Clostridium perfringens Type E Animal Enteritis Isolates with Highly Conserved, Silent Enterotoxin Gene Sequences

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Several Clostridium perfringens genotype E isolates, all associated with hemorrhagic enteritis of neonatal calves, were identified by multiplex PCR. These genotype E isolates were demonstrated to express α and ε toxins, but, despite carrying sequences for the gene (cpe) encoding C. perfringens enterotoxin (CPE), were unable to express CPE. These silent cpe sequences were shown to be highly conserved among type E isolates. However, relative to the functional cpe gene of type A isolates, these silent type E cpe sequences were found to contain nine nonsense and two frameshift mutations and to lack the initiation codon, promoters, and ribosome binding site. The type E animal enteritis isolates carrying these silent cpe sequences do not appear to be clonally related, and their silent type E cpe sequences are always located, near the ε toxin genes, on episomal DNA. These findings suggest that the highly conserved, silent cpe sequences present in most or all type E isolates may have resulted from the recent horizontal transfer of an episome, which also carries ε toxin genes, to several different type A C. perfringens isolates.

Clostridium perfringens is an important cause of enteric and histotoxic disease in both humans and domestic animals (14, 18, 25, 26, 28). The virulence of this bacterium largely results from its ability to produce at least 13 different toxins (19, 23). Each individual C. perfringens isolate carries genes for only a subset of these 13 toxins (9, 10, 20, 27), which provides the basis for a commonly used classification scheme (19) that assigns C. perfringens isolates to one of five types (A through E), depending upon the ability of the isolate to express α, β, ε, and ε toxins. C. perfringens type E isolates produce two of these typing toxins, α toxin, a 42.5-kDa single polypeptide with phospholipase C, sphingomyelinase, hemolytic, and lethal properties (29), and ε toxin, a binary toxin consisting of two noncovalently associated components (named εa and εb) that induce the ADP-ribosylation of actin at Arg-177 (3). Previous epidemiologic studies (see reference 1 for a review) have implicated C. perfringens type E isolates in animal enteric disease, including enterotoxemias of calves, lambs, and rabbits. However, understanding of the molecular pathogenesis of these infections is very limited, i.e., it is unclear whether symptoms of type E animal enteritis result exclusively from the action of the α toxin and ε toxin expressed by all type E isolates, or, since the full repertoire of toxins produced by type E isolates has not yet been determined, if these symptoms might involve one or more additional toxins of C. perfringens.

In the present study, 1,347 C. perfringens animal disease isolates were subjected to routine multiplex PCR diagnostic screening (20, 27) using primer sets designed to identify the presence of genes encoding C. perfringens α toxin, β toxin, ε toxin, and ε toxin, or enterotoxin (CPE). During this screening, 12 isolates (all from different herds) that carry both α and ε toxin genes were identified (representative results are shown in Fig. 1). Consistent with previous epidemiologic studies (1), all 12 of these type E isolates originated from neonatal calves diagnosed with hemorrhagic enteritis. Although the samples submitted to us for diagnostic screening were not necessarily random or representative, the fact that all 12 type E isolates identified in this study originated from neonatal calves suffering from hemorrhagic enteritis (with most of these calves experiencing sudden death) is nevertheless notable, since these type E isolates represented 7% of all C. perfringens isolates submitted from similar clinical cases. This suggests that type E C. perfringens may be an underappreciated cause of hemorrhagic enteritis in neonatal calves and that a rigorous epidemiologic survey is perhaps warranted to better evaluate the importance of type E isolates in neonatal hemorrhagic enteritis of calves.

Multiplex PCR analysis also revealed that these 12 type E animal enteritis isolates, as well as the type E reference strain, NCIB 10748, carry cpe sequences (representative results are shown in Fig. 1). These data expand on recent reports (11, 12, 17) that had identified cpe sequences in a few type E reference strains by suggesting that cpe sequences are present in most, if not all, type E isolates, including those associated with animal enteritis.

