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## GENERAL ARTICLE



# Pathology, infectious agents and horse- and management-level risk factors associated with signs of respiratory disease in Ethiopian working horses

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## Abstract

**Background:** Respiratory disease is a common cause for presentation of working horses to clinics in Ethiopia and a priority concern for owners.

**Objectives:** To identify risk factors for and association of pathogens with respiratory signs in working horses.

**Study design:** Unmatched case-control study.

**Methods:** Cases were those animals recently coughing (last 7 days) or observed with coughing, nasal discharge or altered respiration at the time of examination. A physical exam and respiratory endoscopy were performed including a tracheal wash sample to detect the presence of pathogens and serology performed on blood. An owner questionnaire was administered. Risk factors were determined using multivariable logistic regression.

**Results:** Data on 108 cases and 93 unmatched control horses were obtained. Case horses often had underlying lower airway pathology and were significantly more likely to have *Streptococcus zooepidemicus* detected (OR: 12.4, 95% CI: 3.6-42.4). There was no evidence of a major role for viral respiratory pathogens. Risk factors included completion of strenuous work (OR: 2.7, 95% CI: 1.2-6.3), drinking from stagnant water sources (OR: 2.3, 95% CI: 1.0-5.2) or being housed on a cobbled floor (OR: 2.0, 95% CI: 1.1-3.8). There were increased odds of respiratory disease in young and old horses in this population.

**Main limitations:** Samples for pathogen detection and cytology were only taken from the trachea.

**Conclusion:** *S. zooepidemicus*, a common commensal, may play a role in clinical respiratory disease in this population.

## KEYWORDS

horse, pathology, infectious, asthma, Africa, *Streptococcus equi*, strangles

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## 1 | INTRODUCTION

Respiratory disease is a major cause of time off work and poor performance for equids. In Ethiopian working horses, coughing and nasal discharge are frequently identified at clinics (SPANA clinic data, unpublished). They are a priority health concern for owners<sup>1</sup> and are often 'poorly defined'.<sup>2</sup> Limited diagnostic options mean a presumptive diagnosis is typically based on clinical history and examination alone.

Serological surveys from this study area suggested a role for *Streptococcus equi* subspecies *equi* (*S. equi*)<sup>3</sup> and alphaherpesviruses (equine herpes virus (EHV) 1/4).<sup>4,5</sup> Endemicity of these pathogens occurs in other equine populations and causality in respiratory disease is well established.<sup>6,7</sup> There was no serological evidence of equine influenza virus (EIV) in a previous study,<sup>3</sup> but given its global prevalence and potential impact in a naïve population, its presence was further examined here. Additionally, the picornaviruses, equine rhinitis virus A (ERAV) and B (ERBV), were recognised as frequent contributors to respiratory disease,<sup>8,9</sup> and the role of lesser characterised pathogens of the equine airways was considered in this study given the limited data on respiratory disease in sub-Saharan Africa equines. The gammaherpesviruses EHV-2 and -5 are ubiquitous in equine respiratory secretions, but evidence for their causal role in disease is conflicting.<sup>10,11</sup> *Streptococcus zooepidemicus*, commonly considered a commensal, has also been frequently linked with clinical signs and outbreaks of respiratory disease<sup>12,13</sup> and was therefore investigated here.

Noninfectious respiratory disease includes a spectrum of asthma often presenting with coughing, exercise intolerance and nasal discharge. Severe equine asthma (previously recurrent airway obstruction, RAO) is widely recognised and is likely a hypersensitivity response to inhaled organic dusts where, in contrast to milder forms of equine asthma, an increased respiratory effort is apparent at rest. Mild forms of equine asthma (previously inflammatory airway disease, IAD) tend to affect younger horses. Presentation may be subclinical with a variable inflammatory profile in airways.<sup>14</sup>

This case-control study aimed to investigate the underlying pathology in airways of horses with overt clinical signs; identify any horse- or management-level factors that may increase the odds of disease; and determine associations between infectious agents and respiratory signs using molecular detection of organisms in lower airways and serological evidence for exposure to known pathogens of the equine respiratory tract. This work will aid the therapeutic decisions taken by veterinarians in Ethiopia, and the advice provided for prevention of disease in working equids.

## 2 | MATERIALS AND METHODS

Case and control horses were identified at four SPANA clinic locations (two static and two mobile) in Oromia region, Ethiopia, between September and December 2014. Horses reported with recent coughing (last 7 days) or observed coughing, with nasal discharge

or altered respiration (rate and/or character) at examination were defined as cases. Controls were unmatched and systematically selected as the next horse presenting without a history or signs of respiratory disease that did not require immediate attention on welfare grounds. Horses with suspected African Horse Sickness, Epizootic Lymphangitis, Glanders, severe pleuropneumonia and where epistaxis was the only sign were excluded from the study. A sample size of 94 cases and 94 unmatched controls was estimated<sup>15</sup> to detect an odds ratio of 2.5 or greater with exposures of 20% or more, with 80% power and 95% confidence intervals. Participants were fully informed and gave verbal consent for inclusion in the study and were present throughout the procedure which they could ask to be stopped at any time. Owners also completed an individual verbal questionnaire (Data S1) in either Amharic or Afan Oromo (TA).

