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Equine

Investigation of the Blood Microbiome in Horses With Fever of Unknown Origin

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Keywords: microbiome | molecular diagnostics | pathogen discovery | tick-borne disease

ABSTRACT

Background: Fever of unknown origin (FUO) without a respiratory component is a frequent clinical presentation in horses. Multiple pathogens, both tick-borne and enteric, can be involved as etiologic agents. An additional potential mechanism is intestinal barrier dysfunction.

Objectives: This case–control study aimed to detect and associate microbial taxa in blood with disease state.

Study Design: Areas known for a high prevalence of tick-borne diseases in humans were chosen to survey horses with FUO, which was defined as fever of 101.5°F or higher with no signs of respiratory illness or other recognisable diseases. Blood samples and clinical parameters were obtained from 52 FUO cases and also from matched controls from the same farms. An additional 23 febrile horses without matched controls were included.

Methods: Broadly targeted polymerase chain reaction (PCR) amplification directed at conserved sequence regions of bacterial 16S rRNA, parasite 18S rRNA, coronavirus RdRp and parvovirus NS1 was performed, followed by deep sequencing. To control for contamination and identify taxa unique to the cases, metagenomic sequences from the controls were subtracted from those of the cases, and additional targeted molecular testing was performed. Sera were also tested for antibodies to equine coronavirus.

Results: Over 60% of cases had intestinal microbial DNA circulating in the blood. Nineteen percent of cases were attributed to infection with *Anaplasma phagocytophilum*, of which two were subtyped as human-associated strains. A novel *Erythroparvovirus* was detected in two cases and two controls. Serum titres for equine coronavirus were elevated in some cases but not statistically different overall between the cases and controls.

Main Limitations: Not all pathogens are expected to circulate in blood, which was the sole focus of this study.

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Conclusions: The presence of commensal gut microbes in blood of equine FUO cases is consistent with a compromised intestinal barrier, which is highlighted as a direction for future study.

1 | Introduction

Equine fevers of unknown origin (FUO) are very common and usually are not fully diagnosed, despite studies attributing a wide range of diseases, including infections, non-infectious inflammatory diseases and neoplasm (Minamimoto 2022). In terms of infectious diseases, FUO can be associated with pathogens transmitted by several routes. Equine coronavirus (ECoV) is one common enteric infection connected with FUO in horses and has been detected in faeces obtained from adult horses in several countries, including the United States (Pusterla et al. 2013).

Ticks, especially ixodid ticks (also known as hard ticks), are major vectors transmitting diverse disease-causing agents to humans, domestic animals and wildlife, posing an increasing threat to both human and animal health. Lyme disease and anaplasmosis are the most common tick-borne diseases (TBDs) reported in horses in the United States (Divers et al. 2018a; Tirosh-Levy et al. 2020). Diagnosis of TBDs is usually made according to history of tick bite, clinical suspicion, serology and detection of antigen or pathogen nucleic acid (Subbiah et al. 2021). However, diagnosis of TBDs can be challenging due to non-specific symptoms and the broad spectrum of disease-causing agents that ticks transmit (Pace and O'Reilly 2020). Lyme disease, equine granulocytic anaplasmosis (EGA), tick-borne encephalitis virus (TBEV) and equine piroplasmosis are crucial TBDs in horses with One Health implications. Studies have indicated that tick vectors can transmit more than one agent. In the United States and Europe, for instance, *Babesia* spp., *Anaplasma phagocytophilum* and *Borrelia burgdorferi* are all transmitted by *Ixodes scapularis* (blacklegged tick), *I. pacificus* (western blacklegged tick) and *I. ricinus* (castor bean tick; Cerar et al. 2016; Fleshman et al. 2022; Reye et al. 2010; Swanson et al. 2006). Animals and humans that are infected with multiple agents can have more severe and prolonged clinical illness (Swanson et al. 2006).

The blood microbiome is one of the few systems where consistent taxa are not well defined for any species. Blood was previously thought to be typically sterile, but studies in the past decade have demonstrated that many bacteria, including known pathogenic ones, are able to survive in blood and inside of red blood cells, with differences of taxonomy profiles in each blood fraction being observed (Damgaard et al. 2015; Païssé et al. 2016). The concept of the leaky gut in horses has been gaining momentum, where intestinal microbes may permeate through compromised membranes and circulate in the blood (Stewart et al. 2017). Subbiah linked horse blood microbiome profiles to certain types of illnesses: Phylum proteobacteria made up a higher proportion of blood bacteria profiles in horses that tested positive for tick-borne pathogens when compared with healthy counterparts (Subbiah et al. 2021). Importantly, however, numerous studies have shown the presence of contaminating DNA in lab reagents that could confound results from individuals tested without appropriate controls (Castillo et al. 2019). The goal of our study was to inform clinicians of potential causative factors of FUO, with the hypothesis that a broadly targeted molecular detection approach would identify associated TBD taxa. The finding of typical gut taxa prompted further investigation of potential viral involvement.

2 | Materials and Methods

2.1 | Sample Size Calculation and Recruitment

The primary objectives were to identify etiologic factors/pathogens that predispose horses to develop fever of unknown/undiagnosed origin and examine the role of geographic location and intrinsic factors (age, breed and sex of the horse) in this risk. The main outcome was fever. To address this objective, we used a case–control study design. We assumed the prevalence of individual infectious agents associated with fever was rare. We used the formula for sample size calculations suggested by Schlesselman (1982) to determine the number of cases to be enrolled in the study. Assuming conservatively that a particular pathogen that predisposes to FUO is present at rate of 2% (exposure rate) in the general population (control group) then it is 10-times more likely (odds ratio) to detect it (exposure factor) in fever cases than in non-fever horses. We planned to test this hypothesis at type I error protection of 10% ($\alpha = 0.1$) and a power of 80%. We computed that we needed to identify 116 cases of unknown fever and a similar number of controls. To allow for attrition and sample spoilage, we aimed to increase the number of pairs to 124. The controls were selected randomly from the premise (by the submitting clinician) where a case was reported to control for potential confounding bias due to unknown factors.

