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GENOTYPING OF *Clostridium perfringens* ASSOCIATED WITH SUDDEN DEATH IN CATTLE

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ABSTRACT: Toxigenic types of *Clostridium perfringens* are significant causative agents of enteric disease in domestic animals, although type E is presumably rare, appearing as an uncommon cause of enterotoxemia of lambs, calves and rabbits. We report herein the typing of 23 *C. perfringens* strains, by the polymerase chain reaction (PCR) technique, isolated from small intestine samples of bovines that have died suddenly, after manifesting or not enteric or neurological disorders. Two strains (8.7%) were identified as type E, two (8.7%) as type D and the remainder as type A (82.6%). Commercial toxoids available in Brazil have no label claims for efficacy against type E-associated enteritis; however, the present study shows the occurrence of this infection. Furthermore, there are no recent reports on *Clostridium perfringens* typing in the country.

KEY WORDS: *Clostridium perfringens,* iota toxin, sudden death, PCR, cattle.

CONFLICTS OF INTEREST: There is no conflict.

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INTRODUCTION

The aim of this work is to describe the typing of *Clostridium perfringens* strains isolated from cattle, a widely occurring pathogenic bacterium and certainly the most important cause of clostridial enteric disease (enterotoxaemia) in domestic animals (1).

The pathogenicity of *Clostridium perfringens* is associated with several toxins. The alpha, beta, epsilon and iota toxins are the major lethal poisonous substances produced by the organism and are closely related to its virulence, even though they produce several minor extracellular toxins. Usually, *C. perfringens* has been classified into five toxigenic types (A through E) on the basis of their ability to produce the major lethal toxins (2, 3). Two other major toxins (i.e. enterotoxin and beta-2) can also be produced by all types of *C. perfringens*, although they are not used for its typing (4).

C. perfringens type A is consistently recovered both from the intestinal tracts of animals and from the environment, while others (types B, C, D and E) are less common in animal intestinal tracts (5).

In sheep, type D is widely regarded as the causative agent of fatal enterotoxemia or "overeating disease" (3), but is also important in calves, goats and adult cattle (1). In Brazil, Lobato *et al.* (6) reported the detection of *C. perfringens* type D in a bovine enterotoxaemia case, but *C. perfringens* typing has been rarely described in the country.

Type E is a putatively uncommon cause of enterotoxemia of lambs, calves and rabbits (7). Little is known about the pathogenesis of type E infections, although it is assumed that, in keeping with the pattern set by isolates of other toxin types, iota toxin plays an important role (8). Recently, Songer and Miskimmins (8) reported two cases of bovine enterotoxemia caused by *C. perfringens*, determined as genotype E by PCR analysis.

Bovine enterotoxaemia is characterized by a high case fatality rate, sudden deaths, lesions of hemorrhagic enteritis of the small intestine and, quite often, an absence of other clinical signs (1). In the present trial, among the *C. perfringens* strains isolated from intestinal samples