteristics were grouped into different categories: < 5 years and ≥5 years of age, racing breeds (i.e., Thoroughbreds and trotters) versus others (for instance, saddle horse, pony, etc.), and males versus females.

2.2 | Handling of incoming clinical specimens

Clinical samples sent to LABÉO were processed via a routine workflow, that is., 200 µl of the TW or BAL samples was seeded into 1.8 ml of mycoplasma culture broth (INDICIA) and incubated at 37°C with 5% CO₂ for *Mycoplasma* spp. enrichment during 3–7 days before DNA extraction. For this study purpose, another 200 µl of the sample was seeded in 1.8 ml of culture broth (broth B1) containing phenol red, used as pH indicator, and incubated at 37°C with 5% CO₂ for mycoplasma growth. The B1 broth was examined daily for 72 hours to track any changes in turbidity, pH, and contamination, then further processed as described in Data S1 in order to optimise the isolation of mycoplasma colonies.

2.3 | Real-time PCR (rt-PCR) assays on enriched clinical specimens

A multiplex rt-PCR adapted from previous works^{23–25} was developed and validated to detect *Mycoplasma* spp. and *M. felis* (Data S2). The 20 µl PCR mix was composed of 12.5 µl Taqman Universal PCR Master Mix (ThermoFisher Scientific), primers at 200–300 nM, probes at 50–150 nM, and water. The rt-PCR was performed on a StepOne™-model Applied Biosystems thermocycler. Five µl of DNA or water (negative control) was added to the premix before the run.

Validation of the rt-PCR (i.e., target, non-target species, and limit of detection [LOD_{PCR}]) was done according to French AFNOR standard NF U47-600-2. Briefly, LOD_{PCR} was determined using 6 five-fold

serial dilutions of quantified DNA extracts from three strains, that is., *M. equirhinis* (field strain, ANSES-F13945), *M. felis* (field strain, ANSES-F13444), and *M. pulmonis* (NCTC 10139). Eight replicates of each dilution were tested in three independent rt-PCR runs. LOD_{PCR} at 95% was set as the highest dilution with at least 23/24 positive replicates (interpretation was done using a cut-off value for cycle threshold, Ct, of 40 per default). To convert the LOD_{PCR} from a DNA amount in ng into equivalent genome copy numbers, the following formula was used (Amount [ng] × 6.022 × 10²³) / (Length (bp) × 10⁹ × 660), with genome sizes of respectively 841 695 bp for *M. felis*; 730 410 bp for *M. equirhinis* and 963 879 bp for *M. pulmonis* according to the NCBI databases (<https://www.ncbi.nlm.nih.gov/genome/>). *Mycoplasma* or non-*Mycoplasma* species and strains used for inclusivity and exclusivity testing are listed in Data S3.

The analytical sensitivity of the whole method (from DNA extraction to rt-PCR analysis) was also determined according to guidelines developed by the American Association of Veterinary Laboratory Diagnosticians (AAVLD).²⁶ Briefly, five-fold serial dilutions of three enumerated mycoplasma suspensions (same strains as for LOD_{PCR}) were added to a mycoplasma-negative TW down to a theoretical concentration of 1 CFU/ml each. Then 200 µl of the spiked TW was either analysed directly (after a 4 days' storage at -80°C) and 200 µl were seeded in 1.8 ml of mycoplasma broth and incubated for 4 days (enrichment procedure). DNA of 100 µl of each sample was extracted according to the routine workflow at LABÉO and amplified in three independent runs. The analytical sensitivity was the highest dilution where 3/3 replicates were positive.²⁶

Nucleic acids were extracted from 100 µl of routine *Mycoplasma* spp. enrichment broth using the Adiamag Kit^c and the KingFisher Flex robot (ThermoFisher Scientific). rt-PCR was then performed on an Applied Biosystems thermocycler as described above. An exact Ct cut-off value was defined according to rt-PCR performances (see results section) to discriminate tested samples as positive, doubtful, or negative.

