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The *Clostridium perfringens* enterotoxin from equine isolates; its characterization, sequence and role in foal diarrhoea

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SUMMARY

During a survey of foal diarrhoea between 1991 and 1994, *Clostridium perfringens* was significantly associated with disease with 56% of cases infected [1]. The contribution of enterotoxigenic *C. perfringens* to this association, was assessed by use of the reverse passive latex agglutination test for enterotoxin (RPLA; Oxoid Unipath) and vero cell toxicity neutralized by antitoxin on stored faecal samples and sporulated faecal isolates of *C. perfringens*. Polymerase chain reaction (PCR1) based on the DNA sequence for the whole enterotoxin gene [2] yielded a fragment from an equine isolate of the anticipated size which, cloned into plasmid M13 phage, had a sequence essentially identical to the published sequence. Consequently, all faecal isolates were also tested by PCR1 and for a part of the enterotoxin gene (PCR2).

Significant association with diarrhoea (controls not in contact with cases) was found with positive RPLA tests on faeces (OR = 13, $P = 0{\cdot}002$) and isolates (OR = 4·57, $P = < 0{\cdot}0001$), vero cell toxicity of isolates (OR = 1·78, $P = 0{\cdot}026$), and PCR1 (OR = nd, $P = 0{\cdot}029$) but not PCR2 or vero cell toxicity of faeces. Significant association with diarrhoea was also found for isolates negative by RPLA (OR = 3·91; CI 2·05–7·57; $P < 0{\cdot}0001$) or PCR1 (OR = 4·81; CI 2·84–8·20; $P < 0{\cdot}0001$). Many of the isolates from RPLA positive faeces and verotoxic isolates were PCR negative and no evidence could be found for the presence of the enterotoxin gene in a random selection of RPLA positive/PCR negative isolates by gene probe on chromosomal DNA and PCR reaction product or vero cell toxicity neutralized by specific antiserum. Failure of the vero cell toxicity on faeces to be associated with diarrhoea or for cytotoxicity of cultures and RPLA on cultures to agree with the PCRs was believed to be related to the presence of other cytotoxins, the inherent cytotoxicity of equine faeces and to the poor specificity of the commercial antiserum used in the test.

Enterotoxigenic *C. perfringens* could not account for the overall association of *C. perfringens* with foal diarrhoea because (a) cultures positive by PCR, RPLA or cytotoxicity were not significantly more common amongst isolates from cases than controls; and (b) the proportion of isolates from cases positive by PCR (PCR1 or PCR2) was too small at 9·7%.

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INTRODUCTION

Clostridium perfringens is a cause of enteric disease in a wide range of animals and man [3–15]. However, different strains of *C. perfringens* utilizing different pathogenic mechanisms are often responsible for distinct syndromes. Many of these syndromes result from the elaboration of a variety of toxins in the gastrointestinal tract which have an aggressive action causing severe tissue damage that is often fatal [9, 15–19]. Strains which produce an enterotoxin which stimulates hypersecretion of fluid are a cause of food poisoning [10] and infectious diarrhoea in institutionalized populations [20], which are both milder than the necrotic enteric disease associated with the more aggressive toxins. Only partial sequences of the enterotoxin were initially obtained because of difficulties in cloning the whole gene [21–23]. Recently, however, the whole gene was cloned and sequenced but some areas were not homologous with the previously published sequence for fragments of the gene [2].

A variety of isolation techniques designed to increase the sensitivity of culture of *C. perfringens* [23] identified these bacteria as an important cause of diarrhoea in a recent survey of foals [1]; *C. perfringens* was also associated with the largest proportion of diarrhoea with a fatal outcome. The mechanism of pathogenesis was unknown, however, although it was clear that the majority of disease associated with *C. perfringens* was not life threatening.

The objectives of this study were (a) to establish if isolates from cases of foal diarrhoea could react with the reverse passive latex agglutination test (RPLA) for *C. perfringens* enterotoxin; (b) to attempt to clone and sequence the enterotoxin gene from an equine isolate and assess its relatedness to the gene sequenced from the human isolate; (c) to develop molecular genetic methods for the detection of the enterotoxin gene in isolates from the survey of foal diarrhoea; and (d) to compare the results of these methods with the RPLA.

