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Infectious agents associated with diarrhoea in neonatal foals in central Kentucky: A comprehensive molecular study

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Summary

Reasons for performing study: Diarrhoea caused by infectious agents is common in foals but there is no comprehensive molecular work-up of the relative prevalence of common agents and appearance of coinfections.
Objectives: To determine the prevalence of 9 infectious agents in gastrointestinal (GI)-diseased and healthy foals with ages ranging from 1 to 20 weeks of age and to what degree coinfections are associated with clinical signs of GI disease.
Study design: Retrospective controlled observational study.
Methods: The population consisted of 88 Thoroughbred foals aged 2 days to 17 weeks born on 32 different studfarms in Kentucky. Healthy (n = 37) and GI-diseased (n = 51) foals were identified based on clinical presentation. Faecal samples were analysed for 9 infectious agents by real-time PCR: equine rotavirus, equine coronavirus, *Clostridium difficile* toxins A & B, *Neorickettsia risticii*, *Clostridium perfringens* alpha toxin, *Lawsonia intracellularis*, *Rhodococcus equi*, *Cryptosporidium* spp., and *Salmonella* spp. *Salmonella* was also cultured from overnight selenite enrichment broth.
Results: The prevalence of infectious pathogens under study was between 0% (*Lawsonia intracellularis*) and 34.6% (equine rotavirus). The overall prevalence for any infectious agent was 63.2% in the GI-diseased group and 43.2% in the healthy group. Coinfections were significantly more frequent in the sick group (15 monoinfections vs. 22 coinfections) than in the healthy group (12 vs. 4, respectively, P = 0.0002). Six of the 8 infectious agents were associated with the GI-diseased group, the other 2 were not (equine coronavirus and *R. equi*).
Conclusions: The use of panels rather than individual tests in combination with quantitative toxin gene analysis enables detection of coinfections significantly associated with risk of disease. Several infectious diseases previously not tested for or considered unimportant were found at high prevalence and require further investigation.

Keywords: horse; foal; diarrhoea; infectious; real-time PCR; *Cryptosporidium*; equine coronavirus; coinfection

Introduction

Aetiological diagnoses are considered to be a priority by equine clinicians because this facilitates early decisions on the patient's care and management and allows timely discussion of measures to prevent disease spread. Recently, there have been rapid developments in both the understanding and characterisation of existing and new equine infectious agents and the development of rapid, comprehensive and affordable molecular diagnostic tools [1–3].

Within the first 6 months of life, up to 20% of foals have episodes of diarrhoea caused by infectious agents [4]. There is a limited understanding of the relative prevalence of common agents and the mechanism of coinfections. In this study we used molecular tests to screen for 9 infectious agents or their toxin genes that are able to induce or at least contribute to foal diarrhoea [5] including real-time PCR assays for equine rotavirus (ERV), equine coronavirus (ECoV), *Clostridium difficile* toxin A (CDTA), *C. difficile* toxin B (CDTB), *Neorickettsia risticii* (Potomac Horse Fever, PHF), *Clostridium perfringens* alpha toxin (CPA), *Lawsonia intracellularis*, *Rhodococcus equi*, *Cryptosporidium* spp., and *Salmonella* spp. (PCR on selenite enrichment broth and culture). A subset of samples were also tested for rotavirus using a commercial rotavirus rapid immunoassay with human specificity. These tests were used to determine the prevalence of monoinfections and coinfections in healthy and GI-diseased foals and to explore associations with age, outcome or clinical data.

Materials and methods

Animals and sample collections

Informed consent was obtained from the owner or owner's agent for all foals included in the study. Faecal samples (approximately 5 g) were collected from 88 Thoroughbred foals aged 2 days to 17 weeks born on 32 different studfarms in central Kentucky. Depending on the initial clinical presentation, 2 groups of foals were identified: GI-diseased (n = 51 from 30 farms) and healthy foals (n = 37 from 5 farms). Gastrointestinal-diseased animals were defined as having watery diarrhoea for greater than 24 h of duration, decreased milk consumption and/or ultrasonographic evidence of enterocolitis. Foals with ultrasonographic evidence of excessive fluid in the colon and/or small intestine and a history of fevers, colic or anorexia were classified as GI diseased. Foals were included in the healthy group if they had no clinical evidence of diarrhoea, fever or inappetence before, or at the time of, faecal collection. Five grams of fresh faecal material was stored in faecal containers, kept at 4°C and sent overnight to the diagnostic laboratory on blue ice. Faecal samples were processed immediately at the laboratory upon arrival. Total nucleic acid extractions and real-time PCR was performed as a comprehensive equine diarrhoea panel at a commercial laboratory (Test code 2911: Equine diarrhea RealPCR panel - comprehensive)[a].