2.4 | Identification of rt-PCR-positive extracts and isolated strains

Positive-testing DNA extracts (with Ct < the Ct cut-off value of rt-PCR) were further amplified using 16S rDNA PCR for identification purposes (Data S2). Sanger sequencing of the amplicons using the primer U1/583 (5'-TTGATCCTGGCTCAGG-3')²⁷ was outsourced (Genewiz, Germany). Identification of *Mycoplasma* to species level was done using the leBIBI^{QBPP} online tool (<https://umr5558-bibiserv.univ-lyon1.fr/lebibi/lebibi.cgi>).²⁸ These DNA extracts were also analysed in parallel using two previously-described PCR assays targeting *M. equirhinis*²⁹ and *M. pulmonis*,²³ respectively. These PCR assays were performed in a 20 µl mix containing 5 µl 5X Buffer (with MgCl₂) (Promega), 1 µl dNTP, 0.4 µl Go Taq polymerase (Promega) and primers at 40 µM. All PCR runs were performed on a T100 thermal cycler (Bio-Rad) in conditions detailed in Data S2. Size of the

amplicons (211 bp and 266 bp, respectively) was verified by capillary electrophoresis using the QIAxcel Advanced system (Qiagen).

In the strain isolation process (Data S1), each colony picked on an agar plate was grown in liquid broth using serial dilution to track pH changes, and 200 µl taken from the tube that showed a colour change (from red to orange for acidifying species or red to darker red for alkalising species, with or without additional turbidity depending on the strains) indicative of mid-exponential growth was used for DNA extraction via a QIAamp DNA mini kit (Qiagen). Elution was performed in 50 µl of sterile water, and 5 µl of the extract was submitted to PCR for species identification as described above for positive DNA extracts.

2.5 | Data analysis

Statistical analysis was performed using GraphPad Prism 9.1.12 software. In initial univariable analyses, the prevalence of *Mycoplasma* spp. was compared between groups using a chi-squared test. Explanatory variables that had an individual impact on *Mycoplasma* spp. prevalence were further analysed using a multivariable logistic regression model. *P* values ≤0.05 were considered significant.

3 | RESULTS

3.1 | Validation of our rt-PCR workflow for *Mycoplasma* spp. detection

The LOD_{PCR} at 95% CI for the rt-PCR workflow targeting 16S rRNA gene was 4.2 E-6, 6.0 E-6 and 1.3 E-5 ng/µl for *M. felis*, *M. equirhinis* and *M. pulmonis*, respectively (Data S4). This corresponds to approximately 4.5, 7.5 and 12.3 genome copy numbers per µl, respectively. The LOD_{PCR} for the *M. felis*-specific rt-PCR was similar to that of 16S rDNA, with a mean Ct slightly below the mean 16S rDNA Ct (36.7 vs. 36.9). Linear regression analysis was performed for each rt-PCR assays (Data S4), resulting in comparable, satisfactory PCR efficiencies (82.1%, 90.5%, 81.5%, 81.2% for *M. felis* (16 S rRNA gene and *tuf*), *M. pulmonis*, *M. equirhinis*, respectively) and suitable linearity with 0.96 < R² < 0.98. Since the mean Ct values for LOD_{PCR} were around 37, we set this value as an exact cut-off to interpret positive-testing samples. Samples were subsequently classified into three categories, that is., positive if Ct < 37, doubtful if 37 ≤ Ct ≤ 40, and negative if Ct > 40.

Based on these interpretative criteria, all the target materials, that is *Mycoplasma* species listed in Data S3, were found positive by 16 S rDNA rt-PCR, but only the *M. felis* strain was also positive for the *M. felis tuf* signal. The non-target materials (viruses and bacteria) were all negative (SD 3), which points to very good specificity.

The analytical sensitivity of the rt-PCR-based method, with the Ct cut-off of 37 as set above, varied from <1 to 10 000 CFU/ml without any enrichment and from <1 to 100 CFU/ml after 4-day in-broth enrichment (Table 1, and details in Data S4), showing a clear effect of

TABLE 1 Comparative sensitivity of three methods (culture, rt-PCR, and rt-PCR post pre-enrichment) for detecting three *Mycoplasma* species in spiked TW.