Cases were recruited from equine clinics in three geographic areas including the Northeast, Mid-Atlantic and Great Lakes of the United States. Informed consent was obtained. The case definition was a horse with acute onset fever (>/ = 101.5°F), without respiratory signs or known infectious cause, and no previous antibiotic therapy within 7 days prior to the fever. Blood samples were submitted by participating practitioners in the targeted geographic areas using a supplied collection kit. Participating veterinarians were instructed to obtain blood samples within 48 h of onset of fever. For each animal meeting the case definition, an additional control blood sample was requested from a horse residing on the same premise, free from fever or other signs of infectious disease. Samples from control horses were submitted with 52 cases; no control samples were provided for 23 other cases. Horse-associated ticks and ticks found in the same premise as the horses were also solicited for identification and testing. Samples were received between 9 March 2012 and 27 March 2013. Routine testing results were provided to the participating veterinarians as part of their clinical care of the animals.

A questionnaire was administered to collect information on enrolled horses (Figure S1). Clinical parameters recorded

included lethargy, icterus, cough, limb oedema, anorexia, colic signs and faecal character. Each of these was collected as four qualitative responses scored from 1 to 4 with 1 as least severe and 4 as most severe. Additional questions included presence of ticks on the property, horse transportation within the past 30 days, ticks present on the horse at the time of sampling, and history of ticks attached within the past 14 days.

2.2 | DNA Analyses

A 75 μL aliquot of whole EDTA blood was extracted using the DNeasy Blood & Tissue Kit (Qiagen) according to manufacturer instructions. Ticks were microscopically speciated, dissected and initially homogenised with buffer and proteinase K using a pestle. Nucleic acid was then extracted from the tick lysate using the same kit as for blood. Samples were eluted in 100 μL of buffer from the kit.

Nested polymerase chain reaction (PCR; see Table S1 for primers) was performed for *A. phagocytophilum*, *Neorickettsia risticii* and *Leptospira* species with reactions set up in 25 μL total volumes using a native Taq DNA Polymerase (Invitrogen). PCR products were visualised on a 1.5% agarose gel stained with ethidium bromide. Positive and negative controls were run with each assay.

Metagenomic DNA sequencing was performed using broad-based genus-specific enrichment assays targeting group-specific gene sequences. Primers (Table S1) targeted *Anaplasma*, *Babesia*, *Bartonella*, *Borrelia*, *Ehrlichia* and *Rickettsia* spp. The primers were used to amplify DNA from potential bacteria present in blood from all cases and controls ($n = 127$) by conventional PCR. Appropriate primer pairs targeting pathogen groups known to be associated with specific tick species were also used to identify the presence of these bacteria in tick pools. The following target regions were used to broadly detect members of the different genus groups: group-specific 16S ribosomal RNA gene (*Ehrlichia*, *Anaplasma* and *Rickettsia* spp.), 18S ribosomal RNA gene (*Babesia* spp.) flagellar DNA (*Borrelia* spp.) and 16S-23S ribosomal RNA gene intergenic region (*Bartonella* spp.). Purified amplicons were pooled in equimolar amounts and quantified using a fluorescent nucleic acid stain (Quant-iT PicoGreen dsDNA Assay Kit). Libraries were prepared using NexteraXT (Illumina) and subjected to fragment analysis to assess quality and quantity prior to sequencing using 2 × 250 paired-end chemistry (MiSeq, Illumina). All ticks were tested for *A. phagocytophilum* and *B. burgdorferi* by PCR in addition to metagenomic sequencing.

To enhance the ability to detect potential *Bartonella* infections, aliquots of acid citrate dextrose blood samples were also cultured by inoculation into *Bartonella* alpha proteolytic growth medium (BAPGM) enhanced with sheep whole blood. Upon detection of growth, cultures were mechanically homogenised with zirconia beads. Nucleic acid was then extracted according to manufacturer protocol using an automated magnetic bead-based kit (AM1840, Thermo-Fisher). Samples were eluted in 90 μL followed by real-time PCR for identification.

To subtype *A. phagocytophilum*, we conducted a hybridisation capture followed by next-generation sequencing using a hybridisation panel with ssDNA probes corresponding to the sequences of the seven genes in the Huhn et al. multilocus sequence typing (MLST) scheme (*pheS*, *glyA*, *fumC*, *mdh*, *sucA*, *dnaN*, *atpA*) of *A. phagocytophilum* (Huhn et al. 2014). The DNA previously extracted from the blood samples of three horses (H52, H54, H55) was used to prepare libraries using the Library Preparation Enzymatic Fragmentation Kit 2.0 (Twist Biosciences) and the CD Index Adapter Set 1–96 (Twist Biosciences), following the standard protocol. These libraries were allowed to hybridise with the ssDNA probe panel for 16 h. After hybridisation, the libraries were sequenced in the MiSeq Sequencing System (Illumina) using the MiSeq Reagent Kit v3 (Illumina). The complete or partial sequences obtained from the seven genes were uploaded to the *A. phagocytophilum* database (https://pubmlst.org/organisms/anaplasma-phagocytophilum) of the Public Databases for Molecular Typing and Microbial Genome Diversity (PubMLST; Jolley et al. 2018). This database uses the sequence of the seven genes to identify identical or closely related allelic groups that have already been classified within the database. The sequences of the seven genes obtained from two of the *A. phagocytophilum* (H54 and H55) were concatenated and aligned with those of the concatenated sequences of the closely matched allelic profiles using the Clustal Omega algorithm (Sievers et al. 2011) in Geneious Prime 2023.2.1 (Biomatters Ltd.). This alignment was used to construct a maximum likelihood (ML) phylogenetic tree in MEGA11 (Tamura et al. 2021).

