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A CASE CONTROL STUDY OF FOAL DIARRHOEA, IN PARTICULAR INVESTIGATING THE ROLE OF *CLOSTRIDIUM PERFRINGENS*

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L'analyse multivariée d'une étude sur la prévalence des germes entéropathogènes potentiels chez des poulains de toute race réalisée dans toutes les exploitations du Royaume -Uni entre 1991-1933 et utilisant des témoins sains n'ayant pas eu de contact avec les cas a montré que seuls Clostridium perfringens (58% des cas), rotavirus, Cryptosporidium spp (20%) et Strongyloides westerii (5%) étaient associés à la maladie. Aucune interaction n'a été mise en évidence entre ces germes. C. perfringens a également été retrouvé chez 23% des poulains en faisant la détection de germes indifférentiés de faible valeur pronostique et diagnostique.

Les isolats de cette étude ont été examinés pour un sous-type associé avec la maladie dans le sous-ensemble des poulains positifs à C. perfringens pour identifier s'il pouvait agir comme germe pathogène primaire. Les isolats entéropathogènes détectés par PCR étaient de prévalence trop faible pour jouer un rôle dans l'association globale de C. perfringens avec la diarrhée. La plupart des poulains positifs pour C. perfringens avaient des isolats de type A et seuls 8% des isolats étaient de type B, C, D, ou E. Aucun de ces types n'a été associé à la maladie chez le sous-groupe de poulains C. perfringens.

Les isolats ont été typés par les différences dans les polymorphismes des espaces intergéniques du gène ARN 16S-23S. Ceux-ci ont été clonés et séquencés afin d'obtenir des amorces spécifiques pour la détection par PCR des différents espaces. Quatre sous-types ont été identifiés et l'un d'entre eux, le type 2, était suffisamment prévalent (35% des cas) et était associé avec la maladie chez les poulains positifs à C. perfringens ce qui suggère qu'il pourrait agir comme pathogène primaire et pourrait jouer un rôle dans l'association de C. perfringens avec les diarrhées. Une prévalence particulièrement élevée chez les cas fatals a indiqué que le type 2 de C. perfringens joue un rôle pathogène majeur.

INTRODUCTION

Most foals have at least one episode of diarrhoea before they are 6 months old (Urquhart, 1981) which may cause loss of condition or even be fatal (Palmer, 1985). Although *Salmonella* and rotavirus are recognised causes of foal diarrhoea (Holland *et al.*, 1990; Browning *et al.*, 1991) there are a range of other organisms which act as enteropathogens in other mammals. These have not been assessed for prevalence and relative importance in Thoroughbreds and non-thoroughbreds alike on studs and in other environments and include *Clostridium perfringens*, which has been reported in association with foal enteric disease in case reports (Dart *et al.*, 1988; Montgomerie and Rowlands, 1937 - to cite 2 examples), .

The first objective of the study was to identify the organisms significantly associated with foal diarrhoea and in particular to see if there was any epidemiological evidence for a role for *C.perfringens*. For the first time in relation to foal diarrhoea, we employed multivariate analysis and used as controls healthy foals which were not in contact with cases. This approach avoids analytical reduction of associations caused by passive carriage or incubation/sub-clinical infections in healthy animals in contact with cases. We also used several methods of isolation of *C.perfringens* to enhance its detection which otherwise, if dependent on one method, might favour the recovery only of endospores or vegetative cells.

C.perfringens is one of the first organisms to colonise the neonatal gut and a proportion of any healthy population of mammals can be expected to carry the organism. *C.perfringens* is also phenotypically and genetically highly variable and this is reflected in differences in virulence in other diseases. Consequently, if any association of *C.perfringens* was found with diarrhoea then it is possible that it might be caused by one or more pathogenic subtypes. Our second objective was to type the *C.perfringens* isolates by their possession of different virulence factors and polymorphisms of the 16S-23S intergenic spacer to find evidence of pathogenic subtypes. Analytically, this evidence was sought in two ways. Firstly, a subtype of *C.perfringens* would be unlikely to be acting as a pathogen if its isolation was not associated with diarrhoea. Any subtype that was associated with diarrhoea might be acting as a primary pathogen or might be a component of a multifactorial cause of diarrhoea. The second approach was to test if there was a subtype more prevalent amongst cases than controls positive for *C.perfringens*. Such evidence might be taken as support for a subtype which causing disease more frequently than not when it is present .