METHODS

Bacteria

Up to five isolates of *C. perfringens* per foal were examined by all tests for enterotoxin depending on their recovery from stored culture or re-isolation from stored faeces; numbers of isolates tested were 529 from 184 *C. perfringens* positive foals with diarrhoea and 98 from 66 healthy *C. perfringens* positive foals. *C. perfringens* NCTC8239, a human isolate from which the enterotoxin gene was originally cloned and sequenced, served as a control for the polymerase chain reaction for the enterotoxin gene. *Escherichia coli* JM 105 was used for the plasmid cloning and TG1 for M13 subcloning of the enterotoxin gene.

Purification of *C. perfringens* DNA

An overnight culture of *C. perfringens* in 20 ml FAB (Oxoid) at 37 °C in an atmosphere of 10% (v/v) CO_2, 10% (v/v) hydrogen, and 80% (v/v) nitrogen, was centrifuged at 10000 g for 7 min at 4 °C. The cells were resuspended in 1 ml 8 M guanidine thiocyanate, heated at 100 °C in a bath for 15 min, allowed to cool and then centrifuged at 10000 g for 7 min. The supernatant fraction was purified with the Magic DNA Clean-up System (Promega) primarily according to the manufacturer's instructions, using water for the DNA elution step, but eluting at 90 °C rather than 65–70 °C.

General molecular methods

General methods for M13mp19 phage propagation, purification, phage DNA purification, agarose and polyacrylamide gel electrophoresis, dot-blot, random primer radiolabelling of DNA probe and polymerase chain reaction (PCR) were as described previously [24].

Cloning and subcloning

DNA potentially encoding the whole enterotoxin gene was obtained by polymerase chain reaction using primers (5′ TAT ATG GTT AGT AAC AAC AAT TTA AAT CC and 5′ AT GGA TCC ATA TTA AAA TTT TTG AAA TAA TAT TG) derived from the sequence published for the human isolate and was cloned into pTrc 99A (Pharmacia) and subcloned into the bacteriophage M13mp 19.

Single stranded sequencing

The Sanger dideoxy nucleic acid chain termination method [25] for single stranded sequencing of deoxyribonucleic acid with a Sequenase kit (Amersham) was used. Complementary primers were synthesized upstream of the 3′ end of sequence generated and were used to continue sequencing.

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Enterotoxin detection

Isolates of *C. perfringens* were allowed to sporulate in Duncan and Strong medium [26], the culture supernate was recovered and the enterotoxin was detected by reverse passive latex agglutination (RPLA; Oxoid). All methods for culture preparation and enterotoxin detection followed the manufacturer's recommendations. Cytotoxicity of the culture supernates and faecal suspensions was determined by vero cell cytotoxicity assay as previously described [27], with a polyclonal *C. perfringens* enterotoxin antiserum produced by Biogenesis Limited. However, the intrinsic toxicity of equine faeces for vero cells meant that a starting dilution of 1/100 had to be used.

Preparation of recombinant enterotoxin antiserum

The enterotoxin band at the 35000 Dalton molecular weight position on an SDS-polyacrylamide gel was excised and prepared with Freund's incomplete adjuvant for injection into a rabbit. A rabbit was selected whose serum did not contain neutralizing activity for the cytotoxicity of several isolates, some of which produced toxins which could not be neutralized by the commercial antiserum to enterotoxin. The rabbit was vaccinated twice with an interval of 3 weeks. A serum sample was taken 2 weeks after the last vaccination.

PCR detection of enterotoxin gene and alpha toxin gene

PCR was conducted using a cycle program of; 1 cycle of 94 °C for 1 min; 30 cycles of, 94 °C for 1 min, then 52 °C for 2 min, and 72 °C for 3 min; and finally 1 cycle of 72 °C for 7 min. The 5× reaction buffer contained 300 mM Tris-HCl, 75 mM $(NH_4)SO_4$, and 10 mM $MgCl_2$, at pH 8·5 (at 22 °C). Primers were added to 0·05 μg/50 μl; dNTP mix at a final concentration of 1 mM; Taq polymerase at 1 unit/50 μl of reaction and sample DNA (diluted 1/100) at 1 μl/50 μl of reaction.

During this study a multiplex PCR method for the major toxin genes of *Clostridium perfringens* and the enterotoxin gene was published [28]. The isolates were re-tested for the presence of the enterotoxin gene by this method. The multiplex PCR, which includes primers for both the alpha toxin and enterotoxin genes, was carried out with the primer sequences as published [28] but using altered PCR conditions using a cycle program of; one cycle of 95 °C for 5 min; 30 cycles of, 94 °C for 1 min then 50 °C for 2 min and 70 °C for 3 min; and finally one cycle of 70 °C for 7 min.