Clinical examination and endoscopy of the respiratory tract (to the carina) were performed by a single veterinarian (G.L.). Due to the requirement for horses to work after examination, sedative agents were not administered but a humane nose twitch was applied for a maximum 20 minutes limiting examination to a single naris insertion route, single sample collection point and brief inspection of URT. Full ethical approval was granted with abandonment of examination if animals showed any signs of distress. Secretions at the 'sump' of the trachea were sampled by flushing 20 mL of sterile phosphate-buffered saline (ambient temperature), via a sterile endoscopic catheter. The endoscope underwent a full cleaning routine between animals with biological detergent followed by 20-minute submersion in glutaraldehyde disinfectant.

Fixed slides of tracheal wash sediment were imported to the UK (TARP/2014/163), stained with May-Grunwald-Giemsa and evaluated by a single board-certified clinical pathologist (F.C.) using methods adapted from Whitwell and Greet (1984).<sup>16</sup> This included leucocyte differential count (minimum 200 cells, excluding epithelial cells) and subjective evaluation of mucus, epithelial cells and infectious agents.

Nucleic acid extraction was performed manually on 200 µL of tracheal wash sediment within 4 hours of collection using a commercial kit (Pure Viral RNA Kit, Roche Diagnostics). Samples were stored at -20°C. Freezer storage at -80°C (recommended for RNA) was not available. Therefore, to stabilise RNA samples, 10 µL of extracted nucleic acids was applied to Whatman FTA cards (GE Life sciences)<sup>17</sup> and stored at -20°C until transportation to UK and then stored at -80°C (IMP/GEN/2008/03). Nucleic acids were eluted from FTA cards using methods adapted from Chomczynski<sup>18</sup> and used for detection of RNA viruses: EIV, ERAV/ERBV and equine arteritis virus (EAV). Diagnostic tests are described in Table 1 and Table S1.

### 2.1 | Data analysis

Associations between clinical, endoscopic and cytological variables and case-control status were assessed using univariable logistic regression analyses. Secondly, horse-, owner- and management-level factors were investigated for associations with case-control

**TABLE 1** Diagnostic tests used for detection of multiple pathogens from equine respiratory secretions

Pathogen	PCR test	Details	Methods	Primers
Equine influenza virus	Real time RT-qPCR	Multiplex NP & M	<sup>33</sup>	NP F TTCTGGAGAGGTGAAAATGG NP R CATAAACACAGGCAGGTAGG M F ACCGAGGTCGAAACGTACGT M R CGCGATCTCGGCTTTGA
Equine arteritis virus	Real time RT-qPCR		<sup>34</sup>	F GGCGACAGCCTACAAGCTACA R CGGCATCTGCAGTGAGTGA
Equine rhinitis virus A/B	Real time RT-qPCR	Multiplex	AHT	ERAV target: 5'UTR region ERBV target: 3D Pol region
Equine herpesvirus 1/4	Real time qPCR	Multiplex	AHT	EHV-1 gB gene EHV-4 gC gene
Equine herpesvirus 2	Semi-nested PCR	Round 1 Nested round 2	<sup>24</sup>	F ATGCTCTCTGACAAGAATCACTAC R TGTGTAGACAATGGGAATCTG R2 CGAGACAAACATCTTTTCTCT
Equine herpesvirus 5	Nested PCR	Round 1 Nested round 2	Liverpool Uni.	F ATGAACCTGACAGATGTGCC R CACGTTCACTATCACGTCGC F GATTTCCAGGTGATACAGCTC R ATCGTGGATGGAGTCATCCAG
<i>S. equi</i>	Real time qPCR	Multiplex	<sup>35</sup> ex. SZIC control	eqbE2 F TGGGATTCTGTGCCGATTTT eqbE2 R CAACGCGTAGAAGAACGATCTAAA 2,190 F CAACGCGTAGAAGAACGATCTAAA 2,190 R CCTCCAATTGAGCTTTTTGGTT SZIC F CGCATGCGGGTAGATTATGTAG SZIC R TCCCACGAGAAGGTGCGAGAA
<i>S. zooepidemicus</i>	Real time PCR	Multiplex, E. coli IPC	AHT	Target gene: 8,800 Target gene: 8,940
<i>B. bronchiseptica</i>	PCR assay	Subset 20 cases Multiplex	<sup>36</sup>	F CCCCCGCACATTTCCGAAC TTC R AGGCTCCCAAGAGAAAGGCTT F CACCTGCCCCATCTCC R TGGCGCTGCCCTATC
<i>M. felis</i>	PCR assay	Case samples only	<sup>37</sup>	F CTTGCGACCTAACAAGTCC R GGACTATTATCAAAGCACATAAC
	<b>Serology test</b>	<b>Sensitivity/ specificity</b>		<b>Criteria for positive cut-off</b>
<i>S. equi</i>	Dual Antigen A & C ELISA	93.3%/99.3%	<sup>20</sup>	Positive: OD ≥ 0.5 Borderline: OD = 0.3-0.4
Equine arteritis virus	ELISA, Virus Neutralisation	96.8%/95.6%	<sup>38,39</sup>	OD change ≥0.5 Neutralisation ≥75%
Equine herpesvirus (1/4)	Complement fixation		<sup>22</sup>	Reciprocal dilution titre ≥1:80
Equine rhinitis virus (A/B)	Complement fixation		<sup>22</sup>	Reciprocal dilution titre ≥1:80
Equine influenza virus	ELISA, Haemagglutinin inhibition	95.3%/79%-99%	<sup>40,41</sup>	Percentage inhibition ≥55% Reciprocal dilution titre ≥1:32