Sample preparation

Total nucleic acid extraction protocols were used according to the manufacturer's recommendation (Corbett X-tractor Gene)[b]. An aliquot of the total nucleic acid was used to reverse transcribe the ribonucleic acid (RNA) portion into complementary DNA (cDNA) for the RNA applications (rotavirus and coronavirus) adapted from previously published protocols [6]. Genomic DNA (gDNA) and cDNA were used for real-time PCR according to published protocols [7].

Real-time PCR assay design

All real-time PCR assays were designed and validated to use the same PCR reaction conditions and reagent concentrations to allow analysis of all PCR targets and quality controls on the same 384 well plate [8]. Selection of primers and hydrolysis probes was performed using a design software (PrimerExpress 3.0, Applied Biosystems). Amplification was carried out on a Roche Light Cycler (LC) 480 instrument[c] using the default amplification protocol: 2 min at 50°C, 10 min at 95°C and then 45 cycles of 10 s at 95°C, 20 s at 60°C and 1 s at 72°C. Crossing points (CP) were calculated using the

2nd derivative maximum method analysis module with the high sensitivity algorithm. Real-time PCR tests included PCR primers and a 6-FAM - TAMRA quenched conventional hydrolysis (TaqMan) probe[d] [8]. Commercially available PCR reagents were used for the PCR amplification (Roche LightCycler 480 Probes Master)[c]. Outside primers allowing confirmation of positive PCR results by sequencing of the target region were designed for all PCR tests. These outside sequencing primers were also designed in PrimerExpress to guarantee uniform amplification. Target genes for the real-time PCR tests were the following: ERV: VP4 (GenBank accession number EU717544) and VP6 (L49043), ECoV: M gene (EF446615), CDTA: toxin A gene (X60984), CDTB: toxin B (X60984), PHF: 16 s rRNA (AF194082.1) [9], *C. perfringens*: alpha toxin (L43545), *Lawsonia intracellularis*: aspartate ammonia-lyase (*aspA*, AM180252), *R. equi*: virulence associated protein A gene (*vapA* AF116907) [10], *Salmonella* spp.: invasion A gene (invA, EU348366). Equine specific PCR tests including equine rotavirus and coronavirus and have been clinically validated to have high analytical sensitivity and specificity [5].

Real-time PCR assay validation

Analytical validation of the 10 real-time PCR was carried out as described [5–7,9,11]. Briefly, 8 criteria were evaluated with new primer and hydrolysis probes in order to characterise the analytical performance of the test: 1) amplification efficiency (95–105%); 2) points on the standard curve (5 or more); 3) intrarun coefficient of variation (CV) using cycle point values from the LC480 PCR equipment; 4) intrarun CV of absolute particle numbers calculated from the CP values; 5) inter-run CV using cycle point values from the LC480 PCR equipment; 6) inter-run CV of absolute particle numbers calculated from the CP values; 7) r square value of the standard curve and 8) signal to noise ratio of fluorescent signal generation (equal or higher than 10-fold). For the standard curves generated on clinical material, re-sequencing was performed around the real-time PCR product using outside primers to confirm analytical specificity (Table 1). Real-time PCR tests were redesigned if they did not pass the analytical validation step until they passed all 8 criteria.

Quality controls

Seven quality controls were run with each diagnostic sample, including 1) PCR positive controls, 2) PCR negative controls, 3) negative extraction controls, 4) DNA preanalytical quality control targeting the host ssr rRNA (18S rRNA) gene complex, 5) RNA preanalytical quality control targeting the host ssr rRNA gene complex, 6) an internal positive control spiked into the lysis solution and 7) an environmental contamination monitoring control. These controls assessed the reliability of the PCR protocols (1 and 6), absence of contamination in the reagents (2) and laboratory (7), cross contamination during the extraction process (3), quality and integrity of the DNA and RNA as a measure of sample quality (4 and 5), RT-protocol (5) and absence of PCR inhibitory substances as a carryover from the faecal matrix (6).