	Culture	rt-PCR	rt-PCR post pre-enrichment
<i>M. equirhinis</i>	10	10 000	<1
<i>M. felis</i>	10	10 and <1 ^a	<1 ^a
<i>M. pulmonis</i>	100	1000	100

Note: Lowest concentrations, in CFU/ml, of mycoplasma detected in spiked samples by each of the methods in 3/3 replicates (see details in Data S2). Cultures were considered positive when characteristic ‘fried-egg’ colonies were observed on agar medium. 16S rDNA rt-PCR-positive samples were defined using the Ct < 37 as cut-off.

^aResults are identical using the *M. felis*-specific rt-PCR assays.

TABLE 2 Comparative rates of detection of *Mycoplasma* spp. in 616 clinical specimens using either 16S rt-PCR post-enrichment or culture.

		Culture		Total
		Positive	Negative	
rt-PCR	Positive	41	57	98
	Doubtful	1	5	6
	Negative	5	507	512
	Total	47	569	616

Note: Cultures were considered positive when characteristic ‘fried-egg’ colonies were observed on agar medium. The results of 16S rRNA rt-PCR assays were interpreted as positive if Ct < 37, doubtful if 37 ≤ Ct ≤ 40, and negative if Ct > 40.

a mean 4-day enrichment versus none on efficiency of detection. Post-enrichment rt-PCR-based detection also had better sensitivity than culture-based detection (from 10 to 100 CFU/ml), except for *M. pulmonis* where it was equivalent.

3.2 | Comparative efficiency of culture versus rt-PCR for mycoplasma detection in clinical samples

Of 616 horses, 98 (15.9%) tested positive by rt-PCR targeting 16S rRNA gene for *Mycoplasma* spp. (Table 2), of which four were also positive for the *M. felis tuf* gene. Among the rt-PCR positive horses only 41 were also positive by culture-based detection. Only 6 samples were positive by culture-based detection but negative or doubtful by rt-PCR (Table 2), illustrating the better sensitivity of the rt-PCR-based method and demonstrating that the methods are complimentary for optimal detection.

3.3 | Prevalence of *Mycoplasma* spp.

The overall cumulative (by culture- or rt-PCR-based detection) prevalence of *Mycoplasma* spp. in our population was 16.9% (104/616).

Among the 98 rt-PCR (16 S rDNA)-positive DNA extracts, 93 were available for further sequencing analysis for species-level identification. *M. equirhinis* was identified in a majority of samples (79/93, 85%) whereas *M. pulmonis* was identified just once (1%). The rest of the DNA extracts ($n = 13$) were either not identified further than the genus *Mycoplasma* ($n = 4$, with an ambiguous positioning between different species) or else not identified at all as Sanger sequencing resulted in superposed chromatograms.

Interestingly, of the six samples tested as doubtful by rt-PCR ($37 \leq Ct \leq 40$), two were identified as *M. equirhinis* and one as *M. pulmonis*. The higher proportion of *M. pulmonis* in the doubtful category is consistent with the lower sensitivity of the method for this species. For the four rt-PCR *tuf*-positive DNA extracts, that is., *M. felis* positive, one was identified as *M. equirhinis*, suggesting a potential mix of different species in the clinical specimen, and the other three were among the non-available DNA extracts ($n = 2$) and were either non-sequenceable or resulted in a non-exploitable sequence ($n = 1$).

Out of the 47 culture-positive samples, 7 did not lead to isolation because of viability loss during subcultivation steps and 40 strains were isolated: 39 were identified as *M. equirhinis* (98%) and one as *M. felis* (2%). Note that this *M. felis* strain was isolated from the rt-PCR *tuf*-positive sample for which extracted DNA was identified as *M. equirhinis*.