Abbreviations: qPCR, quantitative PCR; RT, reverse transcription; IPC, internal PCR control; OD, optical density; ELISA, enzyme-linked immunosorbent assay; OIE, World Org. for Animal Health; AHT, Animal Health Trust.

status using univariable then multivariable logistic regression models. Continuous variables were assessed for linearity using generalised additive models (GAMs). All variables with  $P < .25$  were assessed for correlation before inclusion in a multivariable model.

A manual backwards stepwise approach eliminated each variable until only those with a likelihood ratio  $P < .05$  remained, or where their removal resulted in a >25% change in coefficient of another variable. The final model was assessed for two-way interactions,

and Hosmer-Lemeshow statistic was used for goodness of fit. Delta-beta values and influence plots were used to identify the sensitivity of the model to individual observations. Finally, pathogen associations with case-control status were also investigated using logistic regression but adjusted for age as a potential confounder as this demonstrated a biologically plausible and statistically significant association and may also be associated with pathogen exposure. All analysis was performed using R (version 3.1.2, R Foundation for Statistical Computing).

### 3 | RESULTS

Clinical examination and tracheal endoscopy were performed on 108 cases and 93 unmatched controls with nine of those invited to participate declining (this initial communication was done in the local language hence, exact reasons for declining were not recorded) and nine examinations were abandoned for behavioural reasons. Cases presented with one or more signs including: coughing (89% historical and 36% during examination), 54% nasal discharge (48% serous, 52% mucoid or mucopurulent) and one epistaxis episode. A fifth (19%) of cases also showed altered respiratory signs. The majority of cases (87%) were being examined for the first time and participants reported reduced work ability in 54%. The majority of controls presented for preventive treatments or administrative documentation (75%) and lameness (23%). All animals examined were male and breed type did not vary across sample sites. Nearly all cases (96%) and controls (99%) were used as cart horses and presented by the owner (98%).

Univariable analyses of clinical and endoscopic findings are presented in Tables 2 and 3 and Figures S1 and S2. Case horses were significantly more likely to have abnormal lung sounds, increased tracheal mucus and increased neutrophil count and decreased lymphocyte proportions in tracheal wash cytology (with reference to the most widely accepted limits for normal). In addition, case horses were more likely to have intracellular bacteria in respiratory secretions. Both cases (29%) and controls (39%) frequently showed increased respiratory eosinophils but there was no significant difference in cases or controls.

Univariable analysis results of horse, owner and management variables are presented in Table S2 and S3 and the final multivariable model is shown in Table 4. The association of cases with clinic location or date examined was initially assessed as a fixed effect in a univariable logistic regression model but neither proved significant. Age of horse was significant ( $P = .02$ ) and a GAM for estimated age demonstrated a nonlinear relationship with case status (Figure 1); a quadratic polynomial provided the best fit, suggesting young horses and older horses were at increased risk, with the lowest risk for horses 9-10 years. Exercise intensity was significantly associated with respiratory cases, with horses performing strenuous exercise at nearly three times greater risk. The effect was independent of number of hours worked. Cases were also more likely to have recently drunk from a stagnant water source and be housed on a cobbled floor when compared with bare earth or concrete floors. No participants reported using bedding. The housing floor type was retained in the final model despite the likelihood ratio test statistic of 0.06, as its inclusion improved the overall model fit as assessed by the Hosmer-Lemeshow test (there was significant evidence of poor fit if floor type was excluded [Table 4]). Furthermore, floor type was significant (LRS  $P = .04$ ) in the model excluding exercise intensity where there were fewer missing values. There were no significant two-way interactions identified in the final model.

Age-adjusted logistic regression models of associations between pathogens and respiratory cases are presented in Table 5. All samples tested were negative for EIV, EAV, EHV-1, *Mycoplasma felis* and *Bordetella bronchiseptica*. One young case (1-2 years), presenting with serous nasal discharge and pyrexia, was PCR positive for EHV-4. A single case with a historical cough and enlarged submandibular lymph nodes was tested PCR positive for ERAV. A further two cases and two controls were tested positive for ERBV with cases having co-infections with EHV-2 and/or EHV-5 and *S. zooepidemicus*. EHV-2 and -5 were the most frequently encountered pathogens in both cases (55%, 51%) and controls (58%, 55%) but neither were significantly associated with case status.

