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Infectious disease surveillance of apparently healthy horses at a multi-day show using a novel nanoscale real-time PCR panel

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Abstract. In the United States, horses are used for a variety of purposes including recreation, exhibition, and racing. As farm, performance, and companion animals, horses are a unique species from a zoonotic disease risk perspective, and the risks of subclinical infections spreading among horses can pose challenges. Using a nanoscale real-time PCR platform, we investigated the prevalence of 14 enteric pathogens, 11 *Escherichia coli* genes, and 9 respiratory pathogens in fecal samples from 97 apparently healthy horses at a multi-day horse event. In addition, sugar flotation test was performed for fecal parasites. *E. coli f17* was commonly detected, prevalent in 59% of horses, followed closely by *Streptococcus equi* subsp. *zooepidemicus* (55%). Additional pathogens recognized included betacoronavirus, *Campylobacter jejuni, Cryptosporidium* sp., *E. coli* O157, equine adenovirus 1, equine rhinitis B virus, and others. The use of PCR data may overestimate the true prevalence of these pathogens but provides a sensitive overview of common pathogens present in healthy horses. Our results prompt the continued need for practical biosecurity measures at horse shows, both to protect individuals interacting with these horses and to minimize transmission among horses.

Key words: equine infectious diseases; equine surveillance; horses.

Introduction

In the United States, there are >7.2 million privately owned horses, which are used for a variety of purposes including recreation, exhibition, and racing.[1] Participation in these activities means horses are often trailered to new locations and brought into close proximity to unfamiliar horses, raising the potential risk for disease transmission.[35] The spread of infectious disease agents between horses poses threats to animal welfare, increases the risk of lost training or competition time, and highlights the need for enhanced biosecurity.[26] Additionally, each year, it is estimated that ~150 million people attend state and county fairs, where they may come into close contact with horses and other animals.[28] The mixing of people and animals in settings with minimal sanitation undeniably raises concerns for zoonotic diseases caused by pathogens such as *Cryptosporidium* sp., *Clostridioides difficile*, and *Salmonella*.[39] For example, a 1999 outbreak of *Escherichia coli* O157:H7 was traced to a county fair and also led to the identification of several patients with *Campylobacter jejuni*,[30] presumably from fair attendance. In another outbreak of *E. coli*, horse manure and other animal feces were considered the most likely source of the bacteria.[25] Interestingly, in a petting zoo setting, 2 of 12 horses tested positive for *E. coli* O157:H7.[15] In a sample of 25 horses used for 4-H, all of the animals were negative for *Salmonella, Campylobacter*, and enterohemorrhagic *E. coli*; 2 horses, however, were positive for *C. difficile*.[27] Understanding the prevalence of various infectious disease agents in apparently healthy horses has the potential to protect both horses and people in contact with horses as well as aid in the identification and prediction of disease outbreaks.

Questions have been raised regarding whether subclinical viral infections pose risks for equine health and performance.[3] The intent of our study was not to correlate pathogen burden and show performance, but rather to quantify the pathogen shedding via feces in apparently healthy horses. However, all of the horses in our study were subject to travel and other potential stressors, which have the potential to dampen the immune system and allow pathogen shedding.[7] Using a nanoscale real-time PCR (rtPCR) platform, we

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investigated the prevalence of 14 enteric pathogens, 11 *E. coli* genes, and 9 respiratory pathogens in fecal samples of 97 apparently healthy horses at a multi-day horse show. The enteric pathogens included: betacoronavirus, *Campylobacter coli*, *C. jejuni*, *Clostridium perfringens*, *C. difficile*, *Cryptosporidium parvum*, *Cryptosporidium* sp., *Giardia lamblia*, *Listeria monocytogenes*, *Listeria* sp., equine rotavirus A (VP4), bovine rotavirus (VP6), *Salmonella* sp., and *Salmonella enterica* subsp. *enterica* serovar Typhimurium. Specific *E. coli* genes or virulence factors screened for included *O157, sta, stx1, stx2, 987p, cnf1, eae, f17, f41*, and *k99*. Additionally, the presence of *Shigella* and enteroinvasive *E. coli* (*EIEC*) was evaluated. Respiratory pathogens included: influenza A virus, equine adenovirus 1 (EAdV-1; *Equine mastadenovirus A*), equine adenovirus 2 (EAdV-2; *Equine mastadenovirus B*), equine rhinitis A and B viruses (ERBV; *Equine rhinitis A virus*), equine herpesvirus 1 (EHV-1; *Equid alphaherpesvirus 1*), equine herpesvirus 4 (EHV-4; *Equid alphaherpesvirus 4*), *Streptococcus equi* subsp. *equi*, and *Streptococcus equi* subsp. *zooepidemicus*. Many of these pathogens have been associated with disease in horses, for example *E. coli f17* has been suggested as a cause of equine diarrhea[11]; are potential emerging pathogens, such as betacoronavirus[33]; or pose potential zoonotic disease risks.[37] Additionally, using a sugar flotation test, we screened for endoparasites, including strongyles, *Strongyloides westeri*, *Oxyuris equi*, and *Parascaris* sp.