Demonstrating that most or all type E isolates carry cpe sequences is interesting because <5% of all C. perfringens animal isolates carry cpe sequences (16). Further, given the suggested involvement of CPE in animal enteric disease from C. perfringens type A isolates (5, 16, 26), detection of cpe sequences in most, if not all, C. perfringens type E isolates associated with veterinary enteritis could suggest that CPE contributes to the pathogenesis of type E infections. To evaluate this possibility, five isolates, i.e., 51 (isolated in Kansas), 294 (isolated in Missouri), 572 (isolated in Colorado), and B2085 (isolated from two different herds in Wyoming), along with the type E reference strain, NCIB 10748, were characterized for their toxin-producing abilities. By using the reverse CAMP test (13), all six type E isolates produced (data not shown) the arrow-shaped zone of synergistic hemolysis indicative of α toxin expression (13). Further, antibodies raised...
against purified α toxin, but not antibodies raised against purified CPE, completely neutralized the synergistic hemolysis produced by these type E isolates (data not shown). An actin ADP-ribosylation assay (30) demonstrated (data not shown) that fluid thioglycolate (FTG) supernatants from all six type E isolates catalyzed the ADP-ribosylation of actin, which is indicative of \( \text{iap} \) expression (30). The involvement of \( \text{iap} \) in this actin ADP-ribosylation was supported by demonstrating (data not shown) that antibodies raised against purified \( \text{iap} \) completely neutralized this activity in supernatant from isolate 853 and that identically prepared supernatants from the type A control isolate ATCC 3624 (which lacks \( \text{iap} \) toxin genes) did not catalyze actin ADP-ribosylation. Additionally, 10-fold concentrated FTG culture supernatants of all six type E isolates (but not concentrated FTG supernatant from the type A isolate F4969, which is positive for CPE and α toxin) caused (data not shown) the characteristic rounding of Vero cells that has previously been ascribed to \( \text{iap} \) toxin (3), suggesting that these type E isolates express both the \( \text{iap} \) and \( \text{iap} \) components of \( \text{iap} \) toxin. This conclusion received further support from the failure of antibodies raised against purified \( \text{iap} \) toxin or CPE to inhibit the Vero cell rounding induced by the concentrated FTG supernatants of type E isolates (data not shown).

Consistent with previous reports demonstrating that CPE expression by type A isolates is strongly associated with sporulation (7, 8, 16), CPE-specific Western blotting detected no CPE expression during vegetative growth of the cpe-positive type A isolates F4406 and NCTC 10239 (data not shown) but showed that both of these cpe-positive type A strains (but not the cpe-negative type A strain ATCC 3624) produced moderate to high levels of CPE (Fig. 2) when grown in Duncan-Strong sporulation medium supplemented with 1.5% bile and 0.005% theophylline (DS-B). Interestingly, similar Western blot studies of our five representative type E animal enteritis isolates and NCTC 10748 detected no expression of CPE under either vegetative (data not shown) or sporulating (Fig. 2) growth conditions. Poor sporulation cannot explain the lack of CPE expression by these six type E isolates, since these type E isolates all sporulated in DS-B at levels higher (data not shown) than that (2 \( \times \) 10\(^6\) spores/ml) of NCTC 10239, the type A strain producing moderate, but readily detectable, amounts of CPE in Fig. 2.

The failure of these five recent type E field isolates to express CPE strongly suggests that CPE is not involved in the pathogenesis of type E veterinary enterotoxemias and also indicates that the failure of type E isolates to express CPE is not an artifact of mutations accumulating during long-term laboratory cultivation of reference strains. Further, demonstrating that these type E field isolates (as well as NCIB 10748 carrying cpe sequences) express α and \( \text{iap} \) toxins, but not CPE, shows that these type E isolates are not generally deficient in virulence factor expression.