Seropositive status to *S. equi* was found in 19% of cases and 11% of control animals. There were also four cases and one control with *S. equi* organisms detected in respiratory secretions by PCR.

**TABLE 2** Univariable logistic regression results of cardiorespiratory and haematological continuous variables from physical exam associated with respiratory cases in working horses in Ethiopia (n = 201, 108 cases and 93 controls)

Variable	Median case	IQR	Median control	IQR	Odds ratio	Lower 95% CI	Upper 95% CI	Wald P-value
Respiratory rate (br/m)	32	20-44	28	20-40	1.02	1.00	1.04	.08
Heart rate (bpm)	48	44-52	44	40-52	1.02	0.98	1.06	.4
Packed cell volume <sup>a</sup>	30%	28%-33%	32%	30%-34%	0.02	0.00	12.40	.22
Total plasma protein <sup>a</sup> (g/dL)	7.6	7.3-8.0	7.6	7.2-8.0	0.90	0.70	1.20	.5

Note: Due to apparent discordance between the Ethiopian results and published reference intervals, the lack of obvious cut-offs for normal and to avoid loss of data, cardiorespiratory and haematological parameters were analysed as continuous variables.

Abbreviations: IQR, inter-quartile range; CI, confidence interval.

<sup>a</sup>Packed cell volume (PCV) and total plasma protein (TPP) were measured manually.

**TABLE 3** Univariable logistic regression results of physical examination, endoscopic and cytological findings associated with respiratory cases in working horses in Ethiopia (n = 201, 108 cases and 93 controls)

Variable	Case n (%)	Control n (%)	Odds ratio	Lower 95% CI	Upper 95% CI	Wald P-value
<b>Clinical exam findings</b>						
<b>Cranial lymph nodes</b>						
Normal	104 (96)	93 (100)	ref			
Enlarged	4 (4)	0 (0)	3.5	0.4	32.2	.2
<b>Respiratory auscultation</b>						
Normal	53 (50)	84 (90)	ref			
Abnormal	54 (50)	9 (10)	9.5	4.3	20.8	<.01
<b>Pyrexia</b>						
<39.0°C	98 (93)	89 (99)	ref			
≥39.0°C	7 (7)	1 (1)	6.4	0.8	52.7	.09
<b>Demeanour</b>						
Bright & responsive	84 (78)	72 (77)	ref			
Quiet or dull	24 (22)	21 (23)	1.0	0.5	2.0	.5
<b>Blood parameters</b>						
<b>Packed cell volume</b>						
<30%	34 (31)	21 (23)	1.6	0.8	3.0	.2
30%-46%	74 (69)	72 (77)	ref			
>46%	0 (0)	0 (0)	—			
<b>Total plasma protein</b>						
<6.5 g/L	0 (0)	2 (2)	0.7	0.1	7.5	.7
6.5-7.3 g/L	28 (26)	35 (38)	ref			
>7.3 g/L	79 (74)	56 (60)	1.8	1.0	3.4	.05
<b>Neutrophil proportion</b>						
<45%	10 (9)	12 (13)	0.6	0.2	1.7	.3
45%-55%	22 (21)	15 (16)	ref			
>55%	75 (70)	66 (71)	0.8	0.4	1.6	.50
<b>Eosinophil proportion</b>						
≤5%	94 (88)	84 (90)	ref			
>5%	13 (12)	9 (10)	1.3	0.5	3.2	.6
<b>Lymphocyte proportion</b>						
<35%	77 (72)	65 (70)	1.0	0.5	1.9	.90
35%-50%	26 (24)	21 (23)	ref			
>50%	4 (4)	7 (8)	0.5	0.1	1.8	.3
<b>Monocyte proportion</b>						
≤3%	27 (25)	23 (25)	ref			
>3%	80 (75)	70 (75)	1.0	0.5	1.9	.9
<b>Endoscopy findings</b>						
<b>Cough on endoscopy</b>						
None or occasional	37 (36)	81 (87)	ref			
Frequent or severe	65 (64)	12 (13)	11.9	5.7	24.6	<.01
<b>Lymphoid hyperplasia</b>						
Grade 0-2	101 (99)	93 (100)	—			
Grade 3-4	1 (1)	0 (0)	—			

(Continues)

TABLE 3 (Continued)