Materials and methods

Participant recruitment and sample collection

Convenience, noninvasive fecal samples were obtained from 100 horses at a show in August 2018 in the northeastern United States. Permission from owners and/or handlers was obtained orally, and information collected on each horse included age, sex, breed, and number of days at the show. Information regarding most recent deworming, distance traveled, diet, or where individual animals were normally housed was not collected. No information regarding individual farms and/or barns was collected. All horses were considered clinically healthy, as they had met the requirement for exhibition, which included a certificate of veterinary inspection (CVI) if coming from out of state; a rabies vaccine if >105 d old, administered prior to arrival; and a negative equine infectious anemia virus (Coggins) test within the previous year for all horses >6 mo old. Individual fecal samples were collected from the horse's stall and aliquoted into 2 plastic bags. Samples were stored at −20°C for PCR quantification and at 4°C for parasite detection.

Laboratory methods

A routine Wisconsin double-centrifugation sugar flotation test was performed on each sample, following the standard protocol of the Parasitology Laboratory at the New York State Veterinary Diagnostic Laboratory/Cornell Animal Health Diagnostic Center (AHDC; Ithaca, NY).

For direct molecular analysis, a 400-mg subsample of feces from each horse was homogenized in 800 µL of phosphate-buffered saline (PBS). Homogenates were centrifuged for 5 min at 18,000 × *g*, and 175 µL of the supernatants were extracted (MagMAX total nucleic acid isolation kit, Applied Biosystems; Kingfisher Flex, Thermo Fisher Scientific). The manufacturer's instructions were altered with an added mechanical lysis step of 2 × 2.5 min with zirconia beads at 2,100 oscillations per min in a Mini-Beadbeater-96 (BioSpec Products), with a 5-min rest between. Bacteriophage MS2 was added to the lysis buffer as an internal control to monitor for inhibition and extraction efficiency.[9,41] Two negative extraction controls, consisting of PBS, were included on each extraction plate. Nanoliter-scale rtPCR was performed (QuantStudio 12K Flex OpenArray platform; Thermo Fisher Scientific) using a previously described customized respiratory panel[13] and a modified version of another panel[5] (Suppl. Table 1). Positive amplification control pools consisting of in vitro transcribed RNA for betacoronavirus, ERVB, equine rotavirus A, and influenza A virus; genomic DNA from purified *C. parvum* oocysts (Waterborne); genomic DNA from *S. enterica* serovar Cerro, *Campylobacter* sp., and *Shigella flexneri*; and ~450-bp long synthetic gBlock DNA fragments for all other targets (Integrated DNA Technologies) were run on each plate along with a negative amplification control (purified water). Each nanoscale PCR reaction was performed in duplicate (enteric panel) or triplicate (respiratory panel), and samples were considered positive if at least 1 (enteric) or 2 (respiratory) technical replicates produced a properly shaped amplification curve.

