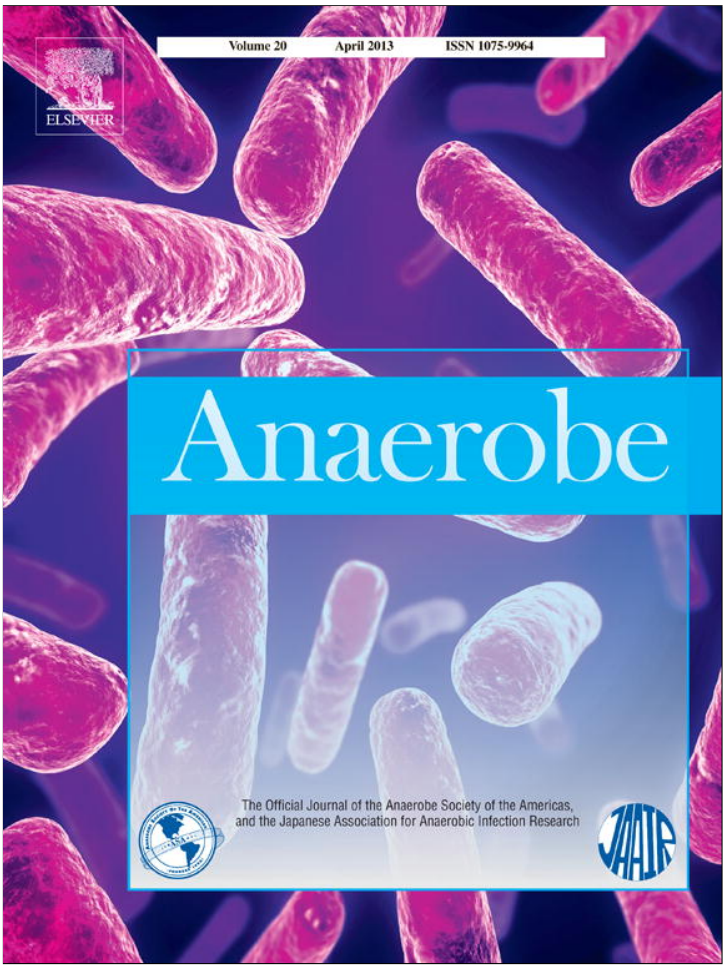

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Clinical microbiology

Sudden death syndrome in adult cows associated with *Clostridium perfringens* type E

L.M. Redondo [a,d], M. Farber [b,d], A. Venzano [a,d], B.H. Jost [c], Y.R. Parma [a,d], M.E. Fernandez-Miyakawa [a,d,*]

[a] *Instituto de Patobiología, Centro Nacional de Investigaciones Agropecuarias, Instituto Nacional de Tecnología Agropecuaria, Calle Las Cabañas y Los Reseros s/n, Casilla de Correo 25 (1712), Castelar, Buenos Aires, Argentina*
[b] *Instituto de Biotecnología, Centro Nacional de Investigaciones Agropecuarias, Instituto Nacional de Tecnología Agropecuaria, Calle Las Cabañas y Los Reseros s/n, Casilla de Correo 25 (1712), Castelar, Buenos Aires, Argentina*
[c] *Department of Veterinary Science and Microbiology, The University of Arizona, 1117 E Lowell Street, Tucson, AZ 85721, USA*
[d] *Consejo Nacional de Investigaciones Científicas y Técnicas, Rivadavia 1917 (1033), Ciudad Autónoma de Buenos Aires, Argentina*

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ABSTRACT

Clostridium perfringens type E is considered a rare toxinotype and an infrequent cause of enterotoxemia of lambs, calves, and rabbits. Until now, only cases of young animal of *C. perfringens* type E bovine enterotoxemia, characterized by hemorrhagic enteritis and sudden death, have been reported. The present report details the genotypic characterization of *C. perfringens* type E isolates obtained from intestinal samples of adult cattle during an outbreak of enterotoxemia in Argentina. The sequences of several housekeeping genes of these isolates were analyzed and compared with those obtained from calves in North America showing a clonal unique lineage.

