



**HAL**  
open science

## Epidemiology and pathogenicity of *M. equirhinis* in equine respiratory disorders

Matthieu Martineau, Elena Kokabi, Anis Taiebi, Stéphanie Lefebvre, Sophie Pradier, Maryne Jaÿ, Florence Tardy, Albertine Leon

► **To cite this version:**

Matthieu Martineau, Elena Kokabi, Anis Taiebi, Stéphanie Lefebvre, Sophie Pradier, et al.. Epidemiology and pathogenicity of *M. equirhinis* in equine respiratory disorders. *Veterinary Microbiology*, 2023, 287, pp.109926. 10.1016/j.vetmic.2023.109926 . hal-04314716

**HAL Id: hal-04314716**

**<https://hal.science/hal-04314716>**

Submitted on 29 May 2024

**HAL** is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



Distributed under a Creative Commons Attribution - NonCommercial - NoDerivatives 4.0 International License



## Epidemiology and pathogenicity of *M. equirhinis* in equine respiratory disorders

Matthieu Martineau<sup>a,b,c</sup>, Elena Kokabi<sup>a,b</sup>, Anis Taiebi<sup>a,b</sup>, Stéphanie Lefebvre<sup>c</sup>, Sophie Pradier<sup>d</sup>, Maryne Jaÿ<sup>c</sup>, Florence Tardy<sup>c,1,2,\*</sup>, Albertine Leon<sup>a,b,1,3,\*\*</sup>

<sup>a</sup> LABÉO, Research Department, Saint-Contest, 14000 Caen, France

<sup>b</sup> Univ of Caen Normandie, Univ Rouen Normandie, INSERM, DYNAMICURE UMR 1311, F-14000 Caen, France

<sup>c</sup> University of Lyon, Anses, VetAgro Sup, UMR Animal mycoplasmosis, 69007 Lyon, France

<sup>d</sup> Jolimont Veterinary Clinic, 31000 Toulouse, France

### ARTICLE INFO

**Keywords:**  
Mycoplasma  
Horses  
Clinical scoring  
Respiratory tract

### ABSTRACT

Mycoplasmas are pathogens involved in respiratory disorders of various animal hosts. In horses, *Mycoplasma* (*M.*) *equirhinis* is the species most frequently detected in clinical respiratory specimens, with a prevalence of 12–16%, but its clinical implication in equine respiratory disorders remains unclear. Here we screened 1948 clinical specimens for the presence of *M. equirhinis*. The samples were both tracheal washes (TW) and bronchoalveolar lavages (BAL) collected by veterinarians in France in day-to-day work between 2020 and 2022. The samples were associated with a standardized form that served to collect key general and clinical information, such as horse age, breed, and living environment. *M. equirhinis* was detected using a combination of culture and post-enrichment PCR. Other diagnostic data included virology and bacteriology as well as neutrophil counts, when available. Prevalence of *M. equirhinis* was examined as a function of a clinical score based on four significant clinical signs (nasal discharge, cough, dyspnoea, and hyperthermia). Multivariate logistic regression analysis was run to identify risk factors for the presence of *M. equirhinis*, and comparative prevalence analysis was used to test for association with other bacteria and viruses. TW and BAL were analysed independently, as we found that TW samples were associated with a higher prevalence of *M. equirhinis*. As prevalence remained steady whatever the clinical score, *M. equirhinis* cannot be considered a primary pathogen. *M. equirhinis* was more frequently isolated in thoroughbreds and trotters and in horses living exclusively stabled compared to other horses or other living environments. *M. equirhinis* was never detected in BAL specimens with a 'normal' neutrophil count, i.e. 5%, suggesting it could be associated with an inflammatory response, similar to that observed in equine asthma. Prevalence of *M. equirhinis* was shown to increase in the presence of other bacteria such as *Streptococcus equi* subsp. *zoepidemicus* (*S. zoo*) or viruses, and *S. zoo* load was higher in *M. equirhinis*-positive samples, suggesting a potential increase of clinical signs in the event of co-infection.

### 1. Introduction

The *Mycoplasma* genus encompasses more than 120 species that are found across various hosts. Some of these species are associated with major infections in humans as well as in livestock and domestic animals

(Brown et al., 2018). Mycoplasmas are significant agents in major respiratory infections such as children's pneumonia due to *M. pneumoniae* or contagious bovine pleuropneumonia caused by *M. mycoides* subsp. *mycoides* (Kudirkiene et al., 2021; Lesnoff et al., 2004). The first report of *Mycoplasma* spp. in the respiratory tract of horses dates back to 1975

**Abbreviations:** BAL, bronchoalveolar lavage; *M.*, *Mycoplasma*; TW, tracheal wash.

\* Corresponding author.

\*\* Corresponding author at: LABÉO, Research Department, Saint-Contest, 14000 Caen, France.

**E-mail addresses:** [Florence.tardy@anses.fr](mailto:Florence.tardy@anses.fr) (F. Tardy), [albertine.leon@laboratoire-labeo.fr](mailto:albertine.leon@laboratoire-labeo.fr) (A. Leon).

<sup>1</sup> Contributed equally to this work.

<sup>2</sup> **Present address of Florence TARDY:** Anses, Laboratoire de Ploufragan – Mycoplasma, Bacteriology and Antibioresistance Unit, BP53, 31 rue des Fusillés, 22440 Ploufragan, France

<sup>3</sup> **Permanent address of Albertine LEON:** LABÉO, 1 route de Rosel, Saint-Contest, 14053 Caen CEDEX 04, France

<https://doi.org/10.1016/j.vetmic.2023.109926>

Received 26 July 2023; Received in revised form 16 October 2023; Accepted 20 November 2023

Available online 22 November 2023

0378-1135/© 2023 The Authors. Published by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

(Allam and Lemcke, 1975), but as mycoplasmas were isolated in both healthy and diseased animals, they did not attract further attention as a focus of diagnosis or research. Several old studies suggested that *M. felis* might be more systematically linked to pleuritis in horses (Ogilvie et al., 1983; Hoffman et al., 1992; Morley et al., 1996) but its prevalence in clinical samples was shown to be very low (0.6%, Martineau et al., 2023). In contrast, recently that *M. equirhinis* has been described as the most frequent *Mycoplasma* species identified in the equine respiratory tract, with a prevalence of 12.3% in the UK, 16.2% in Turkey, and 13.6% in Japan (Mete and Özgür, 2017; Uchida-Fujii et al., 2021; Wood et al., 2005). We recently validated an optimized methodology for the detection of mycoplasmas in equine clinical specimens based on both culture and post-enrichment PCR, and we thus established a prevalence of *M. equirhinis* of 14.1% in France (Martineau et al., 2023). The fact that these non-negligible prevalence values were similar across these four studies, despite different methodologies and different study populations (both in terms of size and quality) shows the need to better understand the role of *M. equirhinis* in equine respiratory disorders.