Enterotoxin gene probe

The enterotoxin gene was amplified by PCR and the product was isolated by gel electrophoresis, labelled by the random primer method, and hybridized with the target DNA immobilized on Hybond N membrane (Amersham) by the dot blot method. Target chromosomal DNA was denatured by heating at 95 °C for 5 min and target PCR product was denatured by heating to 37 °C for 10 min in 0·5 M sodium hydroxide, before application to the membrane.

Statistical analyses

Evidence for a pathogenic role for enterotoxigenic or non-enterotoxigenic *C. perfringens* was sought in two ways. First, 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, with subtypes associated with diarrhoea, was to test if there was a subtype more prevalent amongst cases than controls positive for *C. perfringens*. Such a subtype would then appear to behave as a primary pathogen or, in other words, was causing disease more frequently, than not, when present. 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 χ^2-test using Epi info [29]. 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 this study than were described in the epidemiological study of all agents [1] because all had been investigated for *C. perfringens* even though not all were examined for the other enteropathogens.

RESULTS

Clostridium perfringens was significantly associated with diarrhoea, being isolated from 56% of cases but only 24% of healthy controls not in contact with cases (Table 1); 22 cases were fatal and 68% of these

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Table 1. *Detection of enterotoxin or enterotoxic* C. perfringens *isolates by various tests over the number of foals tested*

Test	Scouring foals	Healthy foals not in contact with cases	Healthy foals in contact with cases	OR (95% CI)*	*P* value*
Isolation of *C. perfringens*	232/414	33/135	33/102	3·94 (2·49–6·62)	< 0·0001
Vero cell toxicity in faeces†	81/414	22/119	30/102	1·06 (0·62–1·83)	0·93
Vero cell toxicity in culture supernates†	120/414	22/120	21/102	1·82 (1·09–3·03)	0·019
RPLA on faeces‡	39/414	1/135	8/102	12·7 (1·86–251)	0·002
RPLA on culture supernates‡	100/411	9/124	8/102	4·11 (1·94–9·01)	< 0·0001
PCR1§	16/414	0/119	2/102	Nd¶	0·029
PCR2‖	18/404	2/129	3/102	2·98 (0·7–26·8)	0·18

* Odds ratio, confidence interval and p value calculated using the healthy controls not in contact with cases.
† Neutralized at least fourfold reduction in titre with antiserum to enterotoxin.
‡ Reverse passive latex agglutination test for enterotoxin.
§ PCR for whole enterotoxin gene.
‖ PCR for part of enterotoxin gene.
¶ Not definable.

yielded *C. perfringens*. Faecal suspensions and culture supernates of isolates of *C. perfringens* giving a positive reaction in the RPLA test were both significantly associated with disease (Table 1). However, isolates giving a negative RPLA result with culture supernatants were equally associated with diarrhoea (OR = 3·91; CI 2·05–7·57; $P < 0·0001$).

Vero cell toxicity of faecal suspensions was not associated with disease (Table 1); this might be explained by the insensitive nature of the test caused by the need to dilute equine faeces (1/100 to overcome its general toxicity for vero cells. Consequently, titres of cytotoxicity of at least 1/400 would be required before a fourfold neutralization of activity by antiserum could be detected.

C. perfringens strain B3550.5, isolated from a foal with diarrhoea, produced large amounts of an antigen that reacted in the RPLA test (reactive titre of culture supernatant > 1/1000) and its culture supernatant fractions were toxic for vero cells; more than 75% of the cytotoxicity could be neutralized by antiserum to enterotoxin. PCR using DNA extracted from strain B3550.5 and primers derived from the human isolates enterotoxin gene sequence, yielded a fragment of approximately 1 kb which was expected of the enterotoxin gene. The PCR product when cloned into pTrc 99A gave recombinants which were toxigenic by the RPLA test and which were verotoxigenic; *E. coli* JM 105 with pTrc 99A without the insert did not react in either test.

The pTrc99A insert was subcloned from the plasmids of three of the recombinants into bacteriophage M13mp19 and each was separately sequenced. All of the sub-cloned fragments had the same sequence (EMBL nucleotide sequence database accession number AJ000766) with the exception of base 894, where, in two of the clones, a cytosine was substituted for the thymidine residue which is present in the previously published DNA sequence and which was in the third of the M13 mp19 recombinants. This substitution did not alter the primary structure. The only other difference in sequence with that published for the human isolate, common to all the sub-clones, was in the first nucleotide of the second codon which was introduced intentionally to facilitate plasmid cloning. Given the almost complete homology otherwise, it seems likely that the native gene would have had the same codon as that published for the human isolate.