3.4 | Prevalence of *Mycoplasma* spp. by horse characteristics or sample types

Age data was available for 554 (90%) horses, which were 190 horses (34%) in the ‘‘under 5 years’’ group and 364 (66%) in the ‘‘5 years and more’’ group. For breed, 524 (85%) horses were classified as Thoroughbreds and trotters ($n = 200$, 38%) or other breeds ($n = 324$, 62%). Sex data was available for 553 (90%) individuals and were 314 males (gelded or not, 57%) and 239 females (43%). Figure 1 charts the prevalence of *Mycoplasma* (positive by at least one method) by horse subgroup or type of sample. On univariable analysis, the prevalence of *Mycoplasma* was significantly higher in young horses ($p = 0.01$). We found a significant association ($p = 0.002$) between breed group and presence of mycoplasmas with prevalence almost two-fold higher among racing breeds (26.0%) than other types (14.8%). There was no significant association between sex and presence of *Mycoplasma* in our horse population. Regarding type of clinical specimens investigated, mycoplasmas were detected in 19.4% of TW versus only 8.4% of BAL ($p = 0.002$). The same associations were also found when we considered only samples in which *M. equirhinis* was identified (Figure 1). For multivariate logistic regression analyses the subpopulation was composed of 515 horses and these analyses confirmed the higher risk of having mycoplasmas and *M. equirhinis* in TW samples and in racing breeds, but an association with age was not confirmed (Table 3).

4 | DISCUSSION

To refine our knowledge on the prevalence of mycoplasmas in respiratory specimens from horses, we developed a mixed-method

FIGURE 1 Prevalence of *Mycoplasma* spp. (●) and *M. equirhinis* (□) in clinical specimens (testing positivity by rt-PCR and/or culture) as a function of (A) age, (B) breed, (C) sex, and (D) sample types. Error bars correspond to 95% confidence intervals. “ns”: not significant, “*”, “**” and “***” refer to *p*-values of ≤ 0.05 , ≤ 0.01 and ≤ 0.001 , respectively, after a Chi-square test. TW: Tracheal washes, BAL: Bronchoalveolar lavages.

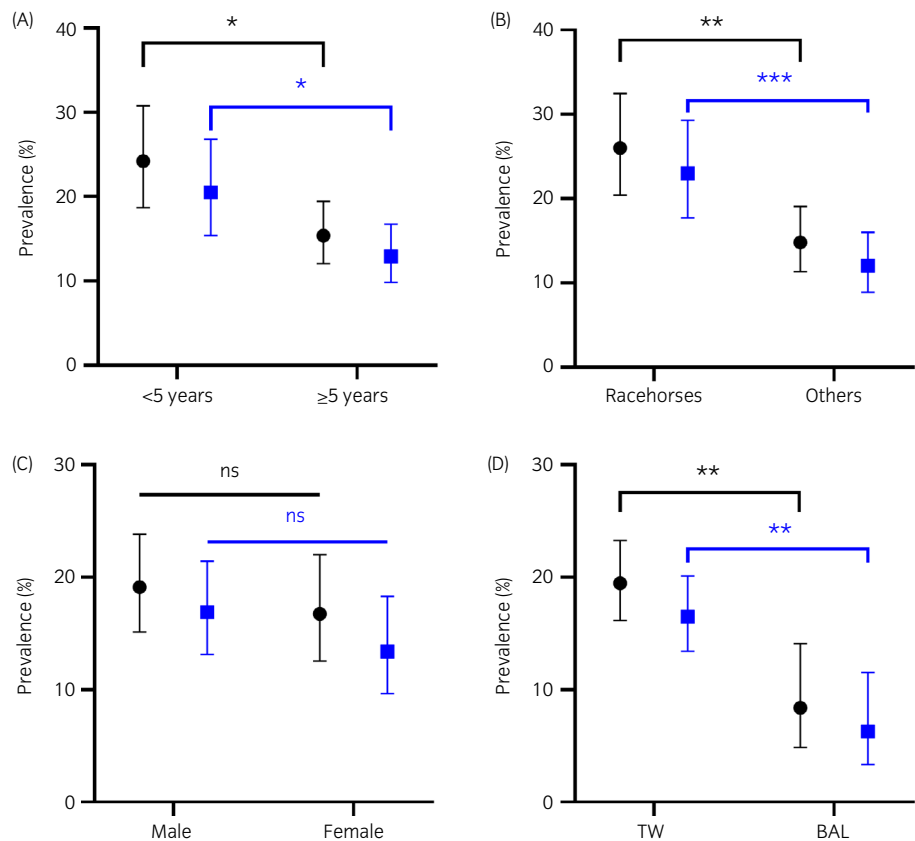


TABLE 3 Multivariable analysis of factors associated with the identification of *Mycoplasma* spp. and of *M. equirhinis*.

