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Foal diarrhoea between 1991 and 1994 in the United Kingdom associated with *Clostridium perfringens*, rotavirus, *Strongyloides westeri* and *Cryptosporidium* spp.

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SUMMARY

A case control study of foal diarrhoea in the United Kingdom was carried out over a 3-year period. *Clostridium perfringens* was significantly associated with foal diarrhoea (Odds Ratio (OR) = 3·0), being isolated from 57% of 421 animals with diarrhoea but from only 27% of 223 healthy foals. Also, *C. perfringens* was significantly associated with fatal diarrhoea (OR = 4·5). About half of diarrhoea with a fatal outcome was attributable to this organism.

The other pathogens significantly associated with diarrhoea were rotavirus (OR = 5·6), *Cryptosporidium* spp. (OR = 3·2) and the nematode *Strongyloides westeri*, which was significant only when present in large numbers (> 2000 eggs/g of faeces: OR = 6·1). *Salmonella* spp. (OR = 14·2) and *Cryptosporidium* spp. (OR = 3·0) were the only other pathogens associated with fatal illness.

Overall, *C. perfringens*, rotavirus, and large numbers of *Cryptosporidium* spp. or *S. westeri* were isolated from 80% of foals with diarrhoea. Thermophilic *Campylobacter* spp., *Yersinia enterocolitica*, *Escherichia coli* and other parasites were not associated with diarrhoea. Carriage of *C. perfringens*, rotavirus and *Cryptosporidium* spp. was significantly greater in healthy foals in contact with cases of diarrhoea than in foals that were not in contact with diarrhoea ($P < 0·05$). There were no statistical interactions between any of the pathogens associated with diarrhoea although separate cases from one location often involved more than one pathogen.

INTRODUCTION

As many as 80% of foals may have one or more episodes of diarrhoea during the first 6 months of life [1]. These may be life threatening if serious dehydration occurs and convalescent foals may be weakened and more susceptible to other infectious diseases [2, 3].

Many microbial causes of diarrhoea in foals have been proposed but their prevalence and significance is unclear. Although there have been many case reports of suspected pathogens, there has not been a comprehensive survey of all potential pathogens other than those affecting thoroughbreds in studs [4–17].

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It was not possible to identify a potential pathogen in 44–78% of cases of foal diarrhoea [10, 11]. Although some of these cases may have been due to non-infectious causes such as overeating, carbohydrate intolerance and/or antibiotic treatment [3], there may be infectious agents that have not yet been recognized as causes of foal diarrhoea.

Salmonella spp. and rotavirus are recognized causes of foal diarrhoea [5, 10, 11, 13, 18]. Potential pathogens, recognized in other animal species, have been isolated from individual cases including 'thermophilic' *Campylobacter* spp., *Yersinia enterocolitica* and *Cryptosporidium* spp. [5, 10, 11, 13]. *Strongyloides westeri* and other helminths infest the equine intestine but their role in diarrhoea is not clear [14]. *C.*

perfringens has been isolated from a large number of individual cases and outbreaks of foal diarrhoea [9, 12, 19–25]. Various attempts have failed to find an association between foal diarrhoea and types of *Escherichia coli* carrying well defined virulence determinants for other mammalian enteric disease [11, 13].

Diarrhoea in foals is usually treated with fluid replacement and antibiotics. Antibiotics have the potential to upset the balance of the intestinal flora and so lengthen or aggravate the existing condition and they are often given without regard to their pharmacodynamic effects in the diarrhoea [3]. There is a clear need to understand the relative importance of the potential infectious causes of foal diarrhoea in order to improve the effectiveness of diagnostic, treatment and control procedures.

The aim of the study was to investigate the associations between foal diarrhoea and various potential pathogens and their relative importance in the United Kingdom. Samples of faeces from foals with and without diarrhoea were tested for the presence of *C. perfringens*, *Salmonella* spp., rotavirus, 'thermophilic' *Campylobacter* spp., *Y. enterocolitica*, undifferentiated *E. coli*, *Cryptosporidium* spp., as well as *S. westeri* and other helminths.