To our knowledge, these type E isolates represent the first report of \( C. \perfringens \) isolates that carry cpe sequences and sporulate at high levels yet do not express CPE. Consequently, the cpe sequences present in these six type E isolates were investigated by restriction analysis. DNA was isolated from \( C. \perfringens \) isolates as described elsewhere (22) and digested to completion with EcoRV (Promega) according to the manufacturer’s specifications. This EcoRV-digested DNA was electrophoresed on a 1% agarose gel and transferred to nylon membranes by capillary action (24). A 233-bp digoxigenin (DIG)-labeled probe corresponding to internal cpe sequences (8) and a 433-bp DIG-labeled probe corresponding to internal \( \text{iap} \) sequences (EMBL accession no. X73562) were prepared by PCR amplification as described in the Genius System User’s Guide (Boehringer Mannheim), with the primer pair 5\'-GGAGATG GTTGGATATTAGG-3' and 5\'-GGACCACGAGTTTGG TAGATA-3' and the primer pair 5\'-ACTACTCTCAGACAAGAC AG-3' and 5\'-CTTTCCTTACTATACTACG-3', respectively. These cpe- or \( \text{iap} \)-specific probes were hybridized to our blots by standard techniques (24), and DNA fragments hybridizing to these probes were detected by using anti-DIG-alkaline phosphatase conjugate and a nitroblue tetrazolium/X-phos-
phate colorimetric substrate (Boehringer Mannheim). Results from these Southern blot studies localized both cpe and iap sequences to an ∼6-kb EcoRV fragment in all six type E isolates (data not shown), strongly suggesting that cpe and iap sequences are physically linked in type E DNA.

Given this result, a computer search was performed on the previously sequenced (21) region of NCIB 10748 DNA containing the κ toxin genes (EMBL accession no. X73562). This search revealed the presence of cpe sequences, in the opposite orientation, about 600 bp upstream of iap in NCIB 10748 DNA. A similar observation was made by Lindsay (17) during the course of this study. To evaluate whether a gene arrangement exists between cpe and the κ toxin genes iap and ibp in the five type E veterinary enteritis isolates, a PCR was performed with primers corresponding to internal cpe (ECPE, 5'-CACCAATCATAT AAAATTACAC-3') and iap (EIOTA, 5'-ATTTGTAAATCTTGTGCATAAG-3') sequences of NCIB 10748 and oriented toward the start codons of these sequences (Fig. 3). This PCR generated a single 1.4-kb product (data not shown) with DNA from each type E isolate. Since this product matches the size predicted from the NCIB 10748 sequence, these primers were apparently amplifying ∼180 bp of iap sequence, ∼670 bp of cpe sequence, and an “intergenic” sequence of ∼600 bp in DNA from all six type E isolates.

To confirm the identity of these PCR-amplified sequences and to investigate the failed CPE expression, both strands of the 1.4-kb PCR products obtained above were sequenced directly with a 373 DNA sequencer (Applied Biosystems, Inc.). This sequencing analysis confirmed that, as in NCIB 10748, cpe sequences in our type E veterinary enteritis isolates lie ∼600 bases upstream, in the opposite orientation, from the 5' end of iap (Fig. 3). Further, the sequences present in all six type E isolates were found to be identical to the previously determined sequence of NCIB 10748 (EMBL accession no. X73562), with the exception of two single-base pair changes (each occurring in a single type E isolate) located in the 3'-end of the 1.4-kb PCR product containing cpe sequences. Specifically, according to the EMBL sequence numbering, nucleotide 281 in isolate 853 is a C rather than a G, while nucleotide 508 of isolate 294 is a C rather than a T. Since the cpe sequence in these 1.4-kb PCR products is incomplete (as is the NCIB 10748 cpe sequence shown in EMBL accession no. X73562), inverse PCR was performed on EcoRV-digested, self-ligated DNA from isolate 853, by using primer CPEIP (5'-ATGCATTAAA CTCA AATCCATGGTGC-3') and primer IOTAIP (5'-ATAC