Immunoassay

Thirty-five samples were tested with a rapid immunoassay with specificity for human rotaviruses (ImmunoCard STAT! Rotavirus)[e] and used according to the manufacturer's package insert instructions.

Microbiological cultures

For the *Salmonella* culture, approximately 1 g of faeces was inoculated into 8 ml of selenite broth and incubated aerobically at 35°C for 18–24 h. Following incubation, subcultures were carried out on Hektoen plates and suspicious black or clear colonies are streaked to Triple Sugar Iron Agar (TSI) and Lysine Iron Agar (LIA). If the biochemical tests were suspicious for *Salmonella*, they were identified on a Vitek[f].

TABLE 1: Results of the analytical validation of the 10 real-time PCR assays for the equine diarrhoea panels using synthetic positive controls and clinical gDNA and cDNA

		Acceptable range								
		95–105%	≥5	<3%	<20%	<3%	<20%	>0.993	≥10	Done
				Reproducibility						
				Intrarun		Inter-run				
Target		AmpEff	Linearity (log)	CV(CP)	CV(ABS)	CV(CP)	CV(ABS)	r^2	S/N Probe	Re-sequencing
Analytical	Equine rotavirus	105%	7	0.21	4.46	0.54	12.18	0.9997	>10	nd
	Equine coronavirus	96%	6	0.28	6.73	0.28	17.89	0.9996	>10	nd
	C. difficile toxin A	95%	5	0.50	7.39	0.76	17.98	0.9996	>10	nd
	C. difficile toxin B	99%	6	0.82	7.02	0.66	15.57	0.9999	>10	nd
	Neorickettsia risticii	103%	9	0.31	3.30	0.75	15.08	0.9988	>10	nd
	C. perfringens alpha toxin	95%	8	0.44	4.89	0.65	14.60	0.9998	>10	nd
	Lawsonia intracellularis	99%	6	0.44	4.89	0.65	14.60	0.9998	>10	nd
	Rhodococcus equi	101%	7	0.57	7.74	0.60	10.29	0.9999	>10	nd
	Salmonella spp.	98%	6	0.96	12.51	0.37	7.81	0.9989	>10	nd
	Lawsonia intracellularis	99%	6	0.44	4.89	0.65	14.60	0.9998	>10	nd
Clinical	Equine rotavirus	105%	7	0.21	4.46	0.26	5.86	0.9997	>10	done
	Equine coronavirus	99%	8	0.55	8.85	0.41	9.27	0.9986	>10	done
	C. difficile toxin A	95%	5	0.27	5.78	0.70	15.08	0.9970	>10	done
	C. difficile toxin B	98%	6	0.80	11.42	0.64	13.63	0.9932	>10	done
	Neorickettsia risticii	95%	5	0.32	5.04	0.46	9.27	0.9990	>10	done
	C. perfringens alpha toxin	102%	7	0.44	9.64	0.59	11.21	0.9988	>10	done
	Lawsonia intracellularis	99%	5	0.81	19.76	0.49	11.21	0.9936	>10	done
	Rhodococcus equi	95%	7	0.39	7.85	0.91	20.37	0.9996	>10	done
	Salmonella spp.	98%	6	0.96	12.51	0.37	7.81	0.9989	>10	done
	Lawsonia intracellularis	95%	5	0.25	5.23	0.32	6.83	0.9999	>10	done

AmpEff = amplification efficiency; CV = coefficient of variation calculated with raw data (CP values) or absolute values (ABS); CP = crossing point; PCR cycle at which fluorescent intensity becomes detectable; ABS = absolute values calculated from CP values by transforming against a standard curve; S/N = signal to noise ratio of the fluorescent signal generation; nd = not done.

TABLE 2: Prevalence of monoinfections and coinfections in 37 healthy foals and 51 foals with gastrointestinal (GI) disease in central Kentucky. The infection per animal was calculated using total recorded infections

	Monoinfection	Coinfection	Ratio M* vs. C	Total infections	Infection per animal
Healthy foals	37%	11%	3:1	**20**	0.63
GI-diseased foals	29%	42%	1:1.6	**69**	1.33

*M = monoinfection; C = coinfection.