1. Introduction

Clostridium perfringens is an important pathogen of humans and animals that is widely distributed in the environment. Isolates of this bacterium are classified according to the production of 4 major toxins (alpha, beta, epsilon and iota) in 5 toxinotypes (A, B, C, D and E) [20]. Each toxinotype is related to specific diseases and distinct epidemiological patterns [13]. Several enterotoxic diseases associated with *C. perfringens* are grouped under the generic name of enterotoxemias, suggesting that the pathogenesis is based on the production of toxins in the gastrointestinal tract which are then absorbed into the general circulation causing systemic effects [13,22]. By definition, *C. perfringens* type E produces alpha and iota toxins [6,13,17] although some type E isolates carries additional virulence genes like beta2 toxin Refs. [1,9], lambda toxin Refs. [8], urease [4] and enterotoxin Ref. [15]. Although it has been demonstrated the effect of some of these toxins individually, the role in the pathogenesis of this toxinotype is not completely understood.

C. perfringens type E enterotoxemia is described as a hemorrhagic enteritis often-fatal syndrome mainly in calves, but also described in lambs and rabbits [7]. Necropsy findings are characterized by acute inflammation with multifocal hemorrhages in abomasum and small intestine [13]. Although normally regard as a rare syndrome in production animals, there are numerous reports suggesting that the type E isolates could be approximately 5% of all *C. perfringens* isolates and that could be associated with 50% of the fatal hemorrhagic enteritis in calves [21].

The present report describes an outbreak of enterotoxemia in adult cattle due to *C. perfringens* type E in Argentina. These isolates were further characterized using Multilocus Sequences Typing (MLST) as genotyping method; the obtained sequences were analyzed and compared with those obtained from calves in North America, revealing significant insight into geographical spread of type E strains.

2. Materials and methods

2.1. Case description

The outbreak occurred in an extensive beef cattle herd in Buenos Aires province – Argentina, in September of 2010, during the following month after the calving season. The rodeo consisted of 300

* Corresponding author. Instituto de Patobiología, Centro Nacional de Investigaciones Agropecuarias, Instituto Nacional de Tecnología Agropecuaria, Calle Las Cabañas y Los Reseros s/n, Casilla de Correo 25 (1712), Castelar, Buenos Aires, Argentina. Tel./fax: +54 11 46210443.
E-mail address: mfernandez@cnia.inta.gov.ar (M.E. Fernandez-Miyakawa).

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cows of around 3 years old, divided into 3 groups of 100 animals each. At the time of the outbreak, feeding was based on a pasture of tall wheat-grass (*Agropyron elongatum*). Ten animals died next to a period of 7 days. All of them belonged to the same group of 100 and did not show any clinical signs of disease. The syndrome was not described in animals of different age.

2.2. Necropsy and histopathology

In one of the affected animals necropsy was performed immediately after death, gross lesions were noted and samples were taken from spleen, liver, kidney and different portions of the intestine, these samples were kept refrigerated and in buffered formalin solution for bacteriological and histopathological diagnosis respectively. Tissue samples were embedded in paraffin, and 5 μm sections were cut and processed routinely, and then stained with hematoxylin and eosin.

2.3. Bacteriology

Tissue and intestinal content samples were processed according to a routine protocol of the Bacteriology Laboratory from INTA Castelar. Briefly, they were inoculated directly onto agar McConkey plates and *Salmonella-Shigella* agar plates, and a pre-enrichment step was done in BHI broth and Tetrathionate at 37 °C for 24 h. For the anaerobes diagnosis, samples were processed similarly, inoculating blood agar plates with 5% defibrinated sheep blood and making pre-enrichment in chopped meat broth (CMM). Inoculated media were incubated in anaerobic jar (Oxoid) in atmosphere with H_2 10%: CO_2 10%: N_2 80% for 24 h. After incubation, colonies were analyzed according to the shape, color, production and type of hemolysis. Bacterial morphology was microscopically assessed in gram-stained smears. Colonies presenting *C. perfringens* characteristics were isolated, cultured in CMM and incubated at 37 °C for 18–24 h. These cultures were submitted to the following biochemical tests for species identification: production of catalase, lecithinase and gelatinase, fermentation of glucose and lactose, and skim milk coagulation. All strains were incubated in CMM, and after 18–24 h of incubation at 37 °C, cultures were stored at room temperature.

2.4. PCR genotyping

Bacterial cells were scraped from plates, resuspended in water and boiled for 20 min.

Cell debris were removed by centrifugation at 13,000× g for 5 min and the supernatant was used as template DNA. For *C. perfringens* toxin type identification multiplex PCR targeting *cpa*, *cpb*, *etx*, *iap*, *cpe* and *cpb2* (Table 1) was performed according to Meer et al. [14].

Table 1
Oligonucleotides used for multiplex PCR.