METHODS

Collection of faecal samples from foals

During 1991, 1992 and 1993, faecal samples were sent by veterinary surgeons as part of a national study of diarrhoea in foals. Samples were requested from thoroughbred and non-thoroughbred foals less than one year old with and without diarrhoea. The distribution of samples collected from different breeds on different types of premises is given in Table 1. Most of the samples from East Anglia were tested on the day they were taken. Samples sent by post from the rest of the country, were tested within two days of collection. *C. perfringens* is sensitive to temperatures just above 0 °C [40] so veterinarians and stud farm managers were asked not to refrigerate samples.

Detection of pathogens

All bacteriological isolation media were purchased from Oxoid Unipath Ltd and made and used according to the manufacturer's instructions. *Escherichia coli* was isolated by culture on CLED medium incubated aerobically at 37 °C for 18 h. Thermophilic *Campylobacter* spp. were isolated on Campylobacter selective medium incubated at 42 °C for 48 h in chambers using the BBL CampyPak microaerophilic system (Becton Dickinson). *Yersinia enterocolitica* was isolated on Yersinia selective medium incubated aerobically for up to 48 h at 30 °C. *Salmonella* spp. were isolated by enrichment in selenite broth with subculture on brilliant green agar and by direct inoculation of desoxycholate citrate agar; enrichment broths were incubated at 42 °C for 24 h before subculture and culture on solid media which was aerobic at 37 °C for 48 h with daily inspection for possible salmonella colonies.

Each sample was cultured for *C. perfringens* by three direct and two enrichment methods to increase the chance of isolating these bacteria in different physiological states. The direct culture methods were: (1) direct culture: serial 10-fold dilutions of faeces in 2% w/v peptone water were spread plate inoculated onto tryptose sulphite cycloserine agar with added (0·1% w/v) lysozyme (TSC) incubated in 10% CO_2 (v/v), 10% (v/v) hydrogen and 80% (v/v) nitrogen at 37 °C for up to 4 days with daily inspection for possible *C. perfringens* colonies; dark colonies with lecithinase activity were enumerated to provide a presumptive number of *C. perfringens* present; (2) heat treatment: pre-treatment of faeces diluted to 10% (v/v) in sterile distilled water at 70 °C for 20 min followed by addition of sodium ethylene diamine tetracetic acid (EDTA) to 20 mM and further incubation at 45 °C for 1 h before culture on TSC plates as described under direct culture and (3) alcohol shock treatment: pre-treatment of faeces diluted to 10% (v/v) in 10% (v/v) ethanol at 70 °C for 10 min before culture on TSC as described above.

The enrichment methods were (1) simple enrichment: suspension of faeces in Robertson's Cooked Medium to 10% (w/v or v/v according to consistency) followed by 18 h anaerobic incubation at 37 °C before subculture on TSC as described above and (2) heat enrichment: as enrichment with the additional step of heating the faeces suspension at 70 °C for 20 min before incubation.

Bacteria were identified using colony morphology and standard biochemical tests [25]. In particular, *C. perfringens* was identified by its characteristic colony formation, its fermentation reactions towards lactose, sucrose, glucose, and salicin, and the production of lecithinase and gelatinase [25].

Rotavirus was detected by Slidex Rota-Kit Monoclonal used according to the manufacturer's instructions (bioMerieux). *Cryptosporidium* spp. oocysts

Table 1. *Distribution of samples between thoroughbred and other foals and those at stud or at other premises*

Premises	Breed	Diarrhoea	No. of foal faecal samples	No. of sampling groups	Range of sampling group size (mean)
Studs	Thoroughbred	+	185	73	1–17 (1·9)
		−	161	37	1–11 (5·9)
	Other breeds	+	39	12	1–16 (2·6)
		−	55	8	1–21 (4·7)
Other premises	Thoroughbred	+	34	33	1–2 (1·2)
		−	2	2	1–2 (1·5)
	Other breeds	+	107	104	1–4 (1·1)
		−	5	3	1–2 (1·3)

Sampling group: samples taken from foals at a particular location separated by a gap of 3 weeks from any other sample taken from that location.

were detected on auramine phenol stained smears and equivocal cases [26–28] confirmed by an immunofluorescence method (DetectIF Cryptosporidium; Northumbria Biologicals). This method of detection was semi-quantitative; less than 5 oocysts per field of view at a magnification of 200× was scored as 1+, greater than 5 oocysts were scored as 2+. Helminth eggs were detected by a modified McMaster method [29].