![FIG. 3. Gene arrangement in type E strains. Schematic representation of the arrangement of sequences in the cpe-iap region of the five type E veterinary enteritis isolates and NCIB 10748. Long arrows indicate the positions and orientations of gene sequences. Short arrows show the positions and orientations of primers used in the PCRs. The EcoRV sites used in inverse PCR are also indicated, and a scale (in base pairs) is shown below the map.](http://iai.asm.org/)
The binding site of the functional type A cpe. Lindsay also noted (17) the presence of nonsense and frameshift mutations, and the lack of an initiation codon and ribosome binding site, in the partial cpe sequence present in the NCIB 10748 sequence EMBL X73562 characterized by Perelle et al. (21) and predicted, but did not show, that this NCIB 10748 cpe sequence should be silent. However, the complete determination of cpe sequences present in NCIB 10748 (and five type E field isolates) in the present study has revealed several previously unrecognized mutations in the 3' portion of this NCIB 10748 cpe sequence, including a number of additional missense mutations, two additional nonsense mutations, and an additional frameshift mutation, as well as correcting several errors regarding the number and location of mutations that Lindsay had identified (17) in the NCIB 10748 cpe sequence. Further, analysis of the NCIB 10748 cpe sequence during our present study has provided a heretofore unrecognized explanation for the lack of CPE expression by NCIB 10748 and other type E isolates, i.e., all three of the recently identified (31) promoters of the type A cpe gene are missing from the cpe sequence of these type E isolates.

Combining these sequencing results with the CPE expression and Southern blot results presented above, it appears likely that our type E animal enteritis isolates and NCIB 10748 carry a single cpe sequence which is silent not only because it lacks promoters, a ribosome binding site, and an initiation codon but also because it contains numerous nonsense and frameshift mutations. This finding is remarkable given recent results (4, 6, 8) demonstrating that not a single base pair variation is present in the cpe ORF of eight different type A isolates, and it confirms that the mutations present in the silent NCIB 10748 cpe sequence are not simply an artifactual consequence of long-term laboratory cultivation.

The single most interesting piece of new information obtained in our study is that the cpe sequences present in five different animal enteritis isolates are highly conserved, if not identical, and closely resemble the cpe sequence found in NCIB 10748. This strong conservation of cpe sequences among the six sequenced type E isolates could indicate that all six type E isolates examined in this study have a common clonal origin. To evaluate this possibility, DNA from each of the five animal enteritis isolates and from NCIB 10748 was digested with either Apal or MluI, and the digested DNAs were then subjected to pulsed-field gel electrophoresis (PFGE), as described previously (2, 5, 6, 15). Results obtained with these MluI- or Apal-digested DNA samples (Fig. 5 and data not shown) did not reveal any clonal relationship between these six type E isolates.

Since some cpe-positive type A isolates carry a chromosomal cpe, while others carry an episomal cpe (5, 6, 15), a well-established (2, 5, 6, 15) PFGE-Southern blot assay was used to determine whether the silent type E cpe sequences of the five type E animal enteritis isolates and NCIB 10748 have an episomal or a chromosomal location. Confirming that our PFGE-Southern blot assay was working correctly, cpe-containing DNA from the type A control strain NCTC 10239, which carries a chromosomal cpe (5), did not enter pulsed-field gels in the absence of restriction enzyme digestion but ran as an ~360-kb DNA fragment following I-CeuI digestion (Fig. 6). In contrast, some cpe-containing DNA from the type A control strain F4969, which carries an episomal cpe (5), entered pulsed-field gels without restriction enzyme digestion, and the DNA markers are shown at the right.
migitation of this episomal cpe-containing DNA was unchanged by digestion with I-CeuI (which does not cut episomal DNA [2, 5, 6, 15]). Similar PFGE-Southern analysis of DNA from NCIB 10748 indicated (data not shown) that the cpe sequences and \( \xi \) toxin genes of this isolate are present on an episome, which is consistent with recent reports (11, 12) indicating that iap is located on a large plasmid in NCIB 10748 and with our present results establishing a physical linkage between the NCIB 10748 \( \xi \) toxin genes and cpe sequences. When similar PFGE-Southern analysis was extended to the five type E field isolates, cpe sequence-containing DNA from these isolates also exhibited behavior consistent with an episomal location (representative results are shown in Fig. 6). As expected given the demonstrated physical linkage between the \( \xi \) toxin genes and cpe sequences in these type E field isolates, PFGE blots stripped of cpe probe were subsequently able to hybridize with an iap-specific probe at the same location previously occupied by the cpe probe (Fig. 6).

Since these PFGE results and the geographically distinct origins of our type E isolates make it unlikely that our type E animal enteritis isolates have a common lineage, additional hypotheses explaining the presence of virtually identical silent cpe sequences in so many type E isolates must be considered. Localization of the highly conserved type E cpe sequences (and the iap and ibp genes) to episomal DNA suggests the possibility that the episome(s) containing cpe sequences and iap and ibp may have been widely distributed among \( C. perfringens \) isolates only fairly recently (hence, relatively few isolate-specific point mutations have accumulated). Since \( \beta \) toxin or \( \epsilon \) toxin genes are not present in type E isolates, type A isolates appear to be the likeliest recipients of the episome(s) containing the silent cpe sequences and \( \xi \) toxin genes. If this hypothesis is correct, it would be notable, since distribution of the episome containing silent cpe sequences and the iap and ibp genes to a number of different \( C. perfringens \) type A isolates would represent one of the first examples of horizontal transfer of virulence genes in \( C. perfringens \).

Regarding the possible origin of the episome(s) carrying silent cpe sequences and \( \xi \) toxin genes, it is also notable that recent studies (5, 6, 15) have revealed that CPE-positive type A isolates can carry cpe either on the chromosome or on a low-copy-number episome. Further, it has been shown that IS1151 insertion sequences are often associated with the episomal cpe of type A strains, while IS1151 sequences are not found near the chromosomal cpe of type A strains (6). Therefore, the presence of IS1151-like sequences ~1 kb downstream of the silent cpe sequences in all type E isolates examined in this study suggests that the cpe sequences present in type E isolates may have originated from a cpe-containing episome rather than from a chromosomal cpe. This could suggest that the type E epimere carrying both silent cpe sequences and the iap and ibp genes arose from interspecies transfer of a genetic element carrying an iap-ibp homolog into a \( C. perfringens \) isolate already carrying a cpe-containing episome; presumably this transfer was then followed by a recombinational or insertional event between the iap- and ibp-containing genetic element and the cpe-containing episome that resulted in the arrangement of type E DNA shown in Fig. 3. Candidate donors for this putative iap-ibp genetic element include \( Clostridium spiroforme \) and \( Clostridium difficile \), which are known to carry toxin genes highly homologous to iap and ibp (21).

Finally, although it appears counterproductive from a pathogenesis viewpoint for an intestinal pathogen to carry a defective enterotoxin gene (especially since CPE is a recognized virulence factor in animal enteric disease), it is possible that a recombinational or insertional event introducing iap and ibp into a cpe-containing episome disrupted the promoter-start codon region of cpe. Once CPE expression was eliminated, preservation of the coding sequence would no longer be selected for, and mutations may then have accumulated in the cpe coding sequence until the episome containing these sequences was rapidly (and recently) distributed from its original \( C. perfringens \) host to other isolates. The retention of these silent cpe sequences by type E isolates may be related to their close proximity to iap and ibp, whose expression could be under selective pressure, making it difficult for type E isolates to shed their defective cpe sequences.

**Nucleotide sequence accession number.** The nucleotide sequences were submitted to the DDBJ, EMBL, and GenBank databases under accession numbers AF037328 (strains NCIB 10748, 51,572, and B2085), AF037329 (strain 294), and AF037330 (strain 853).

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**REFERENCES**