According to the revisited Henle-Koch postulates (Evans, 1976), the causal role of *M. equirhinis* in equine respiratory disorders is ambiguous, as the bacterium was isolated from both healthy and diseased animals (Allam and Lemcke, 1975) and it failed to reproduce the disease in Welsh ponies experimentally infected by nasal route (Hooker and Butler, 1977). Even if the experimental assay in ponies in the 1970s suffered from several drawbacks such as inoculation route and size of the lots, it would not be ethically acceptable today to re-run another challenge study. Over the years, there have been several studies comparing *M. equirhinis* prevalence in diseased versus healthy animals, but most of them were limited in size, with a maximum of 148 individuals tested (Mete and Özgür, 2017; Uchida-Fujii et al., 2021; Wood et al., 2005).

The present study was set up in an effort to better specify the causal role of *M. equirhinis* in equine respiratory disorders using three different approaches. First, we defined a clinical score taking into account several clinical signs routinely registered by veterinarians. A similar approach has already been developed to investigate the role of mycoplasmas in canine infectious respiratory disease (Maboni et al., 2019). Second, building on our previous study that suggested that the prevalence of *M. equirhinis* could vary with type of respiratory specimen and with breed of horse sampled (Martineau et al., 2023), we further explored several variables in an effort to discriminate methodological bias from risk factors for *M. equirhinis* detection. Third, we investigated whether *M. equirhinis* was associated with other microbes, as some equine respiratory diseases are known to stem from co-infections involving multiple agents, bacteria and/or viruses (Reuss and Giguère, 2015).

## 2. Material and methods

### 2.1. Study population and samples

A total of 1948 respiratory samples gathered from 1764 horses were included in the study. The samples were collected between the beginning of January 2020 and the end of December 2022. Most of the samples were collected via the routine diagnostic workflow at LABÉO (n = 1844), with the remainder (n = 104) collected from mares bred at the Jumenterie du Pin (Gouffern-en-Auge, France) that were sampled to increase the number of non- to mildly affected horses included in the study. The samples consisted of tracheal washes/aspirations (TW, n = 1409, 72%) and bronchoalveolar lavages (BAL, n = 539, 28%). Since the rationale for taking samples in the field as well as the nature of these samples (BAL versus TW) depend on the subjective evaluation of the veterinarians we did not sort a priori the samples included in the study in terms of suspicion of infection, inflammation or loss of performance. Samples collected on the same horse within a six-month period were considered as duplicates and only the first sample was taken into account, with one exception, i.e. TW and BAL sampled on a same horse on the same day (n = 123 horses) were both included in the analysis for

purposes of comparison.

### 2.2. Data collection

Data on horse age, breed, sex, use, living environment and region, and clinical signs, and data on sample type (TW vs BAL), time of sampling and shipping temperature were recovered from preformatted record sheets filled in by veterinarians. By default, the sheet recorded four age classes (<6 months,]6–18 months],]18 months- 5 years [, ≥5 years), three sex classes (male, gelding, or female), three living-environment classes (stable, outdoor, or mixed), and two sample shipping-temperature classes (ambient or refrigerated). The clinical signs recorded were spontaneous cough, coughing during TW, epistaxis, tracheal blood, tracheal mucus, dyspnoea, abnormal breath sounds, hyperthermia, and nasal discharge.

Data regarding the detection of microorganisms other than *Mycoplasma* spp. and cytological examinations was recovered from the LABÉO results database, which is filled in regularly as part of the diagnostic workflow in response to veterinarian requests for analyses on respiratory samples. In brief, bacteria were isolated and quantified on appropriate agar media and identified by MALDI-TOF mass spectrometry (Léon et al. 2020), except for *Rhodococcus equi* for which a specific real-time PCR protocol was used (Rodríguez-Lázaro et al., 2006). If more than three bacterial species were detected (excluding *Mycoplasma* spp.), the sample was classified as polymicrobial and the strains present were not identified. Screening for equine respiratory viruses, whenever requested by veterinarians, was performed by real-time PCR (Doubli-Bounoua et al., 2016), and targeted the following viruses: equine herpes virus (EHV) – 1, 2, 4 and 5, equine arteritis virus (EAV), equine rhinitis virus (ERV) –A and –B, equine adenovirus (EAdV) – 1 and – 2, and equine influenza virus (EIV). Cytological analyses were performed by differential leukocyte count as previously described (Orard et al., 2016).

### 2.3. Detection and identification of *Mycoplasma* spp

Full details of the method used to detect and identify *Mycoplasma* spp. from equine TW and BAL samples can be found in Martineau et al. (2023). Briefly, two 200 µL samples were seeded into 1.8 mL of mycoplasma culture broth (INDICIA) and incubated at 37 °C with 5% CO<sub>2</sub> for *Mycoplasma* spp. enrichment during 3–7 days. One broth was used for DNA extraction and real-time PCR to detect *Mycoplasma* spp. and *M. felis*, and the other was used for daily monitoring of mycoplasma growth and further seeding onto agar plates to isolate colonies. Isolates and *Mycoplasma* spp.-positive DNA extracts were identified by endpoint PCRs (*M. equirhinis* or *M. pulmonis*), or if these two PCRs tested negative, by Sanger sequencing of their 16S rRNA.

### 2.4. Data analysis

The raw collected data was further formatted for purposes of analysis. Sex, age, and breed were reassembled into two categories each (male and female, age <5 years and ≥5 years, racehorses and others), as previously described (Martineau et al., 2023). Use of the horses was categorized into two modalities: athletes (e.g. for racing, dressage, training) and non-athletes (e.g. retired, club riding, breeding). The sampling period was split into two semesters. Shipping time of samples was stratified into four classes: [6–12 h],]12–24 h],]24–48 h], and > 48 h.

A cumulative clinical score was calculated for sampled horses with details on nasal discharge, cough, dyspnoea and hyperthermia, which are described as significant clinical signs for respiratory disorders (Couetil et al., 2020; Pusterla et al., 2022; Reuss and Giguère, 2015). These signs were weighted as follows: nasal discharge (scored as 0 if none, or 1 if present), cough (0 or 2), dyspnoea (0 or 3), and hyperthermia (0 or 4). Samples with neutrophil counts > 5% for BAL and >

20% for TW were considered abnormal (Rossi et al., 2018).