Variable	Case	Control	Odds ratio	Lower 95% CI	Upper 95% CI	Wald P-value
	n (%)	n (%)				
Tracheal mucus						
Grade 0-1/3	65 (64)	92 (99)	ref			
Increased ( $\geq 2/3$ )	36 (36)	1 (1)	51.0	6.8	381.1	<.01
Carina appearance						
Normal	90 (90)	82 (89)	ref			
Blunted carina	10 (10)	10 (11)	0.9	0.4	2.3	.8
Blood in wash sample						
Not identified	89 (88)	83 (90)	ref			
Blood present	12 (12)	9 (10)	1.2	0.5	3.1	.6
Tracheal wash cytology <sup>a</sup>						
Neutrophil proportion <sup>b</sup>						
$\leq 19\%$	24 (23)	47 (54)	ref			
$> 19\%$	81 (77)	40 (46)	4.0	2.1	7.4	<.01
Lymphocyte proportion						
$\leq 21\%$	100 (95)	72 (83)	ref			
$> 21\%$	5 (5)	15 (17)	0.1	0.1	0.3	<.01
Monocyte proportion						
$\leq 96\%$	105 (100)	87 (100)				
$> 96\%$	0 (0)	0 (0)				
Eosinophil proportion						
$\leq 2\%$	77 (73)	53 (61)	ref			
$> 2\%$	28 (27)	34 (39)	0.6	0.3	1.0	.07
Mast cell proportion						
0%	99 (94)	84 (97)	ref			
$> 0\%$	6 (6)	3 (3)	1.7	0.4	7.0	.5
Intracellular bacteria <sup>c</sup>						
Not identified	87 (83)	86 (99)	ref			
Present	18 (17)	1 (1)	18.6	2.4	142.3	<.01
Degenerative neutrophils						
Not identified	102 (95)	92 (99)	ref			
Present	5 (5)	1 (1)	4.5	0.5	39.3	.2
Squamous epithelial cells						
Not identified	89 (83)	59 (63)	ref			
Present	18 (17)	34 (37)	0.4	0.2	0.7	<.01
RBC on smear <sup>d</sup>						
Not identified	97 (92)	82 (94)	ref			
Present	8 (8)	5 (6)	1.4	0.4	4.3	.6

Note: Clinical, endoscopic and cytological variables are all likely to be effects of, rather than causes of, underlying respiratory disease and as such were not assessed as potential risk factors in multivariable regression. Missing observations for some aspects of examination due to recording errors or missing data due to horse temperament. Eight tracheal wash samples had insufficient cells to perform a differential cell count.

Abbreviation: CI, confidence interval. ref, reference category

<sup>a</sup>There are no published accepted reference intervals for tracheal wash differential cell counts, so values used in this study were those of Richard et al,<sup>42</sup> who considered a range of studies to establish cut-offs.

<sup>b</sup>Strong neutrophilic inflammation can make it difficult to assess other cell lines accurately.

<sup>c</sup>67% of cases where intracellular bacteria morphology was described (8/12) were cocci.

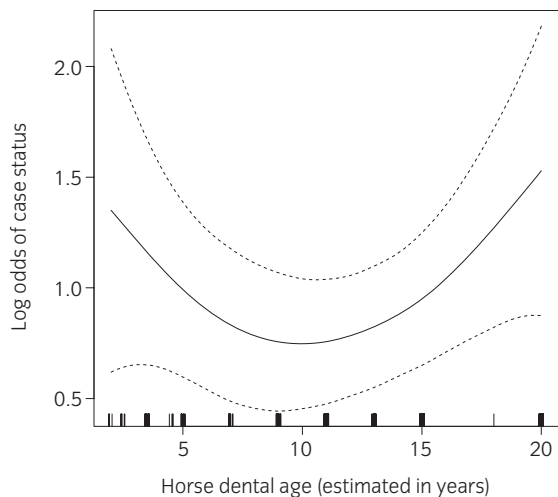
<sup>d</sup>Haemosiderophage, an indicator of previous bleeding in the lungs, was a rare finding and was noted in just one control horse.

**TABLE 4** Multivariable logistic regression model of factors associated with respiratory cases in working horses in Ethiopia (n = 190, 102 cases and 88 controls)

Variable	Coefficient (Std. error)	Odds ratio	Lower 95% CI	Upper 95% CI	LRS P-value
Age centred (y)	0.03 (0.03)	1.03	0.97	1.09	.3
Age centred squared (y)	0.01 (0.01)	1.01	1.00	1.02	.03
Work intensity					
Easy-moderate or variable	ref				.02
Strenuous	0.99 (0.43)	2.69	1.15	6.31	
Drank stagnant water (last 14 d)					
No	ref				.05
Yes	0.82 (0.42)	2.27	1.00	5.20	
Floor type (housing)					
Bare earth/sandy soil	ref				.06 <sup>a</sup>
Flat rocks (cobble)	0.69 (0.33)	1.99	1.05	3.81	
Concrete	-0.31 (0.56)	0.74	0.24	2.23	

Abbreviations: CI, confidence interval; LRS, likelihood ratio statistic.