Data analysis

The overall prevalence of faecal shedding was determined and infection rates for age and farm location calculated. Statistical analysis, contingency tables and (2-tailed) P values (Fisher's exact test) were entered and calculated using GraphPad Prism® 5. The adjusted McNemar's w2-test [12] was used to compare the proportion of positive results and detectability (proportion of valid results) of the 2 sample preparation methods.

Results

Study population

The GI-diseased population had a median age of 6.5 weeks of age (2 days to 17 weeks) and the healthy foal population a median age of 7.5 weeks (3–15.5 weeks, P = 0.4). Of the GI-diseased foals 44% (23/52) presented to the Hagyard Equine Medical Institute for supportive care while 56% (29/52) had supportive care at their breeding farm. Two of the GI-diseased foals had recently undergone celiotomy for correction of small intestine volvulus. Both foals developed diarrhoea 24 h after presentation to the hospital. Overall, 50 of the 51 GI-diseased foals survived (98%) and all healthy foals survived. The majority of the healthy foal population came from 2 breeding farms which accounted for 89% (33/37) of the group. Those 2 breeding farms also had 10 animals classified as GI-diseased. The faecal collection of the healthy foals occurred 2 weeks and 10 days, respectively on these 2 farms before a GI-diseased foal was detected. The foal that did not survive presented at one week of age with severe sepsis, colitis and hypovolaemic shock and died within 2 h of presentation and was PCR positive for CDTA, CDTB and CPA genes in its faeces, the only foal to have all 3 toxin genes identified in a single sample.

Prevalence data

Overall, the presence of infectious agents was significantly associated with the GI-diseased group: in the GI-diseased foals, a total of 69 positive results were recorded (1.33 infectious events per animal) while the healthy animals tested positive in 20 tests (or 0.54 infections per animal; P<0.0001). The most frequent infectious agents isolated from healthy foals were ECoV (n = 10), *R. equi* (n = 5), *Cryptosporidium* (n = 4) and ERV (n = 1). In the GI-diseased group, the most frequent infectious agent was ERV (n = 18) followed by ECoV (n = 15) and *Cryptosporidium* spp. (n = 14) (Tables 1, 2).

Two infectious agents, ERV and *Salmonella* spp., were significantly associated with GI-diseased foals (Table 3). Equine rotavirus had the highest prevalence in the GI-diseased group (35% vs. 3%, odds ratio [OR] 19.6, P = 0.0048; Table 1) followed by ECoV (29% vs. 27%), *Cryptosporidium* (27% vs. 11%), *Salmonella* spp. (14% vs. 0% by PCR and 8% vs. 0% by culture, OR 12.6, P = 0.09), CDTA, CDTB, *E. risticii* and CPA were not statistically associated with disease. *Lawsonia intracellularis* was not expected to be present in the population under study. *Rhodococcus equi* shedding (positive for the virulence gene *vapA*) was found in both groups.

Rates of coinfection

The healthy group had significantly fewer coinfections (11%) than monoinfections (37%, P = 0.04, Table 2) while this was reversed in the GI-diseased group: 29% monoinfections vs. 42% coinfections (total of 71%, P = 0.035). The ratio of mono- to coinfections was 3:1 in the healthy group and 1:1.6 in the GI-diseased group. The GI-diseased foals had significantly more coinfections than healthy foals (42% vs. 11%, P = 0.0018). Coinfections in the GI-diseased group included 10 foals with 2 infectious agents, 8 foals with 3 and one foal with 4 infectious agents.

Viral infections

Of the 18 positive ERV PCR tests in the GI-diseased group, 11 were coinfected with other agents (Table 4). Coinfection with *Cryptosporidium* was the most frequent coinfection, followed by bacterial coinfections. Only one foal was monoinfected with ERV in the healthy group.