## 2.5. Statistical analysis

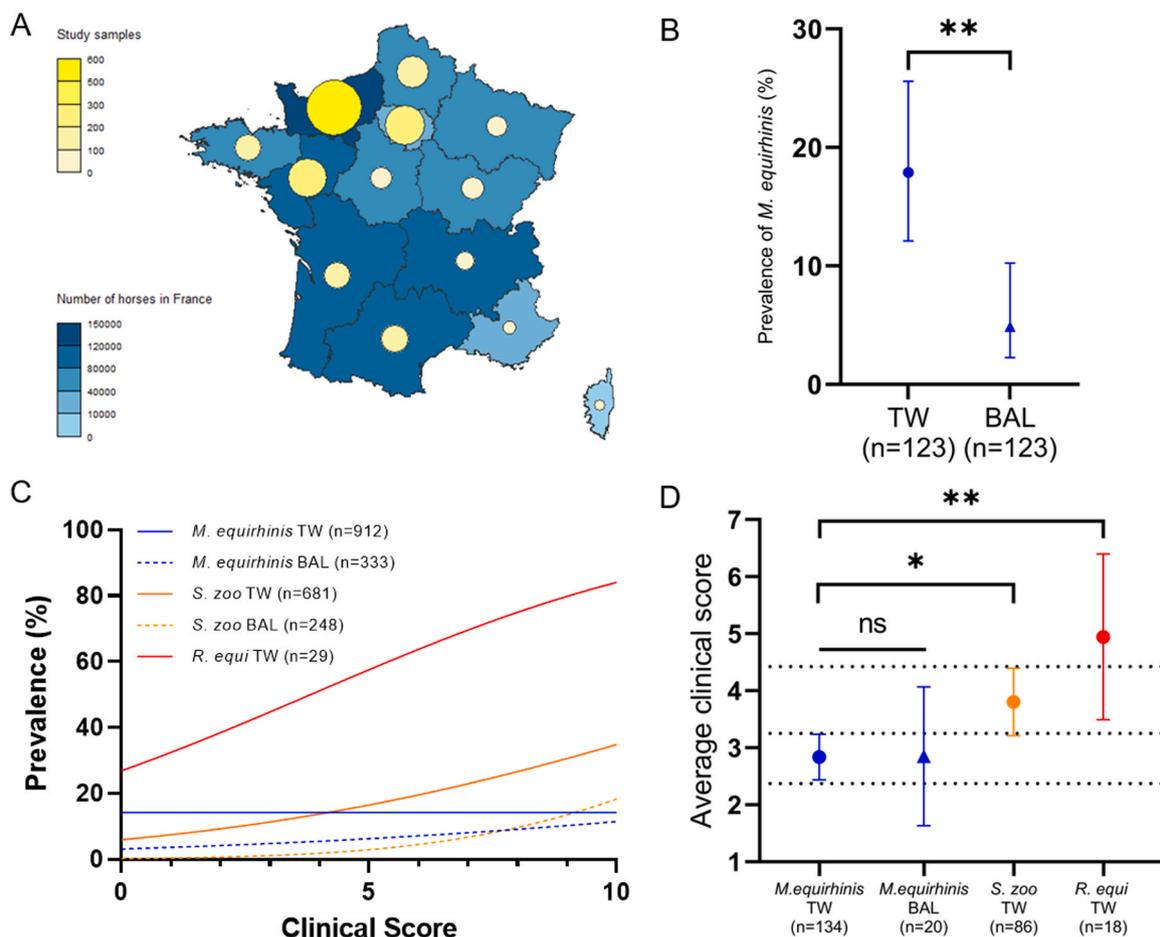
Statistical analyses were performed using RStudio (RStudio, Inc, Boston, MA). Graphed output was produced using GraphPad Prism 9.1.12 (GraphPad, San Diego, CA). A Chi-square test ( $n > 5$ ) or Fisher's exact test ( $n \leq 5$ ) was used to test the independence of a pair of categorical variables. Quantitative variables with a non-normal distribution (tested with Shapiro-Wilk) were tested using the non-parametric Mann-Whitney test. A  $P$ -value  $< 0.05$  was considered significant. A multivariate logistic regression model was built to assess the predictors for presence of *M. equirhinis*. The explanatory variables to be included in the final model were selected using the individual  $P$ -values of several simple logistic regression models. Only significant terms, i.e. individual  $P$ -value less than 0.15 in classical logistic regression (Sun et al., 1996), were retained for the multivariate model. This cut-off was chosen to avoid adding too many variables in the model, which would thus create a final multivariate model with too few individuals, as many horses had missing data. The reference modality was attributed to the majority population (sampling year/semester, population, breed, sex, age, use, living environment, specimen, and shipping temperature) or the best scenario (transport time, clinical score, and neutrophil count). If no *M. equirhinis* was detected in a modality, then Firth's penalised logistic regression was used (Firth, 1993). Odds ratios (OR) and their

corresponding 95% confidence intervals (CI) were calculated for each variable. We used a manual backward stepwise process to construct the final multivariate models, with significance set at of  $P < 0.05$ . Confounding factors and interactions were tested by observing the effect of removing variables on the coefficients of remaining variables. If the coefficient of a significant variable in the model changed by at least 25% following removal of a variable, the removed variable was considered a confounder. Variables suspected of interacting were tested for two-way interactions and, if found significant, were included in the final multivariate model ( $P < 0.05$ ). All the final multivariate models were tested for goodness of fit using the Hosmer-Lemeshow test (Hosmer and Lemeshow, 2000).

## 3. Results

### 3.1. Population analysis

The 1948 clinical samples collected from 1764 horses included in this study originated from different administrative regions of France (30–510 samples per region). The region with the most numerous samples was Normandy, which is also the most horse-populated region of France. However, number of samples per region did not always correlate with equine population per region (Fig. 1A). Note that three samples were collected in the French overseas territories. The overall prevalence of *Mycoplasma* spp. was 16.1% (ranging from 6.6% to 19.2%



**Fig. 1.** Geographic origin of collected samples and comparative prevalence of *M. equirhinis*. (A) Regional distribution of the equine population and samples collected for this study in mainland France. (B) Prevalence of *M. equirhinis* in TW and BAL collected on the same horse, on the same day ( $n = 123$ ). Error bars correspond to 95% confidence intervals. “\*\*”:  $P \leq 0.01$  after a Chi-square test. (C) Prevalence of *M. equirhinis*, *Streptococcus equi* subsp. *zooepidemicus* (*S. zoo*) and *Rhodococcus equi* (*R. equi*) according to the clinical score given to each horse, plotted as a cumulative Gaussian distribution; n, number of tested samples with a clinical score. (D) Average clinical score of horses in which at least one of the agents was detected (showing both TW and BAL samples for *M. equirhinis*). Error bars correspond to 95% confidence intervals. “ns”: not significant. “\*” and “\*\*”:  $P \leq 0.05$  and  $P \leq 0.01$ , respectively, after a Mann-Whitney test.

depending on the region), of which 85.3% were *M. equirhinis*. In the overall population, the prevalence of *M. equirhinis* was significantly different (Chi-square test,  $P < 0.0001$ ) between TW (16.0%) and BAL (7.6%). This difference was confirmed on a subset of horses ( $n = 123$ ) that were sampled at the same time by both TW (17.9%) and BAL (4.8%) (Fig. 1B,  $P = 0.0013$ ).