<sup>a</sup>This variable was retained in the model as it improved the overall model fit (as assessed by Hosmer-Lemeshow test). The Hosmer-Lemeshow statistic for model fit did not indicate the model fit was poor ( $P = .4$ ) when floor type was included but indicated significant evidence of poor fit if floor type was excluded ( $P = .01$ ). Furthermore, floor type was significant ( $P = .04$ ) in the model excluding exercise intensity where there were fewer missing values. Influence and delta-beta plots identified clusters of two and three samples with the highest leverage on the model. Leverage was less than 0.15 for all individuals and the influence exerted to perturb the model was low.

**FIGURE 1** Generalised additive model (GAM) plot to show the nonlinear relationship between horse dental age (estimated in years) and case-control status for respiratory disease

However, neither were significantly associated with respiratory signs in this study.

Horses with *S. zooepidemicus* detected in tracheal wash samples were more likely to present as a case of respiratory disease (OR: 12.4, CI 3.6-42.4). *S. zooepidemicus* was found in combination with other pathogens in several cases: EHV-4 (n = 1), EHV-2 or -5 (n = 20) and in horses seropositive for *S. equi* (n = 8), but was found in isolation in two cases. All three control animals positive for *S. zooepidemicus*, compared with just two of 31 case horses, had squamous epithelial cells identified on cytology suggesting that samples

may be infrequently contaminated from the upper respiratory tract (URT). Fourteen cases also had intracellular bacteria in lower respiratory tract (LRT) macrophages. Fourteen cases had no infectious agents detected either on serology or PCR. Of those, only two had a normal tracheal cytology profile. Further information on all case presentations are in Data S2.

## 4 | DISCUSSION

This is the first study to examine the association between infectious agents and signs of respiratory disease in working horses of Ethiopia. There was a significant association of *S. zooepidemicus* with respiratory cases in our study. Although *S. zooepidemicus* is considered a commensal of the URT, and the method of sampling in this study could be vulnerable to contamination from the URT, the presence of intracellular bacteria on tracheal smears supports a true septic inflammation in the LRT rather than a transient colonisation. Furthermore, any URT contamination due to sampling methodology would affect cases and controls equally.

*Streptococcus zooepidemicus* in the LRT has been implicated as a primary pathogen in respiratory disease<sup>12</sup> and linked to equine asthma (mild) and subclinical respiratory disease.<sup>19</sup> A role in subclinical disease was not identified in this study, however, with very few control horses testing positive. It is unclear whether its role was as a primary pathogen or as a secondary opportunistic infection of the lower airways. This is of interest in this study due to the frequent occurrence of co-infections with other respiratory pathogens. Previously, different strains of *S. zooepidemicus* have been linked with different pathogenic

**TABLE 5** Age-adjusted logistic regression results of serology and PCR results for pathogens associated with respiratory cases in working horses in Ethiopia, adjusted for age as a quadratic term (n = 201, 108 cases and 93 controls)

Variable	Case n (%)	Control n (%)	Odds ratio	Lower 95% CI	Upper 95% CI	Wald P-value
<b>Viral pathogens</b>						
<i>Eq. herpesvirus-1</i>						
PCR-	108 (100)	93 (100)				
PCR+	0 (0)	0 (0)				
<i>Serostatus</i>						
CF-	108 (100)	92 (100)				
CF+	0 (0)	0 (0)				
<i>Eq. herpesvirus-4</i>						
PCR-	107 (99)	93 (100)				
PCR+	1 (1)	0 (0)				
<i>Serostatus</i>						
CF-	108 (100)	92 (100)				
CF+	0 (0)	0 (0)				
<i>Eq. herpesvirus-2</i>						
PCR-	49 (45)	46 (49)				
PCR+	59 (55)	47 (51)	1.03	0.59	1.82	.9
<i>Eq. herpesvirus-5</i>						
PCR-	45 (42)	42 (45)				
PCR+	63 (58)	51 (55)	0.84	0.48	1.49	.6
<i>Eq. arteritis virus</i>						
PCR-	108 (100)	93 (100)				
PCR+	0 (0)	0 (0)				
<i>Eq. influenza virus</i>						
PCR--	108 (100)	93 (100)				
PCR+	0 (0)	0 (0)				
<i>Eq. rhinitis virus-A</i>						
PCR-	107 (99)	93 (100)				
PCR+	1 (1)	0 (0)				
<i>Serostatus</i>						
CF-	108 (100)	92 (100)				
CF+	0 (0)	0 (0)				
<i>Eq. rhinitis virus-B</i>						
PCR-	106 (98)	91 (98)				
PCR+	2 (2)	2 (2)	0.82	0.11	6.09	.9
<i>Serostatus</i>						
CF-	108 (100)	92 (100)				
CF+	0 (0)	0 (0)				
<b>Bacterial pathogens</b>						
<i>S. equi</i>						
PCR-	104 (96)	92 (99)				
PCR+	4 (4)	1 (1)	3.36	0.36	31.10	.3

(Continues)



TABLE 5 (Continued)