Salmonella enrichment PCR was performed on all samples as described previously.[14] *E. coli* culture using gram-negative broth enrichment and eosin methylene blue agar was also performed on the 2 cryopreserved fecal samples that had the highest abundance of the gene encoding the *E. coli* O157 antigen. Whole-genome sequencing (WGS) was performed from a single colony and also attempted from enrichment broth from each sample. DNA was extracted (MagMAX CORE kit; Thermo Fisher Scientific). Barcoded libraries were prepared (Nextera XT library preparation kit; Illumina). Reads from WGS of colonies were assembled using SKESA.[34] Serovar prediction was performed both on assembled and unassembled reads using ECtyper (https://github.com/phac-nml/ecoli_serotyping) and SRST2[19] with the EcOH[18] database, respectively. Virulence factors were searched using the NCBI Pathogen Detection isolates browser (https://www.ncbi.nlm.nih.gov/pathogens/). The raw data from each of the sequenced colonies was uploaded to NCBI under Biosamples SAMN13429602 and SAMN13429521. SRST2 was also used to identify serotype markers in unassembled reads from the enrichment broth sequencing.

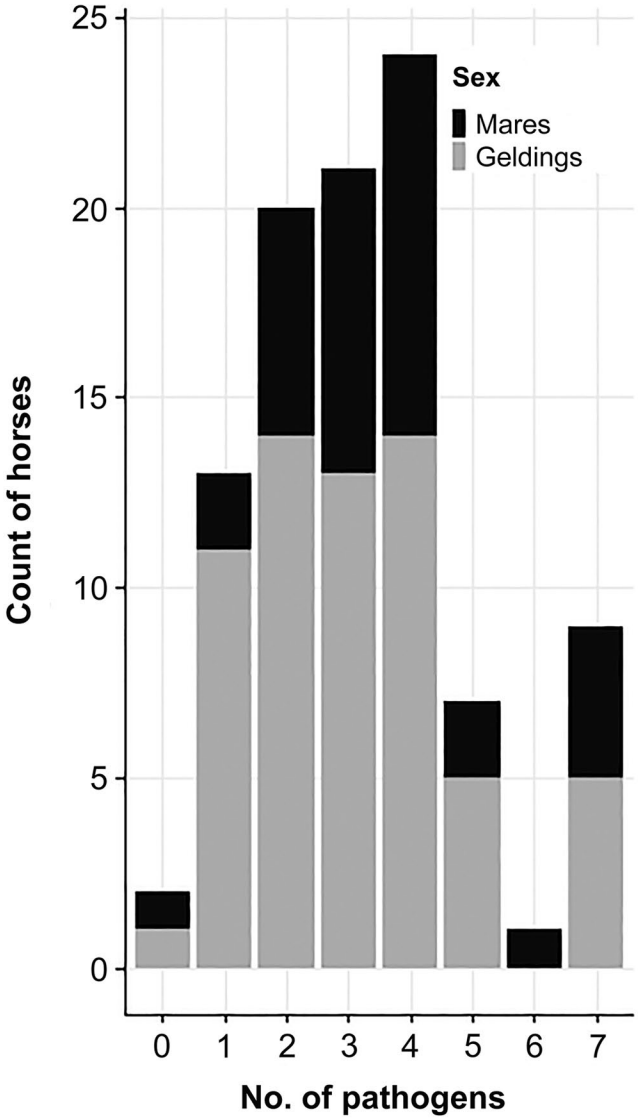

Figure 1. Total pathogen burden in horses participating in a multi-day event ($n=97$). The mean number of pathogens detected was 3.26.

Data analysis

All analyses were performed in R Studio (v.3.6.0; https://rstudio.com/). Three horses, of 100 sampled horses, were eliminated from analysis because of incomplete data. Given the large number of breeds that were present in the study (15), the decision was made to classify horses as draft breed or light breed. The draft breeds included Percherons, Belgians, Shires, and Clydesdales. Light breeds included Miniature Horses, Saddlebreds, Morgans, Quarter Horses, a Thoroughbred, a Shetland Pony, a Paso Fino, a Paint, a Friesian, and 2 mixed breeds. The number of days at the show were grouped into a dichotomous variable, which included ≤3 d and >3 d. The horse ages were also classified as a dichotomous variable, consisting of horses ≤10 y old and those >10 y old. Additionally, all parasites were considered either as positive or below the limits of detection. Likewise, all other pathogens were considered either as positive or not detected. A point prevalence was established for each pathogen, and a 95% confidence interval was determined using the prop.test function in R Studio. Parasite or pathogen presence was additionally stratified by breed classification, sex, the dichotomous days at event variable, and the dichotomous age variable to identify nonrandom associations, with p values calculated using a 2-sided Fisher exact test. A $p \leq 0.05$ was considered significant.