METHODS AND MATERIALS

Faecal samples were collected from Thoroughbred and non-thoroughbred foals less than 6 months of age from throughout Great Britain during 1991-1993. *Salmonella spp*, *Escherichia coli*, *C.perfringens*, *Cryptosporidium spp.*, *Campylobacter spp.*, *Yersinia enterocolitica*, rotavirus and a wide variety of parasites were detected and analysed for associations with disease, as described by Netherwood *et al.*, (1996).

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At least one *C.perfringens* isolate for each of the culture methods giving a positive result for each foal was kept for further study and samples of their DNA were extracted by boiling in 1M guanidine thiocyanate and purification from supernates by Magic DNA clean-up. Enterotoxigenic *C.perfringens* were detected by Reversed Passive Latex Agglutination (RPLA; Oxoid Unipath) and by two polymerase chain reaction (PCR) tests, one using primers based on the termini of the gene (Czeczulin *et al.*, 1993) and the other on internal primers (Daube *et al.*, 1994). Isolates were genotyped by PCR using previously described primers (Daube *et al.*, 1994). The 16S-23S RNA gene intergenic spacers of *C.perfringens* were amplified using a forward primer from the end of the 16S gene sequence described by Barry *et al.* (1991) and a reverse primer designed from the sequence for the 5' end of the *C.perfringens* 23S gene described by Garnier *et al.* (1991). Products in PCR reactions were detected and characterised by gel electrophoresis and ethidium bromide staining. Products from PCR reactions for the intergenic spacer were separately purified from low melting point electrophoresis gels, cloned into pGEM-T (Promega) and sequenced on an ABI PRISM 377 automated sequencer using sequencing primers across the pGEM-T cloning site and associated dye terminator sequencing kit (Perkin Elmer). Following sequence comparison, primers were designed to specifically amplify different intergenic spacer polymorphisms.
The association of different types of *C.perfringens* with diarrhoea and testing of whether each type was more common amongst *C.perfringens* positive cases compared with controls was analysed by c^2 test (Dean *et al.*, 1994). Foals that were originally positive for *C.perfringens* but where stored cultures died and *C.perfringens* could not be re-isolated from stored faeces, were regarded as missing values and were excluded from the analyses. More foals were included in the comparison of *C.perfringens* types than the study of all pathogens as more foals were investigated for *C.perfringens* alone.

RESULTS

Rotavirus, *Cryptosporidium, spp, Strongyloides westerii*, and *C.perfringens* were the only organisms associated with diarrhoea (Table I). Breed, type of premises or part of country did not alter the effect of any of the pathogens but the effect of *C.perfringens* increased with foal age (Table I). *C.perfringens* was the most common of the pathogens associated with diarrhoea, but just over one fifth of controls were also positive.

Table I
Prevalence (%) of pathogens associated with foal diarrhoea in the multivariate model

Organism	Cases (N=365)	Controls (N=124)	Odds ratio (p value)	95% confidence interval
Intercept			1.2 (0.7)	0.3-4.4
Rotavirus	24	2	16.0 (<0.001)	4.0-52
S.westerii	5	0	8.9 (0.04)	1.1-72
Cryptosporidium spp	20	7	3.3 (<0.001)	1.6-5.3
C.perfringens:-	58	23		
in foals <7 days age			2.9 (-*)	0.6-13.0
in foals 2-4 weeks age			3.0 (-*)	1.1-8.4
in foals 5-8 weeks age			2.6 (-*)	1.1-6.3
in foals >8 weeks age			35.6 (-*)	4.5-273

All variables adjusted for the effects of foal age (see Netherwood *et al.*, 1996 for fuller description of methods).
* Based on interaction terms: the main effects of age and *C.perfringens* were also included in model.

Although 43% of cases had enterotoxin RPLA positive *C.perfringens*, only 9.7% of isolates from cases were enterotoxin PCR positive. Tests of an enterotoxin gene probe and verocell cytotoxicity neutralised by enterotoxin antiserum revealed that the RPLA test was reacting non-specifically with isolates providing no evidence of the enterotoxin gene or toxicity. Both isolates that were RPLA positive or negative isolates or enterotoxin PCR positive or negative isolates were significantly associated with diarrhoea ($p<0.05$) but none of these subtypes were more common in *C.perfringens* positive cases compared with positive controls.
Genotyping showed that *C.perfringens* infections were sometimes of mixed types. However, of 220 cases with cultures surviving storage, 88% were positive for type A *C.perfringens*. A similar picture was found with the control foals. However, only 8% of positive foals had types B, C, D or E and less than 7% of positive foals had untypable strains. Only the type A was associated with diarrhoea (OR=4.08; 95%CI: 2.45-6.84; $p<0.0001$) but it was not more prevalent amongst *C.perfringens* positive cases than controls ($p=0.78$).
All of the isolates could be subdivided into one of four types by the presence or absence of three main PCR products of approximately 150, 200 and 350 base pairs (bp). Sequencing of the three different PCR products revealed that all were intergenic spacers with 16S and 23S RNA gene flanking regions and that there were distinct variations in their nucleotide code; these spacers were named X (185bp between the RNA genes), Y (139bp) and Z (217bp). Spacer X had a sequence almost identical to the previously published sequences (Garnier *et al.*, 1991; Barry *et al.*, 1991). Spacers Y and Z had some sequence homology with spacer X, the largest region being at positions 129-157. However, compared with spacer X, spacers Y and Z were largely made up of a combination of additions and deletions of sequence and primers were designed for their specific detection by PCR. A scheme was devised upon which the isolates could be sub-typed by the presence of intergenic spacers Y and Z (since all isolates possessed spacer X). Some isolates possessed only spacer X but, in addition, other isolates also possessed spacer Y, spacer Z or both Y and Z; new intergenic spacer types were thus determined as types 1, 2, 3 and 4, respectively.

Amongst all foals, intergenic spacer type 1 (of all lethal toxin types) was most common (48%), followed closely by type 2 (44%). Types 3 and 4 were relatively uncommon (8.8% combined together; Table II). *C.perfringens* infections were of mixed type in 36% of cases and controls. All types were associated with diarrhoea (Table II), but only type 2 bacteria were associated with diarrhoea in the *C.perfringens* positive subset of cases and controls. In contrast, type 1 isolates were significantly more prevalent in *C.perfringens* positive controls than in positive cases (Table II).

Table II
Distribution of *C.perfringens* intergenic spacer types in foals with diarrhoea and controls

Type	No of 404 positive cases	No of 129 positive controls	Association with diarrhoea			Association with diarrhoea in *C.perfringens* positive subset		
			OR	95%CI	p value	OR	95%CI	p value
All types	220	28	-			-		
Type 1	134	24	2.17	1.33-3.54	0.0013	0.26	0.09-0.78	0.0114
Type 2	143	11	5.88	3.07-11.27	<0.0001	2.87	1.24-6.44	0.012
Types 3 or 4	22	0	15.24	0.92-253	0.0037	6.46	0.38-109	0.147

DISCUSSION

C.perfringens was the most prevalent pathogen significantly associated with diarrhoea but over one fifth of controls were positive as well. Commensal carriage of *C.perfringens* is to be expected and so detection of the undifferentiated organism would be of little diagnostic or prognostic value. Consequently, we set about a search for a subtype of the organism that might be acting as a pathogen. To account for the association of all *C.perfringens* with diarrhoea a pathogenic subtype would have to be (1) highly prevalent itself, (2) be associated with diarrhoea independently of other types of *C.perfringens* and (3) , if it behaved as though it were a primary pathogen, it would be expected to be significantly more prevalent in *C.perfringens* positive cases than controls. Enterotoxigenic *C.perfringens* had too low a prevalence to be the hypothetical pathogenic type creating the association. Genotyping revealed that most isolates were of major toxin type A and although these were associated with diarrhoea they were not more prevalent in *C.perfringens* positive cases compared with positive controls. However, subtyping by the 16S-23S intergenic spacer revealed a new type 2 which fulfilled all of the criteria of prevalence and association suggestive of a major pathogenic role. This is supported by the fact that of the 22 fatal cases studied, 15 were positive for *C.perfringens* and 12 of these were type 2 positive. Further studies are now planned to determine further the significance of the type 2 organism in foal diarrhoea and the diagnostic significance of detecting by PCR a Type 2 organism in a diarrhoeic foal.

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