Variable	Case n (%)	Control n (%)	Odds ratio	Lower 95% CI	Upper 95% CI	Wald P-value
<i>S. equi</i> Serostatus						
ELISA-	87 (81)	82 (89)				
ELISA+	21 (19)	10 (11)	2.02	0.89	4.60	.09
<i>S. zooepidemicus</i> <sup>a</sup>						
PCR-	73 (70)	89 (97)				
PCR+	31 (30)	3 (3)	12.39	3.62	42.42	<.01
<i>Bordetella bronchiseptica</i>						
PCR-	20 (100)	– (–)				
PCR+	0 (0)	– (–)				
<i>Mycoplasma felis</i>						
PCR-	108 (100)	– (–)				
PCR+	0 (0)	– (–)				

Abbreviations: CF, complement fixation; ELISA, enzyme-linked immunosorbent assay; PCR, polymerase chain reaction; CI, confidence interval.

<sup>a</sup>Animals testing positive for *S. equi* on PCR were excluded from analysis due to cross-reaction, and results of *S. zooepidemicus* are limited to presence or absence information.

attributes.<sup>13</sup> It is unlikely that immunity to one strain type is cross-protective to infection with others, and therefore future work should quantify *S. zooepidemicus* in the LRT and examine strain types.

Serostatus for *S. equi*, the causative agent of strangles, was not significantly associated with signs of respiratory disease ( $P = .09$ ); however, 11% of controls and nearly a fifth of case horses were seropositive, hence this may be due to low power to detect an association. The persistence of raised antibody titres following resolution of clinical signs could also affect the level of association between serostatus and clinical signs.<sup>20</sup> Similarly, chronic carriers of *S. equi* may shed bacteria and/or be seropositive for *S. equi* without displaying clinical signs. A previous study in working equids in Lesotho also failed to demonstrate a significant association with clinical respiratory disease<sup>6</sup> despite an overall prevalence of 10.1%, similar to the estimated prevalence of 8% found in this population in a previous cross-sectional study of 350 horses.<sup>3</sup> Low rates of detection of *S. equi* in lower airways, despite many seropositive horses, may be a result of the suboptimal sampling site (*S. equi* is predominantly a URT pathogen found in the nasopharynx or guttural pouches). Alternatively, clearance of the organism after infection could also lead to comparatively low PCR positives compared with serology findings. The small number of cases and resulting low power may also explain the lack of association between current *S. equi* infection and clinical signs. Infection may be of greater importance in particular groups such as young naïve horses.

The overall low rates of detection for viral pathogens are in agreement with a previous seroprevalence study in this population,<sup>3</sup> which found no evidence of EIV and limited evidence of exposure of ERA/BV, EAV and EHV-1/-4. Low prevalence of viral pathogens is in contrast to studies in the United States, for example, where they are commonly detected,<sup>7</sup> but the failure to find significant associations between viral pathogens and respiratory disease is consistent

with another case-control study in the UK.<sup>21</sup> The tracheal sampling site could contribute to low detection rates as it may not be ideal for URT pathogens, such as ERBV or EHV-1/4, but financial and logistical constraints meant a second sample site was not feasible in this study. As reported in a previous study,<sup>3</sup> there was little evidence of EHV-1 and -4 in cases or controls, in contrast to Endebu-Duguma and Getachew et al,<sup>4,5</sup> who suggested exposure to herpesviruses in Ethiopian horses is common. This likely represents a difference in diagnostic approach as previous studies measured virus-neutralising antibodies which can persist for up to a year in contrast to measurement of viral nucleic acids in airways or complement fixing antibodies that last less than 3 months.<sup>22</sup>

ERBV was detected in equal numbers of cases and controls, and in cases it was only found as a co-infection with other respiratory pathogens. There is conflicting evidence for the role of ERBV in respiratory disease,<sup>23</sup> and a limitation of PCR testing is that it cannot discriminate a viable from a nonviable organism. Therefore, viral isolation alongside PCR would have been desirable but was not feasible with current facilities in the field. ERAV was found in isolation in one case horse. This was the first study to look at the presence of gammaherpesviruses (EHV-2/5) in this population; although frequency of occurrence was high, there was no significant association with cases of respiratory disease, in agreement with other studies.<sup>24</sup>

A number of cases with lower airway inflammation had no detectable infectious agent, supporting either a role for noninfectious aetiologies, infection with a novel pathogen or a failure to detect pathogens due to imperfect test sensitivity. This could also indicate an asthma-like chronic inflammatory disease known to be common in other equine populations.<sup>25</sup>

This study confirmed underlying airway pathology in Ethiopian horses presenting with respiratory signs such as increased tracheal mucus and tracheal neutrophilia. Lower airway inflammation leads

to increased mucus secretion that may subsequently lead to coughing. Of those animals with increased tracheal mucus, over half were seen coughing during examination and the remainder all had a recent history of coughing in the last week. These results are in agreement with other studies that coughing is a specific indicator for tracheal mucus<sup>25</sup> and further supports the potential usefulness of owner reporting in this population.<sup>26</sup>

Neutrophilia in respiratory secretions is a common occurrence in equine asthma, found in both mild and severe phenotypes, but also occurs in septic inflammation due to primary or secondary bacterial colonisation.<sup>21</sup> Colonisation can occur with impairment of the mucociliary clearance seen in equine asthma, or following viral insult. Septic inflammation usually presents with a high percentage of neutrophils, a frequent finding in this study, and cases showed significantly more intracellular bacteria in secretions but the presence of degenerative cells, usually found with septic inflammation, was rare.