### 3.2. Clinical scoring

The cumulative Gaussian distribution of the prevalence of *M. equirhinis* as a function of clinical score was compared to that of a primary pathogen [*Rhodococcus equi* (*R. equi*)] of young foals and an opportunist pathogen [*Streptococcus equi* subsp. *zooepidemicus* (*S. zoo*)] (Fig. 1C). For *R. equi*, as no BAL samples were positive, the prevalence curve only plots TW samples. *R. equi* prevalence increased linearly with clinical score, which is the expected pattern for a strict pathogen. The sub-population of clinical specimens, with a clinical score, tested for *R. equi* is small-sized ( $n = 29$  in Fig. 1C) and includes a majority of animals under the age of 18 months, for which *R. equi* can be considered as a pathogen. For *S. zoo* and *M. equirhinis*, prevalence in TW and prevalence in BAL were examined independently. For *S. zoo*, there was a less significant increase compared to *R. equi* in prevalence with clinical score, with TW and BAL curves running almost parallel. For *M. equirhinis*, prevalence was steady (TW) or almost steady (BAL) across the clinical scores, suggesting a non-pathogenic pattern. Mean clinical scores were significantly lower for samples containing *M. equirhinis* than samples containing *S. zoo* or *R. equi* (Fig. 1D). Once again, in the case of samples positive for *R. equi*, the small sub-population ( $n = 18$ ) observed corresponds mainly to young horses. These results suggest that *M. equirhinis* may be considered commensal or opportunist, but not a pathogen.

**Table 1**

Prevalence and univariate analysis of *M. equirhinis* detection by the sampled horse's demographic status. Only variables with  $P < 0.15$  for at least one of the sample types are shown. N = number of samples by modality, n = number of *M. equirhinis*-positive samples, % = prevalence of *M. equirhinis*, OR = odds-ratio, 95% CI = 95% confidence interval 95%,  $P = P$ -value. NA: not available; TW: tracheal wash. BAL: bronchoalveolar lavage.

	TW					BAL				
	N	n	%	OR (CI 95%)	P	N	n	%	OR (CI 95%)	P
All	1409	226	16.0%			539	41	7.6%		
Age										
NA	139					63				
≥ 5 years	897	130	14.5%	1.000		379	22	5.8%	1.000	
< 5 years	373	75	20.1%	<b>1.485 (1.081–2.028)</b>	<b>0.014</b>	97	15	15.5%	<b>2.968 (1.451–5.932)</b>	<b>0.002</b>
Breed										
NA	229					94				
Others	806	124	15.4%	1.000		327	18	5.5%	1.000	
Racehorses	374	75	20.1%	<b>1.380 (1.001–1.890)</b>	<b>0.047</b>	118	18	15.3%	<b>3.090 (1.541–6.201)</b>	<b>0.001</b>
Living environment										
NA	618					246				
Mixed	407	57	14.0%	1.000		174	11	6.3%	1.000	
Outdoor	202	18	8.9%	<b>0.601 (0.335–1.031)</b>	<b>0.074</b>	59	2	3.4%	0.512 (0.079–2.012)	0.404
Stable	182	40	22.0%	<b>1.730 (1.099–2.703)</b>	<b>0.017</b>	60	3	5.0%	0.780 (0.172–2.603)	0.710
Neutrophil (%)										
NA						106				
Normal	71	8	11.3%	1.000		51	0	0.0%	1.000	
Abnormal	604	103	17.1%	1.644 (0.809–3.810)	0.202	382	32	8.4%	<b>9.550 (1.313–1.216E+03)</b>	<b>0.019</b>
Semester										
Spring and Summer	826	116	14.0%	1.000		332	27	8.1%	1.000	
Autumn and Winter	583	110	18.9%	<b>1.423 (1.069–1.894)</b>	<b>0.015</b>	207	14	6.8%	0.819 (0.408–1.577)	0.560
Shipping time										
NA	17					42				
[6–12 h]	163	23	14.1%	1.000		73	7	10%	1.000	
[12–24 h]	772	131	17.0%	1.244 (0.784–2.052)	0.372	249	20	8%	0.792 (0.341–2.030)	0.609
[24–48 h]	305	46	15.1%	1.081 (0.635–1.883)	0.778	101	8	8%	0.806 (0.281–2.321)	0.683
> 48 h	152	23	15.1%	1.085 (0.579–2.035)	0.798	74	0	0%	<b>0.059 (0.0004–0.504)</b>	<b>5.23E-03</b>
Usage										
NA	470					196				
Non-Athlete	512	58	11.3%	1.000		165	7	4.2%	1.000	
Athlete	427	76	17.8%	<b>1.695 (1.174–2.459)</b>	<b>0.005</b>	178	14	7.9%	1.927 (0.780–5.200)	0.168

### 3.3. Statistical modelling

Given the significant difference in proportion of *M. equirhinis* between TW and BAL collected on the same horse at the same time point (Fig. 1B), we ran the risk factor analysis separately on the 1409 TW specimens and the 539 BAL specimens. Categories of predictors containing less than 10% of the population were reassembled, except when analysing the potential impact of origin of samples (LABÉO diagnostic workflow versus 'Jumenterie du Pin'). As the prevalence of *M. equirhinis* did not vary significantly with origin of samples or year of sampling (SD1), we chose to combine these subgroups in order to work on a single population. As the prevalence of *M. equirhinis* showed no change with clinical scores (Figure 1B), we classified these scores into five classes for TW and six classes for BAL (SD1).

In TW samples, age, breed, use, living environment, and semester of collection were significant explanatory variables in the univariate models ( $P < 0.15$ ) (Table 1) and were therefore included in the TW multivariate model (Table 2).