Ten ECoV infections were recorded in the healthy group of which 8 were monoinfections and only 2 coinfections with either *R. equi* or *Cryptosporidium* spp., respectively. In contrast, all 15 recorded ECoV infections in the GI-diseased group were associated with coinfections (P<0.0001). The ECoV coinfections were most frequently associated with *Cryptosporidium* (n = 8), ERV (n = 7) and Salmonella (n = 2). Six of the ECoV infections were triple infections: 4 in combination with ERV and *Cryptosporidium*, one with CPE and *Cryptosporidium* and one with CDTA/B and PHF.

Immunoassay for rotavirus

Analysis of 35 samples with the human specific rotavirus immunoassay resulted in no positive results. Within that group of samples, 13 were positive by real-time PCR. Real-time was confirmed by resequencing VP4 and VP7 genes with outside primers. Sequences showed 98% identities to equine rotavirus isolates deposited in GenBank. These results suggest that the immunoassay with human specificity may not detect some of the equine isolates.

Protozoal infections

Four foals in the healthy group were positive for *Cryptosporidium* spp. (11%) and 3 of these were coinfected: one with ECoV and 2 with *R. equi*. In the GI-diseased group, 14 foals were positive (27%) of which 10 were coinfected with one or 2 additional agents. Of the 10 coinfections, 8 were

TABLE 3: Prevalence of infectious agents found in 37 healthy foals and 51 foals with gastrointestinal (GI) disease in central Kentucky. All infectious agents were detected with equine specific real-time PCR tests; *Salmonella* was also identified by culture. Odds ratio were calculated based on contingency tables and exact P values calculated using Fisher's exact test

	Real-time PCR tests										Culture
	Equine rotavirus	Equine coronavirus	*C. difficile* toxin A	*C. difficile* toxin B	*Neorickettsia risticii*	*C. perfringens* alpha toxin	*Lawsonia intracellularis*	*Rhodococcus equi*	*Cryptosporidium* spp.	*Salmonella* spp.	*Salmonella* spp. culture
Healthy foals	3%	27%	0%	0%	0%	0%	0%	14%	11%	0%	0%
GI-diseased foals	35%	29%	6%	6%	4%	6%	0%	8%	27%	14%	8%
Odds ratio	19.6	1.1	5.4	5.4	7.9	5.4	-	0.5	3.1	12.6	8.9
P	0.0048	0.80	0.27	0.27	0.17	0.27	-	0.39	0.1	0.09	0.14

TABLE 4: Counts of total number of infections and number of mono- and coinfections for each infection agent found in 37 healthy foals and 51 foals with gastrointestinal (GI) disease in central Kentucky. Infectious agents were detected by either real-time PCR (all) and culture (*Salmonella* spp. only)

		Real-time PCR										Culture
Group	Type of infection recorded	Equine rotavirus	Equine coronavirus	*C. difficile* toxin A*	*C. difficile* toxin B*	*Neorickettsia risticii*	*C. perfringens* alpha toxin	*Lawsonia intracellularis*	*Rhodococcus equi*	*Cryptosporidium* spp.	*Salmonella* spp.	*Salmonella* spp.
Healthy	Monoinfection	1	8	0	0	0	0	0	3	1	0	0
	Coinfection	0	2	0	0	0	0	0	2	3	0	0
	Total number of infection calls	1	10	0	0	0	0	0	5	4	0	0
GI-diseased	Monoinfection	7	0	2	2	1	0	0	1	2	3	3
	Coinfection	11	15	1	1	1	3	0	3	12	4	1
	Total number of infection calls	18	15	3	3	2	3	0	4	14	7	4

*Presence of *C. difficile* toxins A and B not considered a coinfection.

with ECoV and 7 with ERV; 7 foals were triple infected with the following combinations: 4 foals with ERV and ECoV, one with ERV and *R. equi*; one with ECoV and CPE; one with ECoV and *Salmonella*.