Polymerase chain reaction using conditions employed for cloning the enterotoxin gene and DNA extracted from eight strains positive by RPLA resulted in only three strains giving a product of the expected size in agarose gel electrophoresis.

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Table 2. *Reaction of isolates positive or negative by RPLA in the PCR, by a gene probe against chromosomal DNA extract, by a gene probe against PCR product and by a test of verotoxicity neutralized by anti-enterotoxin*

Isolates from separate cases of diarrhoea	RPLA (repeat result)	Probe of DNA extracts	PCR	Probe of PCR product	Vero toxic*
1	+(+)	+	+	+	+
2	+(+)	+	+	+	+
3	+(+)	+	+	+	+
4	+(+)	+	+	+	−
5	+(+)	+	+	+	−
6	+(+)	−	−	−	−
7	+(+)	−	−	−	−
8	+(+)	−	−	−	−
9	+(+)	−	−	−	−
10	+(+)	−	−	−	−
From healthy foals ($n = 6$)	−(−)	−	−	−	−
NCTC 8239	+(+)	+	+	+	+

* At least a 75% reduction in toxicity in a duplicate test with antiserum to enterotoxin.

Samples of DNA from the isolates from the survey of foal diarrhoea were re-analysed by PCR using the primers encompassing the complete gene (PCR1) and gel electrophoresis of the product (Table 1). Although far fewer isolates reacted in the PCR test than by the RPLA test, possession of the enterotoxin gene was associated with foal diarrhoea (Table 1). Paradoxically, none of the isolates from cases of diarrhoea with a fatal outcome yielded PCR evidence of the enterotoxin gene. The association of possession of the gene with disease was, therefore, presumably with diarrhoea which was not fatal. Two of the PCR positive isolates were negative by RPLA. The second PCR test, for part of the enterotoxin gene (PCR2), was not associated with disease (Table 1), since additional positive isolates were found amongst cultures from control animals and the total number positive was very small; six extra isolates were found with the gene by PCR2 which also confirmed those previously found to be positive for the gene by PCR1. Just as with the RPLA negative isolates, the PCR negative isolates were associated with diarrhoea at least as strongly as the PCR positive *C. perfringens* (OR = 4·81; CI 2·84–8·20; $P < 0·0001$).

Univariate analysis of isolates with or without a positive RPLA or PCR test failed to identify a subpopulation of *C. perfringens* which was not associated with diarrhoea. A further test of whether a pathogenic subpopulation can be identified is to test if groups of isolates with particular common characteristics, which are associated with diarrhoea, are more likely to have come from a *C. perfringens* positive case than a *C. perfringens* positive control (see methods). Since two of the tests for enterotoxigenic *C. perfringens* identified four subtypes in association with diarrhoea (RPLA+, RPLA−, PCR+ and PCR−), each subtype was tested for a significantly greater prevalence in *C. perfringens* positive cases or positive controls. No such distribution was found (all P values $> 0·05$).

A far larger number of isolates reacted in the RPLA test using culture supernatants than showed evidence of the enterotoxin gene by the two PCR tests. A representative number of isolates were selected that were originally RPLA positive/PCR positive and RPLA positive/PCR negative from separate cases of diarrhoea and from healthy foals. Together with the control enterotoxigenic *C. perfringens* NCTC 8239 these were compared (Table 2) by RPLA, PCR test and reaction of the labelled cloned gene with

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chromosomal DNA and PCR product (whether or not a band could be seen in the product by gel electrophoresis). None of the RPLA negative isolates tested, reacted in any of the genetic tests for the enterotoxin gene. All of the isolates positive by one of the genetic tests were positive by all of the other genetic tests and RPLA; all of these were toxic for vero cells and the activity of three was noticeably reduced by antiserum to the enterotoxin. However, all of the isolates originally classified as RPLA positive/PCR negative failed to react in any of the genetic tests and none possessed vero cell toxicity neutralised by antiserum.

DISCUSSION

The RPLA test for enterotoxin on sporulated culture supernates was significantly associated with diarrhoea in foals being detected in over half of cases with *C. perfringens*. The results of vero cell toxicity tests on cultures supported this prevalence. The apparent importance of enterotoxin for foal diarrhoea led us to establish the presence of its gene in an equine isolate and to compare its relatedness to that published for the human isolate.

The sequence for the enterotoxin gene cloned from an equine isolate proved to be identical to that previously published for a human isolate, with the exception of the intentional substitution in amino acid residue 2. This is the first confirmation of the complete *C. perfringens* enterotoxin sequence from an animal source and indicates that the differences in the previously published partial sequences [21, 22, 30] may be artifactual.