As *M. equirhinis* was not detected in normal-neutrophil-count BAL samples, we used a penalised regression to run an independent analysis on the 'neutrophil count' variable, which showed a significant difference between the two classes (Table 1). *M. equirhinis* was also not detected in samples that took more than 48 h to arrive at the lab for analysis. However, we found a similar prevalence between the two other time-in-transport modalities, showing no linearity between transport time and capacity of detection. Hence, age and breed were the only variables significant enough ( $P < 0.15$ ) to be included in the BAL multivariate model (Table 2).

Due to missing data for some horses, the two multivariate models had 649 TW samples and 439 BAL samples (Table 2). The final multivariate models showed that the significant risk factors for presence of *M. equirhinis* in TW were breed ( $P = 0.049$ ) and living environment

**Table 2**

Multivariate model for TW and BAL samples. EC = estimate coefficient, SE = standard error, OR = odds ratio, 95% CI = 95% confidence interval, P = P-value.

TW (n = 649)					
	EC	SE	OR	CI 95%	P
Intercept	-2.537	0.308	0.079	(0.041–0.138)	<b>2.000E-16</b>
Breed			1.000		
Others			1.000		
Racehorses	0.510	0.259	<b>1.666</b>	<b>(0.993–2.750)</b>	<b>0.049</b>
Living environment			1.000		
Mixed			1.000		
Outdoor	-0.479	0.344	0.620	(0.303–1.180)	0.164
Stable	0.727	0.260	<b>2.068</b>	<b>(1.240–3.440)</b>	<b>0.005</b>
BAL (n = 246)					
	EC	SE	OR	CI 95%	P
Intercept	-3.056	0.273	0.047	(4.140E-05–0.042)	<b>1.776E-15</b>
Age			1.000		
≥ 5 years			1.000		
< 5 years	0.580	0.475	1.786	(0.690–4.481)	0.222
Breed			1.000		
Others			1.000		
Racehorses	1.049	0.456	<b>2.855</b>	<b>(1.151–6.937)</b>	<b>0.021</b>

**SUPPLEMENTARY DATA:**

**SD1:** Prevalence of *M. equirhinis* infection stratified by type of sample (TW or BAL) and the sampled horse's demographic status.

**SD2:** Microorganisms found in TW and BAL samples and association with *M. equirhinis* in TW only. P-values were obtained after a Chi-square or Fisher's exact test according to number of samples analysed. NA: not available.

(P = 0.005 for in-stable), whereas only breed was found to be a significant risk factor in BAL (P = 0.021). Furthermore, the TW multivariate model obtained a good score on the Hosmer–Lemeshow test (P = 0.814), whereas the BAL model obtained a much poorer score (P = 0.431).

**3.4. Interaction between *M. equirhinis* and other agents**

In an effort to reveal any potential association between presence of other agents and presence of *M. equirhinis*, we analysed the presence of *M. equirhinis* as a function of presence of other agents found in TW samples only. Agents present in less than 10 TW were not included in the analysis, for statistical reasons. BAL specimens were ruled out because: 1) BAL is not the recommended respiratory specimen for direct detection

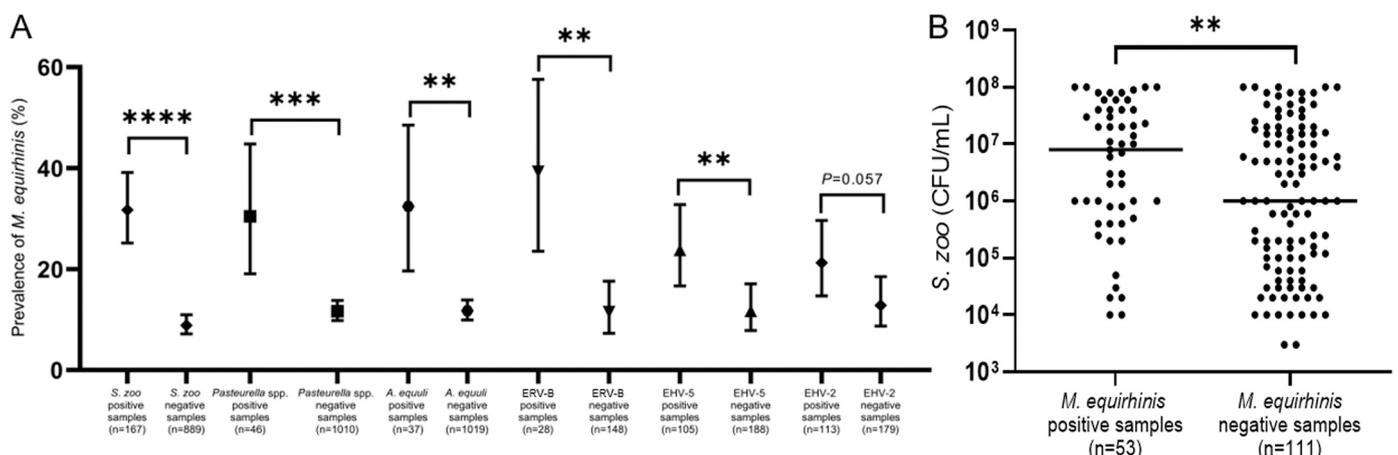
of bacteria, and was sampled less frequently (539 BAL versus 1409 TW) and 2) we had a consistently low rate of detection of other agents of whatever type (SD2). The prevalence of *M. equirhinis* was significantly higher when it was co-isolated with certain other bacteria, such as *S. zoo*, *Pasteurella* spp., or *Actinobacillus equuli*, and certain viruses such as EHV-5 and ERV-B (Fig. 2A), but not with other bacteria and viruses (SD2). Furthermore, by screening our dataset, we were able to show that *M. equirhinis*-positive clinical specimens tended to have a median one log higher *S. zoo* load compared to *M. equirhinis* negative samples, suggesting a positive association between these two bacteria (Fig. 2B).

**4. Discussion**

This study attempted to decipher the clinical role of *M. equirhinis* in equine respiratory disorders.

We first developed a clinical score based on the cumulative occurrence of four clinical signs (nasal discharge, cough, dyspnoea, and hyperthermia). Here we gave the highest weighting to hyperthermia, as an indicator of the infectious process, in contrast to previous studies focusing on inflammatory airway disease, which was recently renamed 'mild equine asthma' (Mete and Özgür, 2017; Wood et al., 2005). Because we had access to more than 1900 clinical specimens, we considered that the potential variability of scoring by several practitioners was smoothed out. However, we ruled out the signs that were too dependent on the veterinarian's subjective evaluation (i.e. presence of tracheal mucus, abnormal breath sounds, and underperformance). The fact that prevalence of *M. equirhinis* remained stable across clinical scores points strongly to a non-pathogenic bacteria, in comparison to other bacterial species with a known clinical role as opportunistic or primary pathogens, i.e. *S. zoo* and *R. equi* (Rodríguez-Lázaro et al., 2006; Waller, 2014). However, this result contradicts a previous study that hypothesised, based on a limited number of observations, that *M. equirhinis* may be implicated in coughs in thoroughbred horses (Uchida-Fujii et al., 2021).

