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, Cryptosporidium, 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 overall prevalence of 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), Cryptosporidium 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).

Sample preparation

Total nucleic acid extraction protocols were used according to the manufacturer’s recommendation (Corbett X-Tractor Gene) [6]. 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 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
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, 2) PCR negative controls, 3) negative extraction controls, 4) DNA preanalytical quality control targeting the host ssr RNA (18S rRNA) gene complex, 5) RNA preanalytical quality control targeting the host ssr RNA 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).

Imunoassay

Thirty-five samples were tested with a rapid immunoassay with specificity for human rotaviruses (ImmunoCard STAT! Rotavirus) 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.

### 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

| Target                  | Acceptable range | Reproducibility            | Target | Acceptable range | Reproducibility |
|-------------------------|------------------|---------------------------|--------|------------------|-----------------|
| Analytical              |                  |                           | Clinical|                  |                 |
| Equine rotavirus        | 105%             | 7                         | Ampeff | Linearity (log)  | CV(ABS)         |
| Equine coronavirus      | 96%              | 6                         | CV(CP) | CV(ABS)          | r²              |
| C. difficile toxin A    | 95%              | 5                         | 0.21   | 4.46             | 0.54            |
| C. difficile toxin B    | 99%              | 6                         | 0.28   | 6.73             | 0.28            |
| Neorickettsia risticii | 103%             | 9                         | 0.50   | 7.39             | 0.76            |
| C. perfringens alpha toxin | 95%           | 8                         | 0.82   | 7.02             | 0.66            |
| Lawsonia intracellularis| 99%              | 6                         | 0.31   | 3.30             | 0.75            |
| Rhodococcus equi       | 101%             | 7                         | 0.44   | 4.89             | 0.65            |
| Salmonella spp          | 98%              | 6                         | 0.44   | 4.89             | 0.65            |
| Lawsonia intracellularis| 99%              | 6                         | 0.96   | 12.51            | 0.37            |

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; S/N = signal to noise ratio of the fluorescent signal generation; nd = not done.
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 diarrhea 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 (3.33 infectious events per animal) while the healthy animals tested positive in 20 tests (or 0.54 infections per animal; P = 0.001). The most frequent infectious agents isolated from healthy foals were ECoV (n = 10), R. equi (n = 8), Equine rotavirus (n = 7) and Salmonella (n = 2). Six of the ECoV infections were multiple infections: 4 in combination with ERV and Cryptosporidium spp., one with CPE and Cryptosporidium and one with CDTA/B and PHF.

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 multiple infections: 4 in combination with ERV and Cryptosporidium, one with CPE and Cryptosporidium and one with CDTA/B and PHF.

Immunobssay 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
with ECoV and 7 with ERV; 7 foals were triply 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 triply infected with ECoV and Cryptosporidium spp. and a second one with Cryptosporidium spp. and ECoV. Clostridium perfingens 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 diarrhea 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 diarrhea 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 coinfected 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 diarrhea, coinfections of rotavirus with other viruses (norovirus, adenovirus and/orastrovirus) were present in 39% of the studied cases [22]. Additionally, pre-existing rotavirus infections increase the risk of bacteraemia in children with nontyphoidal 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 coinfected 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, toxinemia 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

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**TABLE 4: Counts of total number of infections and number of mono- and co-infections 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)**

| Group         | Type of infection recorded | Real-time PCR | Culture |
|---------------|----------------------------|---------------|---------|
| Healthy       | Monoinfection             | 1 8 0 0 0 0 0 0 0 | 0 0 3 1 0 0 |
|               | Coinfection               | 0 2 0 0 0 0 0 0 0 | 0 0 2 3 0 0 |
| Total number of infection calls | 1 10 0 0 0 0 0 0 0 | 0 0 5 4 0 0 |
| GI-diseased   | Monoinfection             | 7 0 2 1 0 0 0 0 0 | 0 0 1 2 3 3 |
|               | Coinfection               | 11 15 1 1 1 1 1 1 1 | 3 0 3 12 4 1 |
| Total number of infection calls | 18 15 3 3 2 3 0 0 4 | 3 0 4 14 7 4 |

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

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**Epidemiological data on infectious agents causing equine diarrhoea is surprisingly little information is available regarding interaction of these infectious agents in horses.**

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Infectious agents associated with foal diarrhoea in central Kentucky

1Qlegen, Valencia, California, USA.
2Roche Applied Science, Indianapolis, Indiana, USA.
3Eurofins MWG Operon, Huntsville, Alabama, USA.
4Meridian Bioscience, Inc., Cincinnati, Ohio, USA.
5Vitek, bioMerieux Inc., Durham, North Carolina, USA.
6GraphPad Prism, San Diego, California, USA.

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