Screening for *Erythroparvovirus* was done on all blood samples. Archived blood samples were treated with 5 drops of ZAP-OGLOBIN II Lytic Reagent per 200 μL at 37°C for 15 min (NC0098316, Beckman Coulter). Treated samples were then extracted using the MagMAX CORE Nucleic Acid Purification Kit (A32700, Thermo-Fisher) following the manufacturer's protocol, with carrier RNA added. DNA fragments were amplified using the Invitrogen Platinum Hot Start PCR Master Mix (2×) kit with all primers at a final concentration of 0.2 μM. The PCR cycle condition was an initial denaturation at 94°C for 2 min; 35 cycles of 94°C for 15 s, 56°C for 30 s and 68°C for 30 s; and a final extension at 68°C for 7 min. Primers for the NS1 gene were designed using ORF Finder on NCBI (Table S1). Samples were visualised through a 1.5% agarose gel. Positive samples were then sequenced via Sanger sequencing. To demonstrate the genetic relationship between the new strain and other parvoviruses, a multiple sequence alignment of 36 NS1 protein sequences obtained from GenBank was performed using MAFFT on Geneious Prime 2023.2.1. A phylogenetic tree was constructed using PhyML with 100 Bootstraps.

2.3 | RNA Analyses

Total RNA was extracted from a separate aliquot of 75 μL whole EDTA blood using the MagMax mirVana Total RNA Isolation Kit (Thermo-Fisher). The cDNA synthesis was performed using Superscript IV Vilo (Thermo-Fisher). Pan-coronavirus nested RdRp PCR was performed as previously reported (Quan et al. 2010; Watanabe et al. 2010). Shotgun RNAseq library preparation was performed using DNAse treatment and rRNA depletion with the RiboZero HMR kit (Illumina), and the NEBNext Ultra II Directional Library Prep kit (New England Biolabs). After quality check, 100 M reads were targeted for each sample using 2 × 150 bp paired-end sequencing on the Illumina NovaSeq 6000 platform.

Analysis was performed using the CZID Illumina mNGS Pipeline v6.8 (Kalantar et al. 2020).

2.4 | Serology

Serum aliquots were frozen at −20°C and analysed in batch for antibody responses to ECoV spike protein, as previously described (Kooijman et al. 2017).

2.5 | Bioinformatics and Statistics

Analysis of clinical parameter scores was performed with GraphPad Prism (San Diego, CA, USA) v.10.3.0. The D'Agostino and Pearson test was used to assess whether ages in years fit a normal distribution. Neither cases nor controls passed this normality test ($p = 0.0014$ and $p = 0.0142$, respectively) and so the Mann–Whitney test was used to compare ages between the groups. Temperatures of *Anaplasma* spp. positive cases versus controls were compared using the Wilcoxon matched pairs signed-rank test for pairs where the case was positive, and by Welch's *t*-test (one-way) for overall comparison including the unmatched cases. Correlations of clinical metadata, which were mostly not normal, were performed with Kruskal–Wallis analysis of variance (ANOVA) with multiple comparisons.

High-quality 16/18S metagenomic amplicon sequences were processed using the Quantitative Insights Into Microbial Ecology tool, QIIME2 (2011). The feature table was constructed using the DADA2 algorithm (Callahan et al. 2016). After removing low-quality and chimeric sequences, the remaining reads were truncated from 0 to 140 bp (for both forward and reverse reads) to exclude sequencing errors from the ends of the reads. We set a minimum similarity threshold of 90% to assign the taxonomy from the SILVA database (Quast et al. 2013; release 128) to these features. The representative sequences, also named 'features' in QIIME2 were generated based on default settings. In order to compare abundance differences between treatment and control group, the count table of these features fit a Poisson model and only significant hits ($p < 0.05$) were used for downstream analysis. Reads for unmatched controls were analysed against the pool of all controls. This statistical analysis was done in the R environment. To confirm results from metagenomics of non-16/18S targets, host-subtracted sequence reads that were trimmed of primers and low-quality segments were assembled de novo using metaSPAdes (Nurk et al. 2017) and analysed by BLAST. To examine relationships between these results and the clinical data, all were first transformed into binary coding (0 or 1). A two-tailed, non-parametric Spearman correlation analysis was then performed.

3 | Results

3.1 | Clinical Case Presentation

A total of 52 matched pairs were obtained in addition to 23 additional cases with no matched control. Due to not meeting the target enrolment numbers, the unmatched cases were included in the study. The majority of cases (42) were from New York state. Other states included Connecticut (3), Massachusetts (1), Maryland (9), North Carolina (1), Pennsylvania (10), Rhode Island (1), Virginia (2) and Wisconsin (5). The ages of cases ranged from 2 to 29 years, with a median of 14 years. The age of control horses ranged from 1 to 35 years, with a median of 13. There was not a significant difference in age between the groups ($p = 0.4830$). The most severe signs observed by the submitting veterinarians were related to anorexia and lethargy (Figure 1). The median body temperature for FUO cases was 103.3°F (Figure 2). Ticks were known to be present on the premises for 76% of cases (56 of 74, one unanswered).

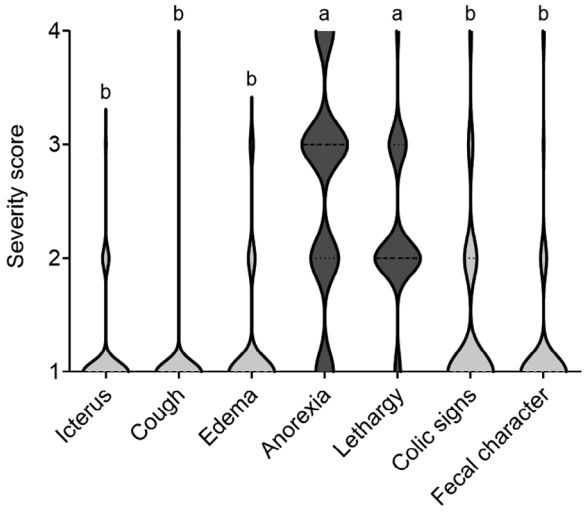

FIGURE 1 | Violin plot of clinical signs observed in fever of unknown origin (FUO) cases. Medians and quartiles are plotted as dashed lines. A score of 1 represents least severe and 4 represents most severe. Letters indicate statistically similar results ($p > 0.05$).