The main limitation of our approach was that we did not design a specific study but instead benefited from available diagnostic laboratory data. First, we did not always have an exhaustive record of clinical signs per horse, which decreased the size of the 'usable' study population (from 1948 to 1245 clinical specimens), although the population was still very robust given the large size of our original database. Second, information on the onset of infection was missing at the time of sampling. Third, we did not include other exams, such as imaging of the deep airways, but those might not have been relevant for our study



**Fig. 2.** Association of *M. equirhinis* with other microbial agents. (A) Prevalence of *M. equirhinis* in TW samples in presence or absence of other specific agents, i.e. *Streptococcus equi* subsp. *zooeidemicus* (*S. zoo*), *Pasteurella* spp., *Actinobacillus equuli* (*A. equuli*), equine rhinitis virus-B (ERV-B) and equine herpes virus- 5 and - 2 (EHV-5 and -2). Error bars correspond to 95% confidence intervals. \*\*, \*\*, \*\*\*\* and \*\*\*\* refer to P ≤ 0.05, P ≤ 0.01, P ≤ 0.001 and P ≤ 0.0001, respectively, after a Chi-square test. (B) Concentration of *S. zooepidemicus* (*S. zoo*) in TW in presence or absence of *M. equirhinis*. Bars correspond to median values. \*\*\*\*: P ≤ 0.01 after a Mann-Whitney test. n: number.

population.

For all these reasons, and to strengthen our hypothesis that *M. equirhinis* acts as a commensal or opportunist agent in the respiratory tract, we investigated further risk factors and co-infection patterns. The screened risk factors were chosen based on previous studies, e.g. period of the year (Uchida-Fujii et al., 2021) or the living environment that might lead to increased exposure to infectious agents due to limited ventilation (Golden et al., 2000).

TW and BAL were analysed separately, as the prevalence of *M. equirhinis* was significantly different in these two types of samples taken from the same animals. Thoroughbreds and trotters were more frequently positive for *M. equirhinis* in both TW and BAL, confirming the findings of our initial study that discussed possible reasons for this increased susceptibility in detail (Martineau et al., 2023). In addition, indoor housing was shown to be associated with a higher risk of detecting *M. equirhinis* in TW samples. Such an association has already been described for equine influenza virus (Gildea et al., 2011). In the absence of canonical virulence factors, the pathogenicity of mycoplasmas is known to come from their ability to escape and modulate the host immune response (Citti and Blanchard, 2013). Note, here, that *M. equirhinis* was only found in BAL specimens with abnormal neutrophil counts, and never in BAL specimens with normal counts. This might explain the poor predictive power (low Hosmer–Lemeshow score) of the multivariate logistic regression model for BAL, from which neutrophil proportion was excluded. Neutrophil imbalance in BAL is one of the parameters used to define asthmatic conditions in horses (Couetil et al., 2020). A previous study also showed that *Mycoplasma* spp. were detected in an asthmatic horse but never in healthy ones, although based on only a limited number of individuals (Manguin et al., 2020). *M. equirhinis* could therefore be associated to asthma, i.e. an inflammatory disorder, as already suggested (Wood et al., 2005), but not to infections (results reported here). This hypothesis is also consistent with the increased prevalence of *M. equirhinis* in horses living indoor in a stable, which is an environment known to promote asthma (Couetil et al., 2016). However, at this stage, we cannot say whether *M. equirhinis* is the cause of this increase in neutrophils, as observed with *M. bovis* or *M. pneumoniae* infection (Allen et al., 1992; Martin et al., 2001), or whether the increase in neutrophils is induced by other agents or environmental sources that go on to facilitate *M. equirhinis* colonisation.

Our co-detection analyses found a significantly higher prevalence of *M. equirhinis* in samples that tested positive for *S. zoo*, *Pasteurella* or *Actinobacillus equuli*. Such a positive association has already been demonstrated at the genus level for *Actinobacillus* (Wood et al., 2005). Furthermore, the concentration of *S. zoo* was significantly higher in samples where *M. equirhinis* was co-detected. Our data did not allow us to conclude on the clinical significance of co-detection of these two species (data not shown), but it did suggest a positive association between the two species. Research on refractory children's pneumonia due to *M. pneumoniae* found that co-detection of *Streptococcus pneumoniae* was frequent and resulted in an increased duration of symptoms in carrier children (Zhang et al., 2018). Further research is warranted to investigate whether co-infection by mycoplasma and streptococci in horses should be considered a cause of disease severity or duration.

TW and BAL samples are not optimal clinical specimens for detecting coinfection with viruses, and nasopharyngeal swabs are usually preferred for viral detection. Nevertheless, here, in TW, we found a higher prevalence of *M. equirhinis* when EHV-5 or ERV-B were also detected. EHV-5 that is very commonly recovered from horses and has a controversial clinical role (Back et al., 2015), whereas ERV-B is associated with coughing in standard bred racehorses at training (Doubli-Bounoua et al., 2016). Mycoplasmas are regularly co-present with other microbes in respiratory disorders of other animal hosts, such as cattle (Grissett et al., 2015), pigs (Saade et al., 2020) and dogs (Maboni et al., 2019), but it remains unclear whether these co-infections could result in worsening clinical conditions in horses. Microbiota studies could help determine the contribution of *M. equirhinis* in what could be

called the 'equine respiratory disease complex', in comparison to what is known in other species, as suggested by Mach et al. (Mach et al., 2021).

## 5. Conclusions

*M. equirhinis* is more often detected in TW than in BAL specimens, but its detection rate is constant whatever the clinical score, pointing to a non-primary infectious agent. *M. equirhinis* could nonetheless play a part in the equine respiratory disease complex, in association with *S. equi* subsp. *zooepidemicus* and ERV-B. Since the presence of *M. equirhinis* is associated with abnormal neutrophil concentrations in BAL, it may act through dysregulation of the host immune response, as is well known in other respiratory complexes. Quantifying the *M. equirhinis* load at the infection site could help define its opportunistic nature, and meta-genomic data would offer a global picture of its contribution to the equine respiratory disease complex.