Case definition

A case was defined as a foal reported to have diarrhoea when sampled. Controls, defined as those foals unaffected by diarrhoea when sampled, were classified into one of two groups depending on whether or not they were in contact with cases of diarrhoea.

Statistical analysis

The data were recorded in Epi-info [30] and transferred to EGRET [31] and LogXact [32] for statistical analysis. Initial univariate assessment of the association between any particular agent and disease was followed by building logistic regression models, initially testing the including of variables associated with disease at $P = 0{\cdot}4$ or less, and then building the model by forward experimentation followed by backward stepwise routine with an acceptance level of $P < 0{\cdot}05$.

Interaction terms between all the main effect variables were considered and the same criteria used to assess the suitability of their inclusion in the models. The models presented were stable and robust, as determined by the lack of significant effect after sequential exclusion from the model of the foals with the largest leverage for each variable.

Models were developed that excluded foals in contact with cases of diarrhoea. Other models included all the controls. Those excluding the controls in contact with cases were considered particularly useful as they were not biased by the possible influence of sub-clinical infections from sick animals which excreted large amounts of pathogens. This classification of controls also allowed estimation of the prevalence of subclinical infection.

One of the variables (*Strongyloides westeri*) was significantly associated with diarrhoea ($P < 0{\cdot}01$) in univariate analysis; however inclusion of this term into maximum likelihood logistic regression models (with controls in contact with cases of diarrhoea excluded) was not possible since the model had no infected controls and therefore would not converge. Similarly, parameter estimates were not provided by exact logistic regression models when the appropriate interaction term was included (although these models did converge when no interaction term was included and all models showed a statistical association between diarrhoea and the presence of *S. westeri*). An approach to minimize bias of parameter estimates was used [33, 34] and the value of the odds ratio estimated using the formula $(a^*(b+1)/(c+1)^*d)$ for *S. westeri*, rather than the more conventional $(a^*d)/(b^*c)$. Since the controls not in contact with cases did not contain a foal positive for *S. westeri*, this was achieved by

artificially giving a positive result to a control foal that was not infected with any of the other organisms in the model. This approach produces a conservative estimate of both the odds ratio estimate and the significance level and allowed the inclusion of an important variable in the final model.

A model that assessed the association of micro-organisms with fatal diarrhoea was also developed although the number of suitable cases was low ($n = 22$). In order to exclude the possibility of post mortem changes in the intestinal flora, microbial isolation data are also presented from foals where samples had been collected during the episode of diarrhoea which immediately preceded death. Due to the small numbers of cases, statistical significance level was set at 0·1 and results were interpreted with caution.

Population attributable fractions (PAF) or attributable proportion were calculated [35]. This was based upon relative risk and prevalence in the healthy population. As this was a case control study of a common disease, it was only possible to calculate PAF for fatal cases of diarrhoea (rare events), when the odds ratio was used as an estimate of relative risk.

RESULTS

Overall prevalence

Rates of detection of pathogens from the faeces of healthy and scouring foals are detailed in Table 2. The most frequently detected were undifferentiated *E. coli*, *C. perfringens*, *Cryptosporidium* spp. and rotavirus. However, a few cases of diarrhoea were associated with *Salmonella* spp. and these were principally isolated from one outbreak in non-thoroughbred horses at a stud. The prevalence of thermophilic *Campylobacter* spp., *Y. enterocolitica* and *E. coli* was little different between foals with diarrhoea and healthy foals. There were no differences associated with breed or geographic location.

Model using as controls only foals not in contact with cases of diarrhoea

The likelihood of diarrhoea in foals, when compared to foals not in contact with other cases of diarrhoea, was significantly associated with rotavirus, *Cryptosporidium* spp., *C. perfringens*, *S. Westeri* and their age. These terms were all included in the model as main effects, as was an age–*C. perfringens* interaction term. The odds ratios and significance levels derived from this model are shown in Table 3. *Cryptosporidium* spp. was modelled as a continuous variable with three levels; foals with large numbers of oocysts detected thus had an odds of diarrhoea of 6·6 (3·3 × 2).

Although the effects of rotavirus, *Cryptosporidium* spp. and *S. westeri* did not appear to change materially with age of foal, the effects of *C. perfringens* were significantly more obvious in the foals over 8 weeks of age (OR *c.* 3·0 for foals < 8 weeks of age versus 36·0 for foals > 8 weeks of age). This reflected the fact that only 2% of the controls 8 weeks of age and older were infected (1/48), compared with 36% of the controls aged less than 8 weeks (27/76). The corresponding rates of infection in the cases were more similar, being 40% and 64% respectively. There was no evidence of interaction between any of the micro-organisms included in the model.

Model using all foals

When all foals were considered in the model, the parameter estimates (or odds ratios) were different to those from the first model that excluded foals in contact with animals with diarrhoea. In particular, the association between diarrhoea and rotavirus was reduced. This is consistent with rotavirus being highly contagious and associated with sub-clinical infection. The effects of *C. perfringens* did not appear to be affected by the age of foal, reflecting the higher prevalence in controls > 8 weeks of age and in contact with cases of diarrhoea (24% *vs.* 2% for foals not in contact with cases). The odds ratios and significance levels derived from this model are shown in Table 4. The association between *S. westeri* and diarrhoea was dependent on the numbers of eggs present, as there was only a positive association with *S. westeri* when more than 2000 eggs per gram of faeces were detected. The association between *Cryptosporidium* spp. and diarrhoea increased with the detection of larger numbers of oocysts in the faeces.

Detection rates of rotavirus and *Cryptosporidium* spp. were significantly greater in controls in contact with cases of diarrhoea than in controls that were not (Table 2: $\chi^2 = 4{\cdot}6$ and 4·3, 1 D.F., $P < 0{\cdot}05$).

Model for diarrhoea with a fatal outcome

Foals were more likely to die when aged less than 1 week old. Multivariate analysis revealed that only *C.*

Table 2. *Prevalence of potential pathogens in faeces of healthy foals and those with diarrhoea*

Organism	Percentage positive faeces overall ($n = 588$)	Percentage positive faeces from cases ($n = 365$)	Percentage positive faeces from controls in contact with cases ($n = 99$)	Percentage positive faeces from controls not in contact with cases ($n = 124$)
Yersinia enterocolitica	< 1	< 1·0	0	0
Salmonella spp.	3	2	8	1
Strongyles	3	1	11	0
Strongyloides westeri	4	5	2	0
Campylobacter spp.	8	8	5	6
Rotavirus	17	24	10	2
Cryptosporidium spp.	17	20	17	7
Clostridium perfringens	46	58	31	23
Escherichia coli	94	96	92	92

Table 3. *Multivariate model of diarrhoea in foals only using controls not in contact with a case of diarrhoea*

Organism	Odds ratio (*P* value)	95% Confidence intervals
Intercept	1·2 (0·7)	0·3–4·4
Rotavirus	16·0 (< 0·001)	4·0–52
Cryptosporidium spp.	3·3 (< 0·001)	1·6–5·3
Strongyloides westeri	8·9 (0·04)	1·1–72
C. perfringens in foals less than 7 days age	2·9 (—*)	0·6–13·0
C. perfringens in foals 2–4 weeks age	3·0 (—*)	1·1–8·4
C. perfringens in foals 5–8 weeks age	2·6 (—*)	1·1–6·3
C. perfringens in foals aged more than 8 weeks	35·6 (—*)	4·5–273

All variables adjusted for the effects of foal age.
* Based on interaction terms; the main effects of age and *C. perfringens* were also included in model.

Table 4. *Multivariate model of diarrhoea, based on all controls*

Organism	Odds ratio (*P* value)	95% Confidence intervals
Intercept	0.9 (0·7)	0·5–1·7
Rotavirus	5·6 (< 0·001)	2·9–10·6
Cryptosporidium spp.	2·1 (< 0·001)	1·4–3·3
Clostridium perfringens	3·0 (< 0·001)	2·0–4·6
Strongyloides westeri (counts of 200–2000 eggs/g)	0·3 (0·05)	0·01–1·0
Strongyloides westeri (counts of > 2000 eggs/g)	6·1 (0·02)	1·3–28

All variables adjusted for the effects of foal age (included in model).

perfringens, *Salmonella* spp. and *Cryptosporidium* spp. were significantly ($P < 0·1$) associated with diarrhoea which resulted in death when samples were taken before or after (Table 5). These organisms were more prevalent in samples taken before death which suggested that the association was not created falsely by their proliferation after death. Attributable fraction calculations suggested that *C. perfringens* was the most important case of foal death (Table 5).

Methods of detection of *C. perfringens*

Each isolation method recovered *C. perfringens* in at least one sample where the other methods failed and

Table 5. *Results from multivariate model of fatal diarrhoea*

Organism	Odds ratios (*P* value)	90% Confidence interval	Population attributable fraction
Salmonella spp.	14·2 (0·06)	1·4–148	8%
Cryptosporidium spp.	3·0 (0·07)	1·1–8·4	11%
Clostridium perfringens	4·5 (0·01)	1·7–11·9	50%
Foal age (> 1 week *v.* < 1 week)	0·3 (0·05)	0·1–0·8	—
Intercept	0·14 (0·006)	0·04–0·4	—

20% of the *C. perfringens* isolations were made by one of the five methods alone. The association of diarrhoea with *C. perfringens* isolated by heat enrichment was greater than with *C. perfringens* isolated by any other method (OR of 3·4 versus the nearest OR of 2·5 for non-heat enrichment; values for $P < 0·001$) indicating the possibility of an association between diarrhoea and heat resistant endospore formation. However, isolation of *C. perfringens* by alcohol treatment was negatively associated with diarrhoea (OR = 0·8, $P < 0·001$). Analysis of isolation methods where viable counts were made did not reveal a particular association between diarrhoea and large numbers of *C. perfringens*.

Just over a quarter of foals with diarrhoea were being treated with antibiotics at the time the sample was taken and, not surprisingly, antibiotic treatment was associated with diarrhoea ($P < 0·001$). However, taking this association into account, there was no relationship (inverse or direct) between *C. perfringens* isolation and antibiotic treatment. Consequently, this data did not provide evidence of the proliferation of *C. perfringens* nor of the elimination of these bacteria in foals treated with antibiotics.

DISCUSSION

This study and other surveys of potential pathogens and foal diarrhoea [5, 10, 11, 13] have revealed an association with rotavirus. Likewise, *Salmonella* spp. were encountered too infrequently to give statistical significance, but when found they were always isolated from severely affected cases or from animals in contact with clinical cases. An association was detected in this survey between isolation of *Salmonella* spp. and fatal diarrhoea.

This study, unlike previous investigations, used multivariate analysis and divided the control group into animals in contact, or not in contact, with cases of diarrhoea. As a consequence associations were detected between some potential pathogens with diarrhoea undetected in previous studies [5, 10, 11, 13] which ignored the statistical effects of sub-clinical infections. Most notable among these were *Cryptosporidium* spp., the presence of *S. westeri*, rather than just large numbers of its eggs, and the age related association with *C. perfringens*.

Cryptosporidium spp. is a cause of diarrhoea in several mammals and it is a suspected cause in immunodeficient foals [5, 26, 36–39]. This study suggests that *Cryptosporidium* spp. may also cause diarrhoea in immunocompetent foals but no association between *Cryptosporidium* spp. and diarrhoea would have been detected if the controls were restricted to foals in contact with cases of diarrhoea (analysis not shown).

The effect of age on the strength of association of *C. perfringens* was only apparent in the model that excluded controls in contact with cases of diarrhoea. The stronger association between diarrhoea and *C. perfringens* in the older age group may be explained by a five times higher sub-clinical infection rate in the younger healthy foals, which declines as the foal matures. In contrast, in the analysis which included controls in contact with cases there was a four times greater prevalence of *C. perfringens* infection in controls older than 8 weeks of age, presumably acquired through contact with cases, than found in healthy foals not in contact with cases of diarrhoea.

The detection of an association between foal diarrhoea and *C. perfringens* was made possible by both the inclusion of controls not in contact with cases but also by the different methods of isolation, which together detected the organism more readily than any single method used here, or, as used in a previous study [13]. Additionally, in the previous study faeces were stored for up to 7 days at 4 °C before testing [13]; *C. perfringens* is sensitive to temperatures just above 0 °C [40] which is why the

veterinarians and stud farm managers were asked not to refrigerate samples collected for this study.

Differences between strains of *C. perfringens* which cause diarrhoea in foals and those isolated from controls may have been reflected in the stronger association of *C. perfringens* isolated by heat enrichment with disease, than seen with any other method, and the negative association of isolation by alcohol treatment, particularly when using the healthy controls not in contact with cases. This supports the possibility of some strains of *C. perfringens* being associated with diarrhoea and others that are not. Currently, it would be difficult to determine if isolation of *C. perfringens* from individual cases was aetiologically significant. We are now testing for an association between diarrhoea and the genes for several virulence determinants of *C. perfringens* using polymerase chain reaction.

C. perfringens was associated with more diarrhoea with a fatal outcome than to any other organism. Unfortunately, details of gross pathology were not available and future studies should determine whether there is severe necrosis in the intestinal mucosa as might be expected in *C. perfringens* enterotoxaemias.

E. coli was isolated from more than 94% of all foals, regardless of health status. However, potentially pathogenic *E. coli* were not differentiated and the possibility remains that most of these isolates were non-pathogenic. In a previous study, *E. coli* isolates possessing known virulence factors for other mammals were found at a low prevalence in both healthy foals and those with diarrhoea [13]. A molecular analysis [41] detected heat labile, heat stable and shiga like toxins in less than 3% of *E. coli* from 63 foals with diarrhoea and 30 from healthy foals but found mannose resistant haemagglutinins, haemolysin production and the attaching and effacing gene in 23%, 11·5% and 11·1% of isolates from cases, respectively; there was no clear pattern of O or H antigen possession. Even though few isolates produced enterotoxins, 42% of those with mannose resistant haemagglutinins had the F41 adhesion and three had K88 or K99. If *E. coli* are a cause of foal diarrhoea it is possible that the pathogenic strains will have adhesins and toxins specific to the horse, as is the case for those strains which are pathogenic for other mammalian species, such as K88 and K99 adhesins for piglets and calves, respectively.

There were too few samples from different breeds of horse and types of premises to justify including these categories in the statistical analyses. Regarding current practice and the treatment of diarrhoea, the results from this survey suggest that a specific bacteriological basis for antibiotic treatment was not sought in most cases. Presently, there is no clear understanding of whether treatment would be helpful in cases in which *C. perfringens* is involved. In man, it has been claimed that treatment with metronidazole is helpful in reducing numbers of *C. perfringens*, reducing enterotoxin in faeces and resolving diarrhoea [42]. Conversely, antibiotic treatment is also thought to be one of the important predisposing factors of human clostridial diarrhoea [43–47] and this may also be true in the foal [48], although our results provide no evidence for this.

There are now important questions regarding whether or not there are virulence determinants of both *C. perfringens* and *E. coli* associated with foal diarrhoea. An initial approach would be to study previously putative virulence determinants to see if there are any which are more strongly associated with diarrhoea than others. It is clearly important that these issues are resolved since this will assist in the management of diarrhoea in the foal.

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