Bacterial infections

Salmonella spp. were only detected in the GI-diseased foal group; 4 were detected by culture and were also positive by PCR. Real-time PCR detected infection in 3 additional foals. Two of the discrepant positive signals were sequenced with outside primers and confirmed to be positive for *Salmonella Typhimurium*. The third sample did not yield sufficient sequence information due to a very low positive signal. Four of the 7 *Salmonella* positive foals were coinfected (57%) with either ERV, ECoV, CPA or *Cryptosporidium* spp. One of the *Salmonella* positive foals was triple infected with ECoV and *Cryptosporidium* spp. and a second one with *Cryptosporidium* spp. and ECoV. *Clostridium perfringens* alpha toxin DNA was found in 4 foals, 3 were coinfected with CDTA&B, one with ECoV and *Cryptosporidium* spp. and one with *Salmonella* spp. and *Cryptosporidium* spp. *C. difficile* toxins A and B were detected in 6% of GI-diseased animals only. Genes for toxins A and B both were present in the positive animals. One *C. difficile* positive foal was coinfected with *Neorickettsia risticii*, one foal was infected only with *C. difficile*. Two foals were infected with *Neorickettsia risticii* and one was coinfected with CDTA&B and ECoV.

Discussion

Many infectious diseases are multifactorial and complex mutual interactions may contribute together to severity and reduce survival [13]. In equine veterinary medicine, coinfections and the consequent interactions contributing to intensify GI disease have not been as well documented.

Epidemiological data on infectious agents causing equine diarrhoea is incomplete and fractionated. This study provides a prevalence overview of 9 infectious agents in foals in central Kentucky. The 9 infectious agents belong to 3 groups of infectious agents including viruses, bacteria and protozoa. Among the viruses, *group A* rotaviruses (GARVs) are thought to be the main cause of diarrhoea in foals up to 3 months of age. Equine GARVs have been identified in the faeces of foals with GI disease in the UK [14], the United States of America [15,16], Australia [17], New Zealand [18], Ireland [19] and Japan [20]. Serological data from several countries, indicate that equine GARVs are ubiquitous. Our ERV prevalence in Central Kentucky of 36% was comparable with a recent report of 20% prevalence in foals in Florida [18]. Concurrent infections of rotavirus and *Salmonella* was described by Eugster *et al*. [21]. This coinfection was also found in the current study (n = 1) but other coinfections were significantly more frequent: rotavirus was mostly associated with equine coronavirus (n = 7) and *Cryptosporidium* spp. (n = 6), which are unique combinations not previously reported in the equine literature. In young children with acute watery diarrhoea, coinfections of rotavirus with other viruses (noro-, adeno and/or astrovirus) were present in 39% of the studied cases [22]. Additionally, pre-existing rotavirus infections increase the risk of bacteraemia in children with nontyphoid *Salmonella* gastroenteritis clearly indicating the type of detrimental interaction coinfecting agents may exert on the host [23]. *Cryptosporidium* coinfections have not been reported in horses but have been described in other animal species or man [24,25]. Surprisingly little information is available regarding interaction of these infectious agents in horses.

Equine samples positive for GRAV by molecular means were negative in an immunoassay with rotavirus specificity in man. The 4 strongest real-time PCR signals, in the range of the limit of detection of other commercially available lateral flow detection systems, were confirmed by sequencing, indicating that lower sensitivity of the human assay cannot explain all discrepant results. Additionally, 3 contamination quality controls were run in parallel with the diagnostic samples indicating that cross-contamination during sample preparation and pipetting of the PCR plates, PCR product carry-over of amplified material and contamination by the inappropriate use of PCR positive controls is unlikely. The discrepant results indicate that equine specific test reagents may have an advantage in detecting equine infectious agents.

Equine coronavirus was isolated and characterised only recently in 2000 [26] but described as an infectious agent in sick foals in 1975 [27]. Although this study and those of others have identified coronaviruses in foals with enteric disease, the pathogenicity and its aetiological role in enteric disease have not been examined. The current prevalence study clearly shows that healthy foals without signs of GI disease are equally often infected with equine coronavirus as foals with GI disease and suggests low pathogenicity of ECoV in foals. However, when analysed as coinfecting agent, ECoV was significantly associated with diseased animals: all ECoV infections in the GI-diseased group were associated with coinfections (15 of 15) while foals in the healthy group were mostly monoinfected (8 of 10). This could indicate that pre-existing coronavirus infections facilitate opportunistic secondary infections through neutropenia and lymphopenia of the local immune environment as is known in other species [28–30] Secondary bacterial and parasitic infections from Gram-negative and anaerobic microflora are described in other species and cause additional complications related to intestinal damage, bacteraemia, toxinaemia and altered mucosal functions [14,30]. Opportunistic infections can be of different origin, including bacterial or protozoal, as shown in this study. Coinfection studies in piglets indicate that coronavirus and bacterial coinfections have a significant effect on the magnitude of the inflammatory immune response and tissue damage compared with monoinfections [29]. In young turkeys, coronavirus and enteropathogenic *Escherichia coli* (EPEC) interact synergistically with severe growth depression and high mortality when compared with monoinfected turkeys [30]. Turkeys infected first with coronavirus and then with EPEC developed greater mortality and increased frequency of attaching and effacing lesions than that observed in turkeys inoculated with EPEC prior to turkey coronavirus or simultaneously inoculated with these agents; these observations, although in different species, could suggest an under-recognised role for coronavirus infections in foals. Clearly, additional studies are needed to determine equine coronavirus virulence factors and the relative importance as a coinfecting agent contributing to GI disease in foals.