## Ethical animal research

CENOMEXA—the Normandy institutional animal care and use committee (CEEA-054) approved this study protocol under authorisation/agreement #APAFIS 2020041612126739 (#26697).

## Authorship

M. Martineau contributed to study design, laboratory analysis, data analysis and interpretation, and co-drafted the manuscript. S. Lefebvre, E. Kokabi and A. Taibi contributed to laboratory analyses. S. Pradier contributed to sampling and the development of clinical scoring for horses. M. Jay contributed to laboratory analyses and data analysis. F. Tardy and A. Léon contributed to study design and data interpretation, and co-drafted the manuscript. All authors read and approved the final submitted manuscript.

## Funding

This work received financial support from the IFCE (Institut Français du Cheval et de l'Équitation, grant number CS-2020–2023–022-MYCO-PAB), the Fonds Eperon (grant number N15–2020), and the GIS (Groupement d'Intérêt Scientifique) CENTAURE (grant number GIS20-SEP-01).

## Declaration of Competing 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.

## Acknowledgements

The authors thank Adélie Colin, Florine Dieu, and Agnès Tricot at the Anses laboratory in Lyon for assisting with strain management. We also thank Coralie Lupo for her support on the statistical analyses, and the LABÉO cytology, bacteriology, and molecular biology diagnostic units for their assistance on analysis of the studied samples.

## Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.vetmic.2023.109926](https://doi.org/10.1016/j.vetmic.2023.109926).

## References

- Allam, N.M., Lemcke, R.M., 1975. Mycoplasmas isolated from the respiratory tract of horses. *J. Hyg. Camb.* 74, 29. <https://doi.org/10.1017/s0022172400046908>.