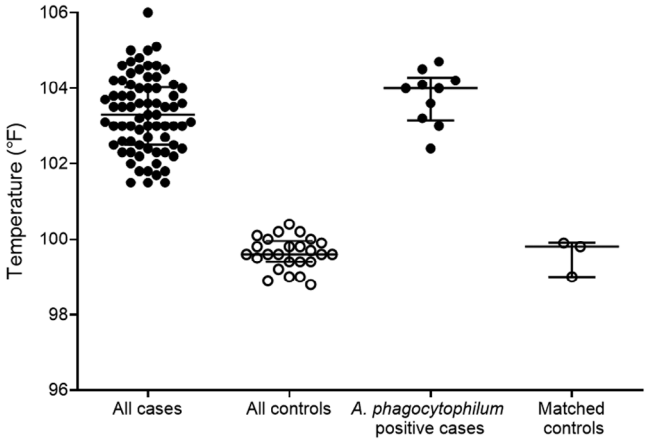

FIGURE 2 | Body temperatures of all cases with fever of unknown origin, all matched controls, only cases positive for *A. phagocytophilum*, and their matched controls. Each data point represents one horse; medians and interquartile ranges are shown as lines.

3.2 | DNA Testing

After subtraction of sequences from their matched controls, 18 cases (24%) had nothing detected by the metagenomics approach

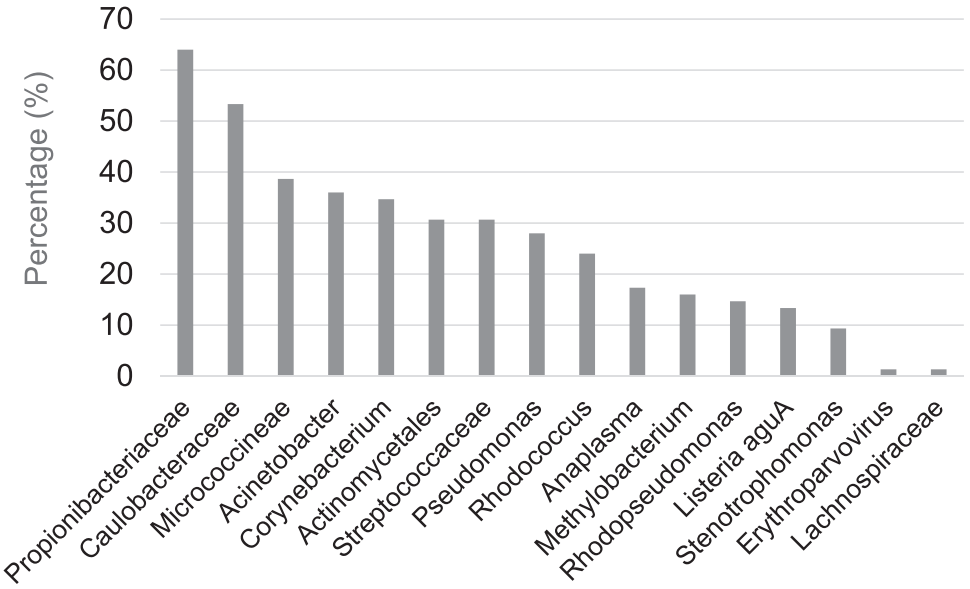

FIGURE 3 | Aggregate analysis of all microbiome data with control sequences subtracted from cases. The percent of cases for which different sequences were detected is plotted for each taxon.

in blood samples. Over half of the control-subtracted cases had sequences from *Propionibacteriaceae* in the blood (Figure 3). The highest fever (106°F) was recorded from a 4-year-old horse in Virginia (145296) with no positive PCR results. Metagenomic analyses did not reveal any significant findings in the blood or the attached tick (Table S2).

Ticks from three paired submissions were positive for *A. phagocytophilum*, and the cases associated with those ticks also tested positive for *A. phagocytophilum* (Table 2). By the metagenomic approach on ticks, *A. phagocytophilum*, *B. burgdorferi* and *Babesia microti* were detected. *Rickettsia* species were all limited to those closely related to the endosymbiont of *I. scapularis*.

On conventional PCR, no tick-borne pathogen DNA was detected in any of the control blood samples submitted. *A. phagocytophilum* was detected by PCR on blood in 10 of the 52 cases. The average temperature for these cases was 103.8 (95% confidence interval [CI], 103.3–104.3°F), compared to 99.6 (95% CI, 98.3–100.8°F) for the controls (Figure 2). Only three of the matched pairs had an *A. phagocytophilum* positive case. When comparing temperatures of all *A. phagocytophilum* positives versus the rest of the cases (no controls were positive), with the hypothesis that *A. phagocytophilum* positives were higher in temperature, the result was marginally significant ($p = 0.046$). Lethargy and anorexia were the most common presentations in the *A. phagocytophilum* positive cases (each with a median severity score of 2 out of 4).

Horses 147074 and 147752 had sufficiently high *A. phagocytophilum* levels that we were able to obtain the complete sequence of all seven genes of the MLST scheme. For sample 145294, we obtained the complete sequence of five genes (*pheS*, *glyA*, *fumC*, *mdh*, *dnaN*) and the partial sequence of two genes (*sucA*, *atpA*). All three horses were from New York state. The allelic profile for samples 145294 and 147074 was identified as ST161 (Table 1). For 147074, all the genes were an exact match to those of ST161. ST64 was identified as closely related to these two samples, with only a different allelic group (gene *sucA*). The allelic profile for sample 147752 was identified as ST218 (Table 1). No other closely related allelic profiles were identified. The pairwise similarity of the sequence of the seven concatenated genes of 147752 and 147074 was 98.1%. The sequences of the five genes obtained for both 145294 and 147074 were identical.

Neither *Leptospira* spp. nor *Bartonella* spp. were detected in any samples. Nested PCR testing identified two cases positive for *Neorickettsia risticii*, one from a matched pair and one unmatched case. The paired case was from Maryland, with a temperature of 102.6°F and anorexia and lethargy noted. The unmatched case was from New York and presented with a temperature of 103°F, anorexia and lethargy. The metagenomic analysis supported this result with a significantly higher number of reads ($p < 0.05$) mapping to the *Neorickettsia* genus present in the case versus control.