	<i>Mycoplasma</i> spp.					<i>M. equirhinis</i>				
	Estimate coefficient	SE	OR	95% CI	<i>p</i>	Estimate coefficient	SE	OR	95% CI	<i>p</i>
Age										
<5 years	0.093	0.277	1.098	(0.635–1.885)	0.8	−0.003	0.298	0.996	(0.553–1.782)	0.9
≥5 years			1.000					1.000		
Breed										
Racehorses	0.559	0.273	1.750	(1.023–2.990)	0.04	0.628	0.293	1.874	(1.053–3.335)	0.03
Others			1.000					1.000		
Sample type										
TW	0.964	0.341	2.623	(1.397–5.385)	0.005	1.097	0.390	2.996	(1.473–6.941)	0.005
BAL			1.000					1.000		

Abbreviations: SE, standard error; OR, odds ratio; 95% CI, 95% confidence interval; P, *p*-value.

diagnostic screening approach based on both culture and duplex rt-PCR after pre-enrichment. The rt-PCR assay, targeting a specific region of *Mycoplasma* spp. 16S rRNA gene, has been described previously²⁵ and is an adaptation of an older end-point protocol.²³ Here we ran a detailed analysis of its specificity, sensitivity, and Ct cut-off for interpretation on three target *Mycoplasma* species already described in horses, that is., *M. equirhinis*, *M. pulmonis*, and *M. felis*.^{14,16–18} This genus-specific rt-PCR assay was run in duplex with an *M. felis*-specific rt-PCR assay targeting the *tuf* gene adapted from previous work.²⁴ By extending the probe by four bases in the 3'

extremity, we were able to modify the acceptable cut-off for positivity to 37 in our experimental setup versus 35 in Söderlund et al.²⁴ Pre-enrichment for 4 days in a *Mycoplasma* growth culture medium of spiked TW before DNA extraction and rt-PCR was shown to improve the sensitivity of the method by a factor of four for *M. equirhinis*, although the effect was more limited for *M. felis* and *M. pulmonis* (due to different growth capacity in our broth medium, data not shown). Note, that this pre-enrichment step hinders the quantitative dimension of the rt-PCR and prevents an estimation of the initial mycoplasma load in the clinical samples. In the routine workflow at LABÉO,

the enrichment time before DNA extraction is not controlled and can vary from 3 to 7 days due to weekend breaks. We showed in our validation procedure that a mean of 4 days of enrichment makes it possible to detect even minute amounts of mycoplasmas, but its benefit is not equivalent for all species. Indeed, overall analytical sensitivity of the method remains lower for *M. pulmonis*. Culture-based detection was shown to be sensitive, although a little bit less than rt-PCR post-enrichment. Given the low positivity rate for *M. felis*, the association of *Mycoplasma* spp. PCR with *M. felis* PCR should be removed as a first-line testing strategy in this epidemiological context. A duplex PCR including *M. equirhinis* detection would be a relevant alternative.

The performance of the method was further evaluated on a set of 616 clinical samples, either TW or BAL, collected on horses with respiratory disorders in France in 2020. Of these 616 samples, 104 tested positive by rt-PCR ($n = 98$) and/or culture ($n = 47$), of which only 41 tested positive by both rt-PCR and culture, thus illustrating the methods were complementary. However, only six samples were detected as positive by culture but not rt-PCR, of which five were *M. equirhinis*, due to its lower sensitivity, as already stated.^{17,30} Non-viable mycoplasmas in the clinical samples can partly explain this difference in detection between culture-based versus rtPCR based assays. Culture-based detection proved time-consuming but provided access to isolated strains, which could be useful for further characterisation.