Bacterial infections including *Salmonella* spp. *C. perfringens* and *C. difficile* were identified in the GI-diseased foal group but significant associations were not found, probably influenced by the small number of

foals in the study. *Salmonella* and *C. difficile* are well recognised causes of diarrhoea in foals [4,16,21,31]. The alpha toxin of *C. perfringens* causes necrotic enteritis in man and colitis in horses in a dose-dependent manner [32]. Nine of 17 bacterial infections in the GI-diseased group were present as coinfections, most frequently with viruses (n = 8) or *Cryptosporidium* (n = 4). *Salmonella* real-time PCR detected more positive samples than culture: only 57% of PCR positive samples were confirmed by culture. This difference was not statistically significant but confirms previous reports in the literature [33]. Resequencing with outside primers confirmed the presence of *S. typhimurium invasion A* gene sequences in 2 of the 3 discrepant samples. Real-time PCR on enrichment broth has shown excellent analytical and diagnostic sensitivity and specificity with the added advantage of shorter turnaround time than culture [34].

The current study addresses prevalence and possible interactions of infectious agents with a comprehensive panel of molecular test in the 2009 foaling season in central Kentucky. We included 3 major groups of GI infectious agents but did not include nematode infections, an aspect for future study. The current study also did not include the occurrence of noninfectious causes of diarrhoea [4,35]. However, while there is a possibility that some of the foals in the study had foal heat diarrhoea, 46 (89%) of the GI-diseased cases were greater than 2 weeks of age.

This study has limitations. Firstly, results may only reflect foals in central Kentucky and not in other geographical regions. Secondly, the majority of the foals in the healthy group came from 2 farms. Therefore the healthy group of foals may not adequately represent the foal population in central Kentucky. Nevertheless, an important result of this study is that it documented a high prevalence of coinfections in foals with GI disease which may have been overlooked by practitioners previously. However, in our study population from central Kentucky, we identified that coinfections occur more frequently in foals with GI disease than in healthy foals and therefore should be considered as a diagnostic marker for infectious GI disease. As with any farm-based study of foal diarrhoea the potential for misclassification of disease status exists but due to our comprehensive clinical criteria for classification, misclassification events in both groups are considered to be small in number.

The study demonstrates that 1) the rate of coinfections in foals has been underestimated, 2) coinfections between viruses and protozoa are more frequent than between viruses and bacteria in this particular study, 3) *Cryptosporidium* and equine coronavirus, infectious agents not considered to be of importance or not tested for in the past, were present at high prevalence and 4) equine specific real-time PCR tests allow rapid diagnosis of infectious agents contributing to diarrhoea in foals. Further research is necessary to establish the importance and role of certain coinfections in the foal, especially equine coronavirus, a newly recognised GI pathogen in horses.

Authors' declaration of interests

C. Leutenegger and M. Estrada are employees of IDEXX Laboratories, Inc.

Ethical animal research

Informed consent for sample collection was obtained from the owner or owner's agent for all foals included in the study.

Source of funding

Analysis of diagnostic sample material was performed at IDEXX Laboratories, Inc., West Sacramento, California, USA.

Authorship

The initiation, conception and planning for this study were by N. Slovis and C. Leutenegger. It was executed by N. Slovis, J. Elam and M. Estrada. The paper was written by N. Slovis and C. Leutenegger.

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