Babesia sp. were identified in 6 blood samples using the amplicon metagenomic approach (Table 2). One case (147752) had a partial 18S sequence that could either be from *B. microti* or *Babesia cf. microti*, thus it was designated as *Babesia microti* group (Harris 2016).

Through metagenomic amplicon sequencing, an *Erythroparvovirus* was detected in sample 150458, a 13-year-old gelding from New York state that had recently travelled to Kentucky. Subsequent untargeted DNA sequencing of the original blood sample produced a 5.1 kb contiguous sequence that confirmed the presence of an *Erythroparvovirus* genome closely related (76.1% similarity) to *Primate erythroparvovirus 1* (*Human parvovirus B19*), and a relatively lower similarity to *Ungulate erythroparvovirus 1* (*Bovine parvovirus 3*), which, despite having a lower e-value (2e-32 compared to 8e-18 for Human B19), showed a slightly less percentage identity of 75.2%. We have proposed to name this new species *Equine erythroparvovirus 1* (Yu et al. 2025). The untargeted shotgun sequencing also revealed a relatively

TABLE 1 | Allelic profiles for *A. phagocytophilum*.

Allelic profile	*pheS*	*glyA*	*fumC*	*mdh*	*sucA*	*dnaN*	*atpA*	Clonal complex	Multilocus sequence typing (MLST) cluster	Match to obtained sequences
145294	42	32	28	18	82	28	26	5	1	
Match	exact	exact	exact	exact	Partial (incomplete gene)	exact	Partial (incomplete gene)			
147074	42	32	28	18	82	28	26	5	1	
Match	exact	exact	exact	exact	exact	exact	exact			
147752	104	78	70	54	95	77	59	12	8	
Match	exact	exact	exact	exact	exact	exact	exact			
Exact or closely related allelic profiles										
ST161	42	32	28	18	82	28	26	5	1	Exact to 145294 and 147074
ST64	42	32	28	18	40	28	26	5	1	Closely related to 145294 and 147074
ST218	104	78	70	54	95	77	59	12	8	Exact to 147752

The exact or closely related allelic profiles found in the database are also shown.

TABLE 2 | Number of sequences from *Babesia* sp., with closest matches in reads per million.

	147752 Case	148293 Case	150458 Case	150462 Control	154817 Case	156668 Case
Babesia microti group	12	0	0	0	0	0
Babesia gibsoni	0	0	12	0	20	0
Babesia odocoilei	0	9	0	22	0	0
Babesia bigemina	0	0	0	0	0	11

Results based on amplicon metagenomics.

high level (5000 reads per million, rpm) of *Enterococcus faecium* in the blood of this case. PCR screening using primers targeting the NS1 gene verified the presence of this putative novel *Erythroparvovirus* in one additional case: (99441, 9-year-old from North Carolina) and two controls (107803, 8-year-old from Maryland and 147754, 15-year-old from the same area of New York as 150458, Figure 4). The first case had a temperature of 101.5°F and no other clinical signs. The second case was reported as having a 102.5°F temperature and moderate limb swelling (tendons not visible); another horse on the property also had limb swelling.

To compare control-subtracted taxonomic groupings and clinical parameters for the cases, a non-parametric correlation analysis was performed on binary-coded results. *Anaplasma* PCR and next-generation sequencing (NGS) results were highly correlated, as expected (p = 3.99E−29, 95% CI, 0.8544–0.9411). *Anaplasma* detection by NGS was less-well correlated with a history of ticks within the last 14 days (p = 0.03, 95% CI, 0.01780–0.4623). The only taxonomic group correlated with any clinical sign was *Rhodopseudomonas*, which had a mildly protective effect for anorexia, lethargy and colic signs (all p = 0.02; widest CI −0.4843 to −0.04624).

3.3 | RNA Testing

Three horses (105431, 121845 and 104975) had faint bands visualised by pan-CoV PCR using the Watanabe et al. assay. RNA sequencing of those samples did not yield coronavirus sequences. Horse 105431 had no remarkable findings. Horse 121845 had high levels of *Candida parapsilosis* (38,728 rpm) and *Rhodococcus fascians* (53,223 rpm). Horse 48 had 23,566 rpm of *Rhodococcus fascians*, 26,786 rpm of *Escherichia coli* and 9373 rpm of *Streptococcus mutans* as well as low levels of Sacbrood virus.

Untargeted, metagenomic RNA sequencing was performed on one additional randomly selected matched pair. The matched pair (148293) had no virus sequences detected in either the case or control. *I. scapularis* sequences were detected in both