Results

Demographics

We included 97 horses in our study. The mean age was 10.2 y (SD = 7.2). Most horses were <10 y old (63%); 34% were ≥10 y old. Geldings were more common (65%) than mares (35%). The mean days at the event was 3.8 (SD = 1.4), with 43% of the horses present at the event ≤3 d and remainder present >3 d. The most common breeds included Percherons (20), Belgians (19), Miniature Horses (17), and American Saddlebreds (12); 49% of the horses were draft breeds and 51% were light breeds. The breed classification and dichotomous age variable were highly associated ($p = 0.00007$). In the draft breeds, 87% were classified in the younger age category, compared to the light breeds in which only 45% were in the younger age category. The average age among the draft breeds was 6.5 y; the average age of the light breeds was 13.8 y. No other demographic variables were considered to be significantly associated ($p > 0.05$).

Pathogen burden

Most of the horses in our study were positive for at least one pathogen (Fig. 1). On average, each horse had 3.26 pathogens (SD = 1.73), with the median number of pathogens being 3. A small cluster of horses was positive for 7 pathogens.

Four parasites were detected via sugar flotation: *Eimeria leuckarti*, *Anoplocephala* sp., strongyles, and *Parascaris* sp. *Cryptosporidium* sp., *Strongyloides westeri*, *Oxyuris equi*, and *Dictyocaulus* sp. were not detected in any of the samples. Strongyles were the most common parasite identified, detected in 46 of 97 horses (95% CI: 37.3–57.8%). Among the samples in which strongyles were detected, the average egg count was 912 (SD = 1,110) eggs/g (epg) and the median fecal egg count was 318 epg. Strongyles were more common among those in the younger age category ($p = 0.011$). Strongyles were also detected more frequently in mares (odds ratio [OR] = 2.46), but this association was not significant ($p = 0.055$). Adjusting for breed classification, age was weakly associated with strongyles (Mantel–Haenszel OR: 2.79; 95% CI: 1.06–7.37). *E. leuckarti* was identified only in

Table 1. Prevalence of enteric pathogens detected in 97 equine fecal samples by nanoscale real-time PCR.

Enteric PCR target	Count	95% CI
Betacoronavirus	1	0.05–6.4
Campylobacter coli	1	0.05–6.4
Campylobacter jejuni	16*	10.1–25.7
Clostridium perfringens	2	0.4–8.1
Cryptosporidium sp.	1	0.05–6.4
Giardia lamblia	17	10.8–26.9
Equine rotavirus (VP4)	1	0.05–6.4
E. coli O157	31†	23.1–42.3
E. coli Sta	1	0.05–6.4
E. coli cnf1	13	7.6–22.2
E. coli eae	18	11.7–28.0
E. coli f17	57‡	48.3–68.5

All samples were negative for *Clostridioides difficile, Cryptosporidium parvum, Listeria, Salmonella, Shigella*, and *E. coli stx1, stx2, 987p, f41, k99*, and LT.
* 7 of 16 were positive in 1 of 2 replicates.
† 1 of 31 was positive in 1 of 2 replicates.
‡ 1 of 57 was positive in 1 of 2 replicates.

4 geldings (95% CI: 1.3–10.8%), and an association between sex and *E. leuckarti* was apparent ($p = 0.013$). *Parascaris* sp., identified in 10 horses (95% CI: 5.3–18.6%) was associated with draft breeds ($p = 0.0077$), with 9 of the positive animals classified as draft breeds. Adjusting for age, however, this association was not statistically significant ($p = 0.066$). *Anoplocephala* sp. was identified in 5 horses (95% CI: 1.9–12.2%) and did show any apparent associations with factors assessed.