Gene	Primer	Sequence	Amplicon size
Cpa	F	GCTAATGTTACTGCCGTTGA	324
	R	CCTCTGATACATCGTGTAAG	
Cpb	F	GCGAATATGCTGAATCATCTA	196
	R	GCAGGAACATTAGTATATCTTC	
Etx	F	GCGGTGATATCCATCTATTC	655
	R	CCACTTACTTGTCCTACTAAC	
iA	F	ACTACTCTCAGACAAGACAG	446
	R	CTTTCCTTCTATTACTATACG	
Cpe	F	GGAGATGGTTGGATATTAGG	233
	R	GGACCAGCAGTTGTAGATA	
Cpb2	F	AGATTTTAAATATGATCCTAACC	567
	R	CAATACCCTTCACCAAATACTC	

For genotyping the *C. perfringens* isolates obtained from this outbreak we used the Multilocus Sequence Typing (MLST) scheme developed by Jost et al. [10], (oligonucleotides are listed in Table 2). PCR products were submitted for purification and sequencing to Genomic Unit from the Institute of Biotechnology, INTA (DNA Analyzer ABI3130XL from Applied Biosystems). The nucleotide sequences of seven housekeeping gene fragments and one virulence factor were determined on each DNA strand using the amplification primers.

2.5. Multilocus sequence typing analysis

We used nucleotide sequence from the *C. perfringens* MLST scheme (8 genes from 132 isolates) kindly provided by Jost to develop an MLST database under MySQL, an open source relational database management system. For each sample that was genotyped using the MLST procedure, a pipeline using open source programs (Phred, CAP3) and custom scripts were built up. It was used to rapidly collate paired reads, determines sequences and identify alleles to end up with a haplotype called sequence type (ST), using the *C. perfringens* database for comparison. Both the MLST pipeline and the database are accessible after system administrator manager authorization at http://bioinformatica.inta.gov.ar/mlst_pipeline/.

2.6. Nucleotide sequence accession numbers

Each sequence analyzed in this study has been uploaded to the GenBank database (http://www.ncbi.nlm.nih.gov) under the following accession numbers: JX874985 and JX874993 for *ddlA*, JX874986 and JX874994 for *dut*, JX874987 and JX874995 for *glpK*, JX874988 and JX874996 for *gmK*, JX874989 and JX874997 for *plc*, JX874990 and JX874998 for *recA*, JX874991 and JX874999 for *sod*, JX874992 and JX875000 for *tpi*.

3. Results

3.1. Necropsy findings and histopathology

Post-mortem examination revealed multifocal mucosal hemorrhage in abomasum and small intestines, the content of these organs was watery and blood-stained fluid. No traces of toxic plants were found in rumen. Histopathologically, the principal intestinal lesion was superficial mucosal hemorrhagic necrosis at the jejuneileum (Fig. 1). Many Gram-positive bacilli were found adhering to the necrotic mucosal surface in parts of the intestinal tract. Streaks

Table 2
Oligonucleotides used for MLST.

Gene	Primer	Sequence	Amplicon size
plc	plcF	ATATGAATGGCAAAGAGGAAAC	544
	plcR	AGTTTTTCCATCCTTTGTTTTG	
ddlA	ddlAF	ATAATGGGGGATCATCAGTTGC	429
	ddlAR	TTATTCCTGCTGCACTTTTAGG	
dut	dutF	TTAAGTATTTTGATAACGCAAC	441
	dutR	CTGTAGTACCAAATCCACCACG	
glpK	glpKF	TGGGTTGAGCATGATCCAATGG	547
	glpKR	CACCTTTTGCTCCAAGGTTTGC	
gmk	gmkF	TAAGGGAACTATTTGTAAAGCC	475
	gmkR	TACTGCATCTTCTACATTATCG	
recA	recAF	GCTATAGATGTTTTAGTTGTGG	475
	recAR	CTCCATATGAGAACCAAGCTCC	
sod	sodF	GATGCTTTAGAGCCATCAATAG	475
	sodR	AATAATAAGCATGTTCCCAAAC	
tpi	tpiF	AAATGTGAAGTTGTTGTTTGCC	451
	tpiR	CATTAGCTTGGTCTGAAGTAGC	