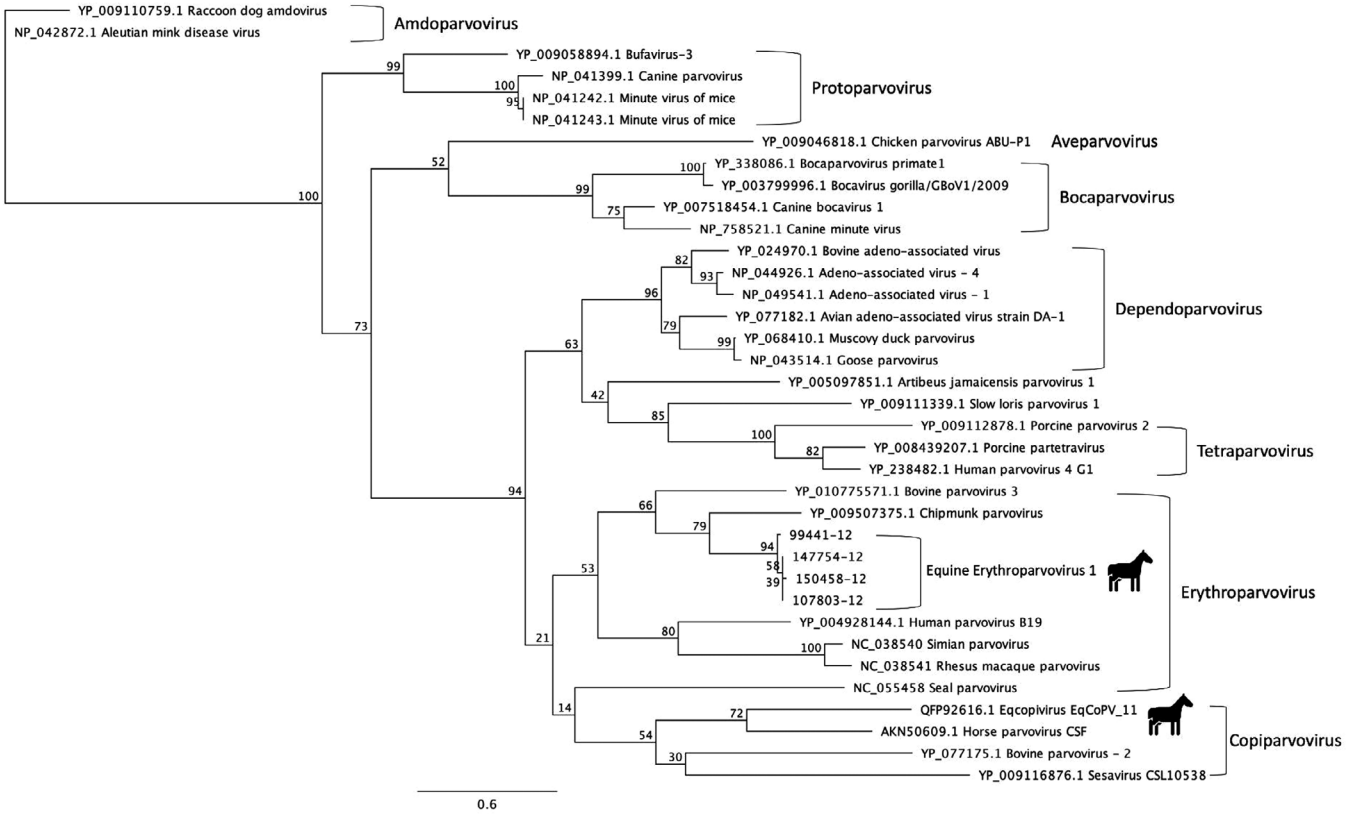

FIGURE 4 | Codon-aware phylogeny of Parvovirus NS1 with amino acid sequences of the four blood samples from this study and 32 sequences from other genera of viruses in the family *Parvoviridae* using PhyML method with 100 Bootstrap resamplings. Bootstrap values are indicated at each node, and the scale bar indicates genetic distance. Horse icons are placed next to sequences from equines.

of these blood samples. The case had very high levels of *A. phagocytophilum* (298,747 reads per million, rpm), 672 rpm of *Streptococcus pneumoniae*, 64 rpm of *Ehrlichia chaffeensis* and 54 rpm of *Clostridium tetani*. The highest taxon abundance in the matched control was an uncultured *Streptococcus* sp. (11,749 rpm). The control also had a much lower level of reads matching *A. phagocytophilum* (23 rpm).

3.4 | ECoV Serology

Serum titres for ECoV were not significantly different between groups. The highest OD450 values (3.095 and 2.936) were obtained from cases 105431 and 120435, both 23-year-old horses. Using the OD cutoff of 1.958 reported by Kooijman et al., 21 cases and 14 controls (30% and 26%) were seropositive. Using run-specific cutoffs, 46 cases and 43 controls (65% and 80%) were seropositive (Figure 5).

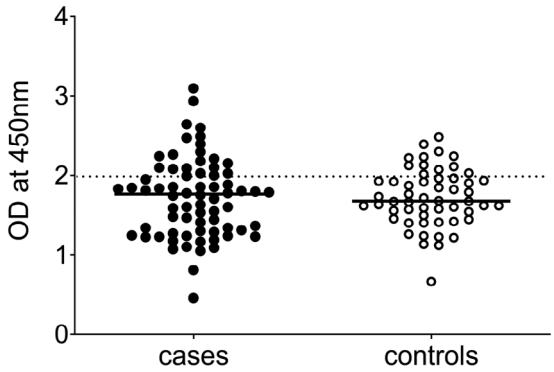

FIGURE 5 | Serum titres for equine coronavirus (ECoV). Individual animals are plotted with medians (solid bars) and the 1.958 OD450 nm cut-off value (dashed line).

4 | Discussion

Our hypothesis that TBD taxa would be primarily identified in FUO cases turned out to be only partially correct. By using a very broadly targeted approach for bacterial pathogens and endoparasites, we sought to identify novel or unexpected pathogens. Initial findings prompted additional testing of the banked samples for coronaviruses. While several unexpected taxa were identified that merit further study, determining the underlying cause of gut microbe signatures in the blood was limited by our only having blood samples available. Future studies would benefit from collecting faecal samples and urine in addition to blood, as not all pathogens replicate sufficiently in peripheral blood to be detectable by molecular methods. Paired serology would also be very beneficial in order to assess recent infectious that could have contributed to the pathology of the acute fever.

Fevers are commonly reported by horse owners in areas with high tick prevalence, especially in temperate regions such as the Northeastern United States (Divers et al. 2018a; Magnarelli et al. 2000; Rochlin and Toledo 2020). Animals (or humans) having

FUO do not always have a history of tick bites or attachment, which is consistent with our observations here. In veterinary medicine, there is no standardised definition for FUO, and the temperature ranges for case definition may vary by species (Grobman et al. 2018; Mair et al. 1989). Many horses present acutely with non-specific clinical signs of partial to complete anorexia, icterus, lethargy and leg oedema, with a fever ranging from 102 to 106°F. Other horses present with less commonly described signs such as central nervous system or gastrointestinal signs. Although gastrointestinal illness was not included in our case definition, and indeed no cases had diarrhoea, we interestingly noted an association of microbial taxa in the blood with abnormal faecal character. This is consistent with a perturbation of the intestinal barrier as a cause of FUO.