We then managed to identify 86% of the rt-PCR-positive samples and 100% of the isolated strains down to species level. Consistent with two previous studies,^{17,18} the commonest species detected was *M. equirhinis*, representing 98% of the isolated strains and 85% of the rt-PCR-positive DNA. The proportion of the two non-horse-specific species was very low, with only 4 positive DNAs and one isolated strain for *M. felis* and one positive DNA for *M. pulmonis*. This low proportion of *M. felis* might accurately reflect the prevalence in clinical samples, but we cannot rule out a bias due to the weaker sensitivity of our method for *M. pulmonis*. Another hypothesis is that *M. pulmonis* very rarely colonises the lower respiratory tract, as it has not been recovered in recent studies.^{16–18,22} As *M. pulmonis* is essentially a rodent pathogen, it may get transmitted to horses through sharing the same habitat and thus be isolated from horse nasal cavities, but not deeper in the respiratory tract.

Thirteen rt-PCR-positives samples were not identified by sequencing. These failures might be due to i) non-specific amplification, a well-known phenomenon with the 16 S rRNA gene targets, or ii) the presence of different mycoplasma species in the DNA extract. This second hypothesis is supported by both the situation in other animal hosts where species mixtures are frequent³¹ and by the fact that in one sample we evidenced both *M. felis* and *M. equirhinis*.

Our results showed a significantly higher prevalence of *Mycoplasma* spp. or *M. equirhinis* in Thoroughbreds and trotters than in other breeds, and in horses under 5 years of age than in older horses but the latter association was not confirmed in multivariate analysis. The majority (71.6%) of the young horses (<5 years) were Thoroughbreds and trotters and breed was retained in the multivariable model. Several hypotheses can be put forward to explain this higher

prevalence in Thoroughbreds and trotters, most of which are likely to be racehorses: 1) racehorses attract close scrutiny for health issues, 2) racehorses frequently contact with horses from other locations during race meets, 3) repeated strenuous exercise and frequent transport might impact the immune system and increase susceptibility, and 4) heavy breathing during strenuous exercise might enhance mycoplasma colonisation of the lower respiratory tract.

Interestingly, the prevalence of *Mycoplasma* spp. and *M. equirhinis* was higher in TW than in BAL samples. TW is recommended by some clinicians when infectious disease is suspected as it gives a more complete picture of the whole lung since secretions accumulate in the trachea. Conversely, BAL is often preferred for diagnosis of non-infectious lower airway inflammation.³² However, the choice of the most suitable sample type remains a matter of clinician preference and, to further understand the impact of sample technique on *Mycoplasma* spp. detection would require parallel tests on the two types of samples, as has been done for other purposes.³²

Now that we have optimised the methods for detection, future studies should aim to decipher the actual clinical role of mycoplasmas and more specifically the prevalent *M. equirhinis* species in equine respiratory infections. This could be done through systematic detection of mycoplasmas in horses with no clinical signs versus horses with respiratory disorders. It would also be helpful to gain further insight into the genomic diversity of *M. equirhinis* strains currently in circulation, as there is still too little genomic data available.

AUTHOR CONTRIBUTIONS

Matthieu Martineau contributed to study design, laboratory analyses, data analysis and interpretation, and preparation of the manuscript. Sophie Castagnet contributed to study design and laboratory analyses. Elena Kokabi and Agnès Tricot contributed to laboratory analyses. Maryne Jaÿ contributed to laboratory analyses, data analysis, and preparation of the manuscript. Albertine Léon and Florence Tardy contributed to study design, data interpretation, and preparation of the manuscript. All authors approved the final manuscript.

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CONFLICT OF INTEREST

None of the authors of this paper has a financial or personal relationship with other people or organisations that could inappropriately influence or bias the content of the paper. No competing interests have been declared.

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The peer review history for this article is available at <https://publons.com/publon/10.1111/evj.13918>.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ETHICS STATEMENT

Research ethics committee oversight not required: retrospective analysis of clinical specimens.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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