The homology of the sequences provided the justification to assess the potential for enterotoxigenicity by PCR independent of sporulation. We expected that possession of the gene would be more common than detection of the enterotoxin which is dependent on efficient sporulation and is notoriously unreliable *in vitro*. PCR1 was used to detect the whole enterotoxin gene, since isolates which gave a positive reaction were more likely to be toxigenic than those in which a PCR test detected only part of the gene and where there may have been only remnants of the gene present in the chromosome. PCR2 detected a relatively small part of the gene and was used because of a potentially greater sensitivity stemming from the smaller product that the reaction might more efficiently amplify. In short, combination of the results of PCR1 and 2 gave more assurance about the probable toxigenicity of isolates and the sensitivity of detecting the occurrence of toxigenic isolates. In practice, although a few more isolates were detected using PCR2, there were not many more than those already detected by PCR1. However, considerably fewer isolates reacted in the PCR test for the enterotoxin gene than reacted in the RPLA immunoassay or test for vero cell toxicity neutralized by specific antiserum. More than 87% of isolates were positive for the alpha toxin by PCR on the same DNA preparations used here using alpha toxin specific primers (unpublished results), suggesting that PCR inhibitors probably could not explain the relatively low enterotoxin gene detection rate compared with rates of cytotoxicity or a positive RPLA.

Confirmation of the PCR test by other genetically based tests showed that the RPLA was frequently positive without evidence for the enterotoxin gene. These results possibly demonstrated the potential for the RPLA to detect other *C. perfringens* antigens. It is our experience with purified bacterial antigens that there are often very minor contaminants which cannot be visualised by silver staining of SDS-PAGE gels but which nonetheless stimulate excellent antibody responses when they are used to immunise rabbits, for example. Furthermore, since the RPLA gave a false positive reaction with cultures then a plausible explanation for the association of an RPLA positive reaction on faeces with disease is that *C. perfringens* is associated with diarrhoea and the RPLA is detecting *C. perfringens*. Alternative explanations are that the antiserum used to make the RPLA test is detecting some other antigen or that antibodies to the enterotoxin are cross-reacting with another antigen in both *C. perfringens* cultures and faeces. Neither of the latter explanations account for the association of a positive RPLA on faeces with disease.

It might be argued that the RPLA and cytotoxin tests are detecting an enterotoxin which is antigenically functionally related to the enterotoxin *sensu stricto* but which varies genetically sufficiently to cause the PCR tests to fail. However, the gene probe would be a less stringent genetic test than PCR and would have shown some reactivity to a toxin with a small but significant amount of genetic difference with the enterotoxin gene. This it failed to do on RPLA positive/PCR negative isolates.

C. perfringens is often one of the first bacteria to colonize the mammalian gut and at any time a

proportion of healthy animals can be found to be infected apparently harmlessly. However, there is considerable phenotypic and genetic variation within *C. perfringens* and some variants possess virulence factors and markers not possessed by others. Some of the major typing toxins and the enterotoxin are cases in point. Consequently, it could be hypothesised that the association of *C. perfringens* with foal diarrhoea is dependent on a pathogenic sub-population with characteristics distinguishing them from isolates found in healthy animals. One level of evidence for this would be the identification of a subtype which is not associated with diarrhoea when other subtypes are. There was no evidence of this kind on the basis of testing by PCR or RPLA. Another line of evidence for a pathogenic subtype would be a significantly higher frequency of the virulence marker amongst isolates from *C. perfringens* positive cases compared with positive controls. Whether or not the RPLA and cytotoxin tests are detecting a virulence factor distinct from the enterotoxin, isolates positive by either of these tests or the PCR tests were not significantly more common amongst isolates from cases compared with isolates from healthy controls not in contact with cases. Consequently, isolates reacting in any of these tests do not explain the overall association of *C. perfringens* with foal diarrhoea; PCR positive isolates were in any case too infrequent to explain the overall association.

Although no strong evidence can be provided to date for a role for enterotoxigenic *C. perfringens* in foal diarrhoea, its undoubted significance as a cause of mild disease in man, compared with other *C. perfringens* diseases, and the generally self resolving diarrhoea seen in foals in which these bacteria do occur, mean that a minor contribution cannot be ruled out. We are now testing the hypothesis that the association of foal diarrhoea with *C. perfringens* may be related to an as yet uncharacterized pathogenic mechanism possessed by a sub-population of the bacteria by searching for molecular markers which are significantly more common amongst isolates from cases than from isolates from healthy not in contact controls.

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