Viruses can disrupt the gut lining, and the presence of very high antibody titres to ECoV in some of our cases is suggestive of recent infection. ECoV is a positive-stranded RNA virus, belonging to the genus of *Betacoronavirus*, causing pyrexia, anorexia and lethargy predominantly in adult horses older than 2 years of age (Kambayashi et al. 2021; Miszczak et al. 2014; Pusterla et al. 2013, Pusterla et al. 2018; Zhang et al. 2007), while colic, diarrhoea and other gastrointestinal signs can also be observed in approximately 10% of clinically affected horses (Pusterla et al. 2013, Pusterla et al. 2018). In this study, approximately 80% of the disease group were reported to have anorexia and lethargy by the submitting veterinarians. While ECoV can cause viremia, it is typically not detected in the blood. RNA extraction from frozen blood samples could also be problematic, for instance, due to degradation by enzymes released from destroyed blood cells through the thawing process of frozen EDTA blood samples (Beekman et al. 2009), or long-term storage at −80°C (Shabihkhani et al. 2014).

Parvoviruses are widespread among many animal species (François et al. 2016; Allison et al. 2013). Following the serendipitous detection of an *Erythroparvovirus* in one blood sample, presumably due to it having a high enough level in the blood that carried through the metagenomic bacterial and parasite-targeted amplifications, we specifically targeted its NS1 gene for PCR screening of all blood samples from this study. Among the four positive samples identified, two originated from the control group and two from horses presenting with FUO. The presence of this virus in both the control and case groups provides insufficient evidence to link the virus directly to any particular clinical sign. The virus is closely related to human Parvovirus B19, which has not been reported in horses (Figure 4). It is not closely related to the equine parvovirus hepatitis virus (EqPV-H), which is in the *Copiparvovirus* genus (Divers et al. 2018b). The symptoms of B19 in humans include fever, rash, sore throat and joint pain. Further studies are needed to characterise the potential pathogenicity and epidemiology of the virus identified here.

Serological tests are adopted to detect antibodies induced by infection and vaccination, and are important tools for seroepidemiological surveillance throughout disease outbreaks. Immunoglobulins of the G class (IgG) are the most frequently selected targets for serological tests and they are usually produced by the organism after approximately 2 weeks since the contact with the pathogen or vaccine antigen. Common serological testing techniques include enzyme-linked immunosorbent assay (ELISA), indirect immunofluorescence assay (IFA) and Western blot (Theel 2016). The low positive predictive value and inherent time window between the host's exposure to a pathogen and a detectable immune response makes serologic testing less useful during an acute onset fever in horses, as in human (Divers et al. 2018a; Liu et al. 2020).

The agents that cause FUO in humans are mostly well known, including infectious and non-infectious ones. The FUO agents in equines, however, are mainly discussed within the scope of infectious agents, tick-borne pathogens and ECoV included. In some cases, fever may be a result of tick bite inflammation. The most prevalent tick-borne pathogen detected here, in close to 20% of cases, was *A. phagocytophilum*. This is a zoonotic pathogen of a wide variety of mammalian hosts (Atif 2015). Allelic profile ST161, found in two horses, matches three human granulocytic anaplasmosis isolates reported in 1997 and 1999 (Langenwalder et al. 2020; Dunning Hotopp et al. 2006; Huhn et al. 2014). The closely related ST64 contains 15 isolates reported in the United States between 1995 and 2016 in humans and a dog with granulocytic anaplasmosis, as well as in two rodents (meadow jumping mouse, *Zapus hudsonius* and chipmunk, *Tamias striatus*) without any clinical signs (Langenwalder et al. 2020; Huhn et al. 2014; Scharf et al. 2011; Majazki et al. 2013; Goodman et al. 1996; Kolbert et al. 1997; Munderloh et al. 1999; Barbet et al. 2013. In contrast, ST218 contains a single isolate from an *I. scapularis* tick collected in 2003 in the United States (Al-Khedery and Barbet 2014; Langenwalder et al. 2020) Infections with *A. phagocytophilum* in horses lead to EGA, a transient infection featuring fever, lethargy, inappetence, ventral oedema, petechiae, icterus, ataxia, recumbency, muscle stiffness and occasionally even death (Butler et al. 2008; Russell et al. 2021). Although readily detected in blood by molecular tests, EGA is prone to be under-diagnosed because sick horses are likely to recover without treatment, and there are resemblances of clinical signs between EGA and other diseases (Russell et al. 2021). We found evidence of co-infection of *A. phagocytophilum* and *Babesia* sp., although the low relative abundances suggest that the *Babesia* infections may have been chronic rather than acute (e.g., horse 147752 with 738,000 rpm *A. phagocytophilum* vs. 12 rpm *Babesia microti*).

Species of *Babesia* cause piroplasmosis/babesiosis in horses, cattle, dogs, sheep, camels and humans (Sang et al. 2021). Although there is one report of *B. gibsoni* in a horse in India (Karnik et al. 2022), the other species detected here have not been reported in horses to our knowledge. Additional targeted surveillance is warranted to confirm these findings. In humans living in the eastern United States, co-infection with *B. microti* and *B. burgdorferi* is most commonly observed, followed by *A. phagocytophilum* and *B. burgdorferi* (Belongia 2002; Hersh et al. 2014). Neuroborreliosis, uveitis and cutaneous pseudolymphoma can occur in horses infected with *B. burgdorferi* (Divers et al. 2018a). In reported equine cases of neuroborreliosis, neurological disorders, such as spinal cord ataxia and paresis, are often observed, while fever is usually absent (Divers et al. 2018a). Horses do not develop characteristic manifestations such as erythema migrans rash as humans do in early stages of infection of *B. burgdorferi*. Therefore, any clinical illness does not become apparent until spirochetes have disseminated. Finally, horses in these targeted geographic areas are also commonly infected with *Neorickettsia*, yet only three cases with this specific agent were identified. This may have been due to vaccination,

although we did not collect vaccination histories as part of this study.