No samples were positive for *Salmonella* either on direct feces or from enrichment. A total of 7 enteric pathogens were detected: betacoronavirus, *C. coli*, *C. jejuni*, *C. perfringens*, *Cryptosporidium* sp., *G. lamblia*, and equine rotavirus A. Pathogens that were tested for, but not amplified, included *C. difficile*, *C. parvum*, *Listeria* spp., bovine rotavirus, and *Salmonella* sp. Additionally, 5 *E. coli* virulence genes were identified via direct fecal PCR (Table 1). Specific pathogens or genera included *sta*, *cnf1*, *eae*, and *f17*, and the O-antigen gene encoding *E. coli* serotype O157. *E. coli* factors that were tested, but not amplified, included *stx1*, *stx2*, *987p*, *f41*, and *k99*. *Shigella*/EIEC was not detected. *E. coli f17* was commonly detected; 59% of horses tested positive. The O157 serotype was also detected frequently, with matches in 32% of the horses. Days at event did not appear to impact shedding of any of the enteric pathogens (all $p > 0.18$). *E. coli cnf1* was associated with draft breeds ($p = 0.0075$; age-adjusted $p = 0.023$). The exogenous internal control was detected in all samples, with a SD of 1.5 detection cycles.

E. coli colonies from the 2 horses with the highest O157 detection from feces (horses 23 and 55 with relative cycle threshold values of 13.9 and 12.2) did not match the O157 serotype by WGS. Instead, they were predicted to be O115:H10 and O93:H19, respectively. Similarly, a sequence corresponding to the O157 serotype was not detected in the aerobic enrichment broth incubated with feces from each of these horses. Both isolates encoded *lpfA* and *espX1*, and one also had a partial match to *fdeC*.[29] No other *E. coli* strains in the NCBI Pathogen Detection database clustered phylogenetically with these 2 isolated strains.

Three pathogens with respiratory tropism were detected in feces directly: EAdV-1 in 1 horse (95% CI: 0.05–6.4%), ERBV in 38 horses (30.5–50.7%), and *S. equi* subsp. *zooepidemicus* in 53 horses (44.2–64.7%). Additional pathogens that were tested for, but not detected, included influenza A, EAdV-2, equine rhinitis A, EHV-1, EHV-4, and *S. equi* subsp. *equi.* No relationship between age, days at event, or sex was identified. *S. equi* subsp. *zooepidemicus* was associated with light-breed horses ($p = 0.042$; age-adjusted $p = 0.023$).

Discussion

The novel application of nanoscale rtPCR for molecular enteric pathogen detection in feces allows for efficient assessment of a large group of pathogens and to assess the prevalence of these pathogens in apparently healthy horses. The top 3 breeds in our study were Percherons (20 of 97), Belgians (19 of 97), and Miniature Horses (17 of 97), whereas the most common horse breeds in the United States are Quarter Horses, Paints, and Thoroughbreds.[21] Thus, this was a unique sample to understand potential pathogen differences in less common breeds of horses, including breeds that may be used for special purposes. Draft breeds appeared to be associated with higher levels of the gene encoding *E. coli* cytotoxic necrotizing factor 1 (age-adjusted OR: 6.92; 95% CI: 1.2–38.9) and potentially lower levels of *S. equi* subsp. *zooepidemicus* than the light breeds in our study (age-adjusted OR: 0.15; 95% CI: 0.03–0.81). The only horse that had betacoronavirus detected was an 8-y-old draft mare, present at the event for 3 d; this was also the only animal positive for *Cryptosporidium* sp. and was additionally positive for strongyles, ERBV, *C. jejuni*, and *S. equi* subsp. *zooepidemicus*. Whether this high pathogen burden was the result of increased exposure or an underlying immune deficiency is unknown.

Sex did not appear to be highly associated with fecal pathogen shedding in our study. Geldings may be more likely to have *E. leuckarti* (OR: 5.2). Interestingly, the *E. leuckarti* prevalence observed in our study is considerably lower than previously reported prevalences of 27.5–59.1% in studies conducted in Kentucky and Montana.[10] The prevalence of *E. leuckarti* of 4.12% that we observed follows most closely the studies in Albania, the Czech Republic, Greece, and Turkey.[10] These differences may be the result of a variety of factors, including age.

Although the Wisconsin double-centrifugation sugar fecal flotation test is an excellent method for identifying most parasites that void their life stages in host feces, limitations exist for detecting certain parasites including *Giardia*. Our use of nanoscale rtPCR is apparently more sensitive than classical

parasitology techniques, and it amplified genetic material from *Cryptosporidium* sp. and *G. lamblia*, both of which are of zoonotic significance.

S. equi subsp. *zooepidemicus* has often been considered a commensal respiratory pathogen in horses, although it can be present in high levels in respiratory fluids.[2] A specific strain was implicated in one outbreak of upper respiratory disease.[24] Additionally, concerns have grown about the potential spread of this subspecies from horses to people.[31] The high prevalence of *S. equi* subsp. *zooepidemicus* may prompt a commitment to minimizing group housing across species of animals. *S. equi* subsp. *zooepidemicus* has previously been reported to cause disease in an alpaca,[16] cats,[4] dogs,[32] and dairy goats.[22]

Of 11 total *E. coli* genes that we analyzed in direct feces, 5 were detected. *E. coli f17* was the most common, with a 59% prevalence. Enteropathogenic *E. coli* in horses was initially characterized as associated with F41-type pili.[38] *F17* and other fimbriae are associated with pathogenic infections in ruminants. In a small study, 3 of 10 horses with diarrhea and 0 of 14 healthy horses were *f17* positive.[11] Importantly, that study characterized isolated colonies as opposed to direct PCR on feces.[11] A wide diversity of *E. coli* strains is expected to exist in the healthy gut, as opposed to clinical cases that may have a predominant strain causing disease. Even multiple colonies chosen from a plate may not be representative of the overall diversity of this highly variable species in one host. At the time of sampling, no horses were experiencing diarrhea. If involved in pathogenesis of *E. coli* diarrhea in horses, our findings suggest that *f17* may not act alone to elicit diarrhea and may require comorbidities.

Other *E. coli* genes detected included O157, *cnf1*, *eae*, and *Sta*. Genes that were not detected included *stx1*, *stx2*, *987p*, *f41*, *k99*, and *Shigella*/EIEC. In our study, 32% of the horses were positive for the gene encoding *E. coli* O157; given that H antigen detection was not performed, it is unclear what proportion of these samples (or proportion of strains within each animal) were positive for *E. coli* O157:H7. Colonies selected for further characterization serotyped differently, which was not unexpected. Sampling of horses in Ohio for *E. coli* O157:H7 demonstrated a low prevalence of the pathogen (~0.4%).[23] The clinical significance of *cnf1* remains unknown; this virulence factor has been noted in both a healthy horse and a horse experiencing diarrhea,[11] but also in an equine patient with bronchopneumonia.[8] In humans, *E. coli cnf1* is often considered uropathogenic.[36] The *eae* gene encoding the adhesin intimin has been reported in up to half of isolates from diarrheic foals, but also in some healthy horses.[6,17,20] The presence of *E. coli* O157 harboring *eae* but lacking the Stx-encoding bacteriophage is a potential zoonotic concern, especially if clinical testing is focused solely on detection of the *stx* genes.[12]

Most of the draft horses in our study were ≤10 y old, compared to the lighter breeds, which were more evenly aged. This may in part be the result of the competition purposes for which these different breeds are used. Draft breeds in our study were most likely to be participating in driving and pulling events and thus handled by adults. Light breeds participated in a range of events, including events in which youth participants may be competing and for which it would be desired to have an older, calmer horse. Understanding the individuals who may be interacting with specific breeds or certain aged horses may be important when considering the risks of zoonotic disease potential. This may be even more important to consider for those who are immune compromised. The basics of biosecurity and personal hygiene may be the best method to protect other horses and individual people, regardless of the scenario.

We did not detect EHV-1, EHV-4, or influenza A virus. Although these are common equine pathogens, their presence in fecal samples is not expected. The respiratory picornavirus ERBV, however, is acid-stable and can be found in feces.[40] Additionally, the use of voided fecal matter is a convenient opportunity for sampling; however, the use of a nasal swab or wash, utilizing nanoscale rtPCR, may be better suited for diagnostic evaluation. We did not detect *Salmonella*, indicating that subclinical shedding may be infrequent. Nonetheless, in the event of concerns about any of these pathogens, appropriate testing would be warranted. Although our study sample is meant to be representative of healthy horses, the prevalence of these pathogens may vary between geographic locations. The presence of other animals, water sources, individual immune status, and travel history could be other factors contributing to prevalence differences.

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Supplementary material

Supplementary material for this article is available online.

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