- Allen, J.W., Viel, L., Bateman, K.G., Rosendal, S., Shewen, P.E., 1992. Cytological findings in bronchoalveolar lavage fluid from feedlot calves: associations with pulmonary microbial flora. *Can. J. Vet. Res.* 56, 122–126.
- Back, H., Ullman, K., Treiberg Berndtsson, L., Riihimäki, M., Penell, J., Ståhl, K., Valarcher, J.-F., Pringle, J., 2015. Viral load of equine herpesviruses 2 and 5 in nasal swabs of actively racing Standardbred trotters: Temporal relationship of shedding to clinical findings and poor performance. *Vet. Microbiol.* 179, 142–148. <https://doi.org/10.1016/j.vetmic.2015.06.002>.
- Brown, D.R., May, M., Bradbury, J.M., Balish, M.F., 2018. *Bergey's Manual of Systematics of Archaea and Bacteria*, 1st ed., Wiley, <https://doi.org/10.1002/9781118960608>.
- Citti, C., Blanchard, A., 2013. Mycoplasmas and their host: emerging and re-emerging minimal pathogens. *Trends Microbiol* 21, 196–203. <https://doi.org/10.1016/j.tim.2013.01.003>.
- Couetil, L., Cardwell, J.M., Leguillette, R., Mazan, M., Richard, E., Bienzle, D., Bullone, M., Gerber, V., Ivester, K., Lavoie, J.-P., Martin, J., Moran, G., Niedźwiedz, A., Pusterla, N., Swiderski, C., 2020. Equine asthma: Current understanding and future directions. *Front. Vet. Sci.* 7, 450 <https://doi.org/10.3389/fvets.2020.00450>.
- Couetil, L.L., Cardwell, J.M., Gerber, V., Lavoie, J.-P., Léguillette, R., Richard, E.A., 2016. Inflammatory airway disease of horses—Revised consensus statement. *J. Vet. Intern. Med.* 30, 503–515. <https://doi.org/10.1111/jvim.13824>.
- Doubli-Bounoua, N., Richard, E.A., Léon, A., Pitel, P.-H., Pronost, S., Fortier, G., 2016. Multiple molecular detection of respiratory viruses and associated signs of airway inflammation in racehorses. *Virology* 13, 197 <https://doi.org/10.1186/s12985-016-0657-5>.
- Evans, A.S., 1976. Causation and disease: The Henle-Koch postulates revisited. *Yale J. Biol. Med.* 49, 175–195.
- Firth, D., 1993. Bias reduction of maximum likelihood estimates. *Biometrika* 80, 27–38. <https://doi.org/10.1093/biomet/80.1.27>.
- Gildea, S., Arkins, S., Cullinane, A., 2011. Management and environmental factors involved in equine influenza outbreaks in Ireland 2007–2010. *Equine Vet. J.* 43, 608–617. <https://doi.org/10.1111/j.2042-3306.2010.00333.x>.
- Golden, V., Turner, L.W., Coleman, R.B., Gates, R.S., 2000. Equine response to environmental factors: a literature review and recommendations for design and environmental modification. ASAE.
- Grissett, G.P., White, B.J., Larson, R. I., 2015. Structured literature review of responses of cattle to viral and bacterial pathogens causing bovine respiratory disease complex. *J. Vet. Intern. Med.* 29, 770–780. <https://doi.org/10.1111/jvim.12597>.
- Hooker, J.M., Butler, M., 1977. The development of complement-fixing antibody in horses after infection with *Mycoplasma equirhinis*. *J. Comp. Pathol.* 87, 281–286. [https://doi.org/10.1016/0021-9975\(77\)90015-9](https://doi.org/10.1016/0021-9975(77)90015-9).
- Hoffman, A., Baird, J.D., Kloeze, H.J., Rosendal, S., Bell, M., 1992. *Mycoplasma felis* pleuritis in two show-jumper horses. *Cornell Vet.* 82, 155–162.
- Hosmer, D.W., Lemeshow, S., 2000. *Applied logistic regression*. Wiley Series in Probability and Statistics, 2nd ed., Wiley, New York.
- Kudirkienė, E., Aagaard, A.K., Schmidt, L.M.B., Pansri, P., Krogh, K.M., Olsen, J.E., 2021. Occurrence of major and minor pathogens in calves diagnosed with bovine respiratory disease. *Vet. Microbiol.* 259, 109135 <https://doi.org/10.1016/j.vetmic.2021.109135>.
- Léon, A., Castagnet, S., Maillard, K., Paillot, R., Giard, J.-C., 2020. Evolution of in vitro antimicrobial susceptibility of equine clinical isolates in France between 2016 and 2019. *Animals* 10, 812. <https://doi.org/10.3390/ani10050812>.
- Lesnoff, M., Laval, G., Bonnet, P., Abdicho, S., Workalemahu, A., Kifle, D., Peyraud, A., Lancelot, R., Thiaucourt, F., 2004. Within-herd spread of contagious bovine pleuropneumonia in Ethiopian highlands. *Prev. Vet. Med.* 64, 27–40. <https://doi.org/10.1016/j.prevetmed.2004.03.005>.
- Maboni, G., Seguel, M., Lorton, A., Berghaus, R., Sanchez, S., 2019. Canine infectious respiratory disease: New insights into the etiology and epidemiology of associated pathogens. *PLoS ONE* 14, e0215817. <https://doi.org/10.1371/journal.pone.0215817>.
- Mach, N., Baranowski, E., Nouvel, L.X., Citti, C., 2021. The airway pathobiome in complex respiratory diseases: a perspective in domestic animals. *Front. Cell. Infect. Microbiol.* 11, 583600 <https://doi.org/10.3389/fcimb.2021.583600>.
- Manguin, E., Pépin, E., Boivin, R., Leclere, M., 2020. Tracheal microbial populations in horses with moderate asthma. *J. Vet. Intern. Med.* 34, 986–995. <https://doi.org/10.1111/jvim.15707>.
- Martin, R.J., Chu, H.W., Honour, J.M., Harbeck, R.J., 2001. Airway inflammation and bronchial hyperresponsiveness after *Mycoplasma pneumoniae* infection in a murine model. *Am. J. Respir. Cell Mol. Biol.* 24, 577–582. <https://doi.org/10.1165/ajrcmb.24.5.4315>.
- Martineau, M., Castagnet, S., Kokabi, E., Tricot, A., Jaÿ, M., Léon, A., Tardy, F., 2023. Detection of *Mycoplasma* spp. in horses with respiratory disorders. *Equine Vet. J.* evj.13918. <https://doi.org/10.1111/evj.13918>.
- Mete, A., Özgür, N.Y., 2017. Investigation of the presence of *Mycoplasma* as an etiologic agent of inflammatory airway diseases in thoroughbred racehorses in Istanbul Province. *Turk. J. Vet. Anim. Sci.* 365–371. <https://doi.org/10.3906/vet-1606-90>.
- Morley, P.S., Chirino-Trejo, M., Petrie, L., Krupka, L., Schwab, M., 1996. Pericarditis and pleuritis caused by *Mycoplasma felis* in a horse. *Equine Vet. J.* 28, 237–240. <https://doi.org/10.1111/j.2042-3306.1996.tb03779.x>.
- Ogilvie, T.H., Rosendal, S., Blackwell, T.E., Rostkowski, C.M., Julian, R.J., Ruhnke, L., 1983. *Mycoplasma felis* as a cause of pleuritis in horses. *J. Am. Vet. Med. Assoc.* 182, 1374–1376.
- Orard, M., Depecker, M., Hue, E., Pitel, P.-H., Courouc-Malblanc, A., Richard, E.A., 2016. Influence of bronchoalveolar lavage volume on cytological profiles and subsequent diagnosis of inflammatory airway disease in horses. *Vet. J.* 207, 193–195. <https://doi.org/10.1016/j.tvjl.2015.09.027>.
- Pusterla, N., James, K., Barnum, S., Bain, F., Barnett, D.C., Chappell, D., Gaughan, E., Craig, B., Schneider, C., Vaala, W., 2022. Frequency of detection and prevalence factors associated with common respiratory pathogens in equids with acute onset of fever and/or respiratory signs (2008–2021). *Pathogens* 11, 759. <https://doi.org/10.3390/pathogens11070759>.
- Reuss, S.M., Giguère, S., 2015. Update on bacterial pneumonia and pleuropneumonia in the adult horse. *Vet. Clin. N. Am.* 31, 105–120. <https://doi.org/10.1016/j.cveq.2014.11.002>.
- Rodríguez-Lázaro, D., Lewis, D.A., Ocampo-Sosa, A.A., Fogarty, U., Makrai, L., Navas, J., Scotti, M., Hernández, M., Vázquez-Boland, J.A., 2006. Internally controlled real-time PCR method for quantitative species-specific detection and *vapA* genotyping of *Rhodococcus equi*. *Appl. Environ. Microbiol.* 72, 4256–4263. <https://doi.org/10.1128/AEM.02706-05>.
- Rossi, H., Viritala, A.-M., Raekallio, M., Rahkonen, E., Rajamäki, M.M., Mykkänen, A., 2018. Comparison of tracheal wash and bronchoalveolar lavage cytology in 154 horses with and without respiratory signs in a referral hospital over 2009–2015. *Front. Vet. Sci.* 5, 61. <https://doi.org/10.3389/fvets.2018.00061>.
- Saade, G., Deblanc, C., Bougon, J., Marois-Créhan, C., Fablet, C., Auray, G., Belloc, C., Leblanc-Maridor, M., Gagnon, C.A., Zhu, J., Gottschalk, M., Summerfield, A., Simon, G., Bertho, N., Meurens, F., 2020. Coinfections and their molecular consequences in the porcine respiratory tract. *Vet. Res.* 51, 80. <https://doi.org/10.1186/s13567-020-00807-8>.
- Sun, G.-W., Shook, T.L., Kay, G.L., 1996. Inappropriate use of bivariable analysis to screen risk factors for use in multivariable analysis. *J. Clin. Epidemiol.* 49, 907–916. [https://doi.org/10.1016/0895-4356\(96\)00025-X](https://doi.org/10.1016/0895-4356(96)00025-X).
- Uchida-Fujii, E., Kinoshita, Y., Niwa, H., Maeda, T., Nukada, T., Ueno, T., 2021. High prevalence of *Mycoplasma equirhinis* in thoroughbred horses with respiratory symptoms in autumn 2018. *J. Vet. Med. Sci.* 83, 1907–1912. <https://doi.org/10.1292/jvms.21-0163>.
- Waller, A.S., 2014. Equine respiratory disease: A causal role for *Streptococcus zooepidemicus*. *Vet. J.* 201, 3–4. <https://doi.org/10.1016/j.tvjl.2014.04.013>.
- Wood, J.L.N., Newton, J.R., Chanter, N., Mumford, J.A., 2005. Association between respiratory disease and bacterial and viral infections in British racehorses. *J. Clin. Microbiol.* 43, 120–126. <https://doi.org/10.1128/JCM.43.1.120-126.2005>.
- Zhang, X., Chen, Z., Gu, W., Ji, W., Wang, Y., Hao, C., He, Y., Huang, L., Wang, M., Shao, X., Yan, Y., 2018. Viral and bacterial co-infection in hospitalised children with refractory *Mycoplasma pneumoniae* pneumonia. *Epidemiol. Infect.* 146, 1384–1388. <https://doi.org/10.1017/S0950268818000778>.