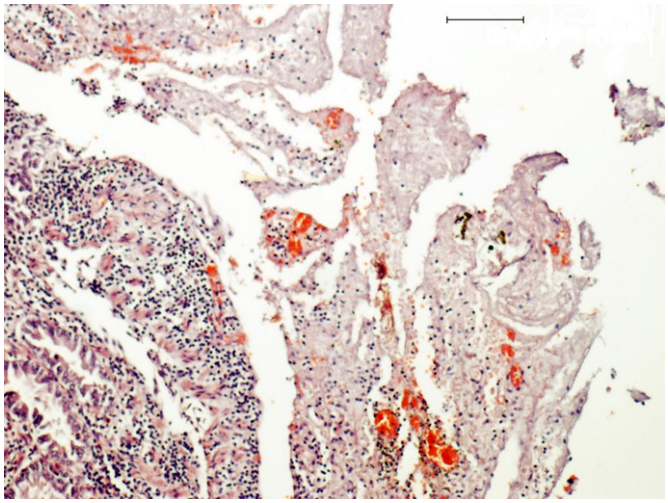

Fig. 1. Sample section of ileum of one of the affected animals. Tissue is autolytic but is possible to observe epithelium detachment, villi atrophy and the presence of hemorrhagic foci. In the lamina propria mononuclear infiltrate is evident. Hematoxylin and eosin. Bar = 100 μm.

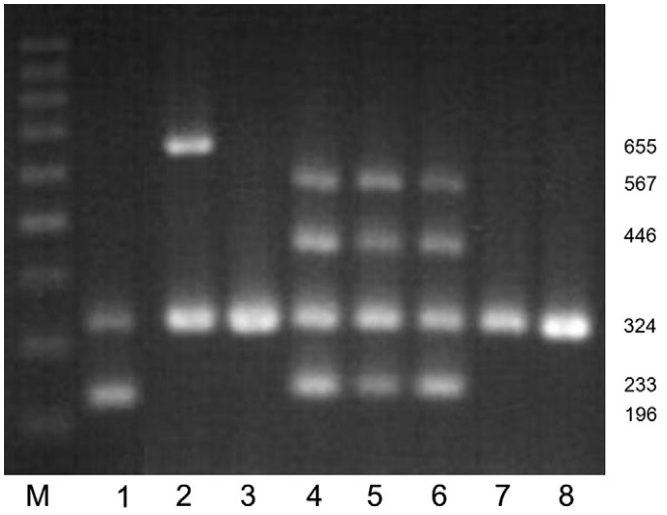

Fig. 2. PCR genotyping. M, 100bp ladder; lane 1, Type C reference strain; lane 2 Type D reference strain; lane 3 Type A reference strain; lane 4 Type E reference strain; lanes 5–6, Type E isolates from outbreak; lane 7–8 Type A isolates from outbreak.

of inflammation were seen on the mucosal surface of the spiral colon, and mesenteric lymph nodes were edematous and inflamed. Microscopic examination of tissues revealed mild autolytic change in segments of jejunum, ileum, and colon. Some areas of ileum appeared necrotic, with inflammation of some villous tips, and signs of lymphadenitis were evident.

3.2. Bacteriology

Large numbers of short gram-positive rods with blunt ends were observed in smears of duodenum, ileum, and colon. No growth was observed in the aerobic or in the anaerobic cultures of the spleen, liver and kidney. In the intestinal inocula, only few mucoids colonies were observed in aerobic cultures, although almost pure cultures of *C. perfringens* were obtained from the anaerobic cultures. At least three colonies of each processed sample were kept in chopped meat broth for biochemical and molecular characterization.

3.3. PCR genotyping

PCR analysis determined that most isolates belonged predominantly to type E toxinotype (Fig. 2) because of the detection of the *cpa* and *itx* genes. In addition, a type A isolate was identified in the intestinal content of one animal. Like most of the type E isolates from animal enteritis cases and the reference strain (NCBI 10748), the analyzed isolates of the present study carry *cpe* and *cpb2* sequences too.

3.4. Multilocus sequence typing analysis

Since all the existing reports of disease caused by *C. perfringens* type E in cattle only describe it in neonates, we investigated the genetic background of the isolates obtained in this outbreak using MLST analysis following the scheme developed by Jost and collaborators [10]. The ST of each isolate was defined after sequencing and analysis of the corresponding genes, and then it was compared with the respective STs of other type E isolates obtained from different bovines. All of the outbreak isolates were assigned a unique ST which resulted to be identical to the ST assigned to most of the bovine type E isolates, including strain NCBI 10748.