Nevertheless, 20% of cases had neither increased tracheal mucus nor neutrophilia, suggesting that respiratory disease may involve other pathologies, or that some animals were incorrectly classified as cases. Furthermore, an absence of clinically apparent signs did not rule out airway inflammation,<sup>19</sup> and some control horses were found to have abnormal cytology results, inclusion of which could bias results towards the null if misclassified.

Eosinophilia was a frequent finding in cases but also in controls and was not associated with presentation for respiratory disease, as found in other studies. This may suggest subclinical airway disease or severe asthma during remission.<sup>27</sup> Eosinophilia may also be associated with lungworm, but it was not possible to assess prevalence in this study.

In this population, there was increased likelihood of younger and older animals presenting as cases, an age pattern found in other populations.<sup>28</sup> where increasing immunity to pathological challenges, or a build-up of tolerance to other aetiological agents occurs as horses reach adulthood. The risk in older animals may reflect the development of hypersensitivities and chronic respiratory disease, as seen in ageing populations elsewhere.<sup>25,26</sup> Other risk factors included a cobbled floor ('flat rocks') in housing. Stabling has been identified as a risk factor for disease through increased exposure to dust and allergens especially in straw or hay, but no participants reported using bedding of any sort or feeding large quantities of hay. Increased odds could be associated with difficulties keeping cobbles clean but may also be a proxy for another management practice not identified, and this warrants further investigation.

Horses that were described by owners as performing strenuous work were at increased risk of disease in this study. High intensity or strenuous exercise can lead to airway contamination and immunocompromise,<sup>29</sup> and has been identified as a risk factor for disease in other populations.<sup>30</sup> Additionally, strenuous exercise in a dusty environment can lead to mucosal damage, and dust storms have also been associated with increased invasive bacteria in the LRT of humans.<sup>31</sup> Dust exposure during work was not measured during this

study, but horses commonly worked on dirt roads in dry environmental conditions. It is also possible that respiratory signs may be more apparent in animals exercising intensely and therefore, they may be more likely to attend clinics as a case.

Stagnant water has not been previously identified as a risk factor for equine respiratory disease and a direct causal link cannot be determined from the current study and it may be a proxy for unmeasured variables. However, stagnant water may be contaminated with potentially harmful organisms, such as *S. equi*, which can survive up to a month in drinking water.<sup>32</sup>

The broad case definition criteria in this unmatched case-control study could represent a range of aetiologies, potentially making the identification of syndrome-specific risk factors more challenging. Controls were selected at the regional clinics, hence it is also possible that they were not 'healthy' and could have been suffering from other disease leading to bias. Although respiratory disease in working horse populations is recognised as a problematic syndrome,<sup>2</sup> there is a lack of published information to compare these results to and they may be specific to this region of sub-Saharan Africa.

In conclusion, horses presenting with coughing, nasal discharge and/or dyspnoea at clinics in Ethiopia often do have changes in the LRT, in particular neutrophilia and tracheal mucus. Colonisation of LRT with *S. zooepidemicus* was significantly associated with clinical signs of respiratory disease. The role of *S. zooepidemicus* as a primary cause of respiratory disease should be further investigated and interventions to limit exposure are recommended. Evidence from this study suggests that there is only a minor role, if any, played by viral pathogens in this population. The only vaccination available and used in this area is against African Horse Sickness. Thus, the susceptibility of the population to the introduction of a novel viral pathogen should be considered. Further work is also needed to understand the role of and risk factors for equine asthma in this and other working equid populations.

## ETHICAL ANIMAL RESEARCH

Ethical approval for this study was granted by the Ethics Committee at the University of Liverpool (VREC143) and University Addis Ababa (VM/ERC/001/06/014).

## OWNER INFORMED CONSENT

Owner consent for participation was obtained verbally due to assumed high levels of illiteracy.

## DATA ACCESSIBILITY STATEMENT

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

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### CONFLICT OF INTEREST

No competing interests have been declared.

### AUTHOR CONTRIBUTIONS

G. Pinchbeck, R. Christley and A. Stringer conceived the study, G. Laing, R. Christley and G. Pinchbeck designed the study; G. Laing, T. Ashine and N. Aklilu conducted fieldwork; G. Laing and F. Cian conducted laboratory analysis and G. Laing drafted the manuscript. All authors contributed to writing the manuscript.

### PEER REVIEW

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#### SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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