As bacterial culture and sequencing methods are constantly advancing, the characteristics of the blood microbiome are beginning to be elucidated (Castillo et al. 2019). Viable bacteria have been identified in the blood fractions of plasma and RBCs in freshly drawn blood (Damgaard et al. 2015). One disadvantage of PCRs is the restricted number of agents detected for each set of PCRs due to the high specificity of primers. However, metagenomics sequencing techniques have made it possible for researchers to define and quantify the taxonomic profiles of microbiomes, which has ameliorated the problem that most microorganisms cannot be grown in the lab (Hugenholtz and Tyson 2008). Hence, microbiomes present in various systems and tissues within human and animal bodies have been defined and characterised in recent decades, usually achieved through metagenomics methods. There are two methods generally used to characterise taxonomic profiles by metagenomics. The first method is shotgun sequence-based, through which all DNA or RNA recovered from a sample is sequenced, thus demonstrating the compositional properties of the origin genomes (Venter et al. 2004). Shotgun sequencing of circulating cell-free DNA from the blood is now commonly used to identify infectious pathogens in liquid biopsy specimens and inform cancer treatment (Chen et al. 2021; Poore et al. 2020; Tong et al. 2022). The other method is focused on the sequencing of amplicons corresponding to partial to complete phylogenetic marker genes, for example, 16S rRNA (Mande et al. 2012). The conservation of the 16S rRNA gene sequence was first noted in 1965 (Dubnau et al. 1965), and it has been used for decades as the gold standard for bacterial taxonomic purposes due to its universality in bacteria, allowing the comparison of differentiated organisms at the genus level (Clarridge 2004). Being clustered together into operational taxonomic units (OTUs) at 97% similarity, the sequence-based clustering of 16S rRNAs can often provide information on microbial differentiation below the genus level (Huse et al. 2012).

One major problem common to targeted and untargeted metagenomics sequencing to evaluate the microbial components is the presence of contaminants, which could lead to inaccurate taxonomic analysis and data interpretation (Haas et al. 2011; Janda and Abbott 2007). Bacterial DNA contamination can disproportionally affect the data interpretation of microbial communities with low biomass (Davis et al. 2018; Glassing et al. 2016; Salter et al. 2014). Another challenge is the noisy background created by host genetic material and colonising microorganisms in blood samples, making it challenging to identify the true pathogens (Zhang et al. 2022). These limitations are significant when attempting to adopt these techniques for routine clinical diagnostics. With appropriate controls, we were able to successfully remove experiment-wide background noise as evidenced by some cases having no detectable microbial taxa.

Cell-free DNAemia and certain bacterial DNA have been observed and well described in healthy blood (Gosiewski et al. 2017; Kowarsky et al. 2017; Nikkari et al. 2001), attributed to the translocation of bacteria that previously existed in the gastrointestinal tract, the oral cavity or outside of the body (Benítez-Páez et al. 2013; Li et al. 2012). The translocation routes are mainly through Peyer's patches, aggregated lymphoid follicles that allow microbes to translocate between gut epithelium and the blood system (Jung et al. 2010). While the term 'dysbiosis', also known as 'dysbacteriosis', refers to significant gut microbiota changes that result in microbiota imbalance, the term 'atopobiosis' more accurately indicates the presence of microorganisms in the wrong place (Potgieter et al. 2015). Both dysbiosis and atopobiosis of the gut microbiome can be associated with chronic, inflammatory diseases and cancer progression in humans (Capuco et al. 2020; Coker et al. 2018; Liu et al. 2020; McDonald et al. 2016; Potgieter et al. 2015). Gut dysbiosis in COVID-19 patients has been linked to secondary bloodstream infections by gut bacteria, resulting from the altered gut epithelium and the immunocompromising effects of SARS-CoV-2 infection (Bernard-Raichon et al. 2022).

As there is no common healthy blood microbiome (Tan et al. the Assistant Secretary of Defense for Health Affairs through the Tick-Borne Disease Research Program, endorsed by the Department of Defense under Award No. W81XWH-22-1-0891 to LBG and MD. Opinions, interpretations, conclusions and recommendations are those of the authors and are not necessarily endorsed by the Department of Defense 2023), at least for humans, dysbiosis is very challenging to assess directly from blood. The results from this study suggest that tick-borne agents are present in the bacteriome of approximately one-third of febrile horses from the regions studied. Environmental surveillance of ticks from affected areas can supplement clinical testing to help guide treatment decisions, as well as monitoring for viral agents for which diagnostics are available. As horses are exposed to many of the same pathogens as their owners, this study provides foundational data and methods for continued development of pathogen discovery approaches that could benefit both people and animals.

Author Contributions

Yining Sun: Investigation, Formal analysis, Writing—original draft. **Y. Tina Yu**: Data curation, Investigation, Writing—original draft, Formal analysis. **Ximena Olarte Castillo**: Formal analysis, Writing—review & editing, Supervision. **Renee Anderson**: Investigation, Methodology, Formal analysis, Data curation. **Minghui Wang**: Methodology, Formal analysis. **Qi Sun**: Methodology, Supervision, Formal analysis. **Rebecca Tallmadge**: Investigation, Methodology, Formal analysis, Writing—review & editing. **Kelly Sams**: Supervision, Writing—review & editing, Project administration. **Guillaume Reboul**: Formal analysis, Writing—review & editing. **Jordan Zehr**: Formal analysis, Writing—review & editing. **Joel Brown**: Formal analysis, Writing—review & editing. **Xiyu Wang**: Formal analysis, Writing—review & editing. **Nicholas Marra**: Formal analysis, Writing—review & editing. **Bryce Stanhope**: Investigation, Data curation. **Jennifer Grenier**: Formal analysis, Supervision. **Nicola Pusterla**: Methodology, Formal analysis, Writing—review & editing. **Thomas Divers**: Conceptualisation, Writing—review & editing. **Linda Mittel**: Conceptualisation, Funding acquisition, Writing—review & editing, Methodology. **Laura B. Goodman**: Writing—original draft, Supervision, Resources, Visualisation, Investigation, Conceptualisation.

Ethics Statement

No research animals were used in this study, and all data were collected for clinical care with informed consent. The authors declare that they have adhered to the Principles of Veterinary Medical Ethics of the AVMA.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are openly available in NCBI BioProject PRJNA1084068.

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Supporting Information

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