4. Discussion

Since first description, more than 50 years ago [7], the iota enterotoxemia has been considered an uncommon cause of enteritis in calves, lambs and rabbits [11]. However, recent reports which analyzed more than 1000 isolates from different regions of the United States, revealed that approximately 4% of the isolates obtained belong to type E and that they represent almost 50% of the isolates obtained in cases of fatal enteritis in calves [21]. Type E isolates were also obtained from other animal species with clinical signs of iota enterotoxemia as goats, deers and peccaries [19]. In Brazil, *C. perfringens* type E has been also isolated in a relatively important proportion, with a prevalence of iota toxin in nearly 30% of samples from healthy and diarrheic calves [16] and 9% of samples submitted from calves with sudden death [5]. Although these studies do not analyze a representative sample, they might suggest that this toxinotype is more common than expected, at least in certain regions. In Argentina, one report mentions the isolation of *C. perfringens* type E from processed raw meat foods [23] although information about the previous isolation of this toxinotype from animals in this country is not available.

C. perfringens type E enterotoxemia has been characterized by hemorrhagic enteritis or sudden death, and has been described only in calves and lambs with often - fatal hemorrhagic enteritis [7,13]. The lesions observed at necropsy are hyperemia and edema in the mucosa of intestine and abomasum, with foci of hemorrhage, acute inflammation and submucosal edema [21,22]. The outbreak of *C. perfringens* type E enterotoxemia of the present report involved adults cows and it was characterized by sudden death in peri-partum with necropsy and histopathological findings similar to those reported in cases of calves. This presumptive diagnosis was confirmed by bacteriological and molecular identification of *C. perfringens* type E. According to our knowledge, this is the first report of this disease in adult cattle, suggesting that *C. perfringens* type E could be potentially dangerous for animals of any age. Further studies are needed to clarify the mechanisms involved in the pathogenesis of enteritis caused by this toxinotype, and identify the predisposing factors, along with a complete characterization of the strains involved in these outbreaks and the role of iota toxin during the disease.

Although traditionally *C. perfringens* isolates are classified according to the production of major toxins, this scheme does not

include potential virulence factors which could be present in several isolates undifferentiated within the classical toxinotype scheme. Also, another drawback is that it not allows defining relationships between strains of different origins. Because of this, numerous methods have been used to infer relationships among bacterial isolates [12,18] and to differentiate disease causing strains; these methods have included the MLST, which is currently considered the 'gold standard' for bacterial typing [24]. This method analyzes sequences of several house-keeping genes and performs a profile of alleles that determine the sequence type (ST) of an isolate, through which it is possible to infer relationships between different isolates. Therefore, for a more complete genetic characterization of the isolates of the present study, a comparative MLST analysis all of these strains was performed. The results shows that the type E isolates obtained from the intestine of animals affected by the outbreak have the same ST, which is coincident with a type E reference strain (NCBI 10748). According to Jost [10], this ST belong to a clonal complex (or cluster), which includes several type E isolates, all from diseased bovines, together with a few type A isolates from porcine and canine origin. A previous report which describes the analysis by Pulsed Field Gel Electrophoresis (PFGE) of some of the strains belonging to this cluster, suggest that these strains would not be clonal [2]. Although these apparently contradictory results would be related to the fact that PFGE analysis includes not only DNA of chromosome but also from plasmids, the MLST results suggest that at least these Type E isolates share a conserved genetic background, which could be related to a phenotype which is particularly successful within an ecological niche in the bovine intestine.

The analysis of the sequences of several chromosomally located genes in this work and others [10] from various bovine isolates belonging to type E, suggests that the presence of the plasmids carrying the genes *iap/ibp* is mainly associated to a defined subset of *C. perfringens*. Further studies with a larger number of strains are needed to clarify whether this is due to the maintenance of the mentioned plasmid by chromosomally encoded factors, or that the combination of certain plasmid genes together with chromosomal genes, produces an increased fitness of the bacteria in cattle [10]. Other groups demonstrated that groups of isolates with a common gene pool could be linked to specific diseases in determined host species [3,15].

Enteric diseases associated with *C. perfringens* type E occurs in a variety of animal species and ages, including adult cow. Frequently, many of the diagnoses of *C. perfringens* outbreaks, like the one presented in this report, are based on bacteriological identification assuming that the digestive syndromes are due to the apparently more common types A and C. Therefore, the genotyping of the isolates of *C. perfringens* during routine diagnostic must be considered essential and also the inclusion of iota toxin detection in the tests for enterotoxemia, Further studies are necessary to determine the role of toxinotype E in digestive diseases of cattle to design health plans based on complete knowledge of the epidemiology and pathogenesis of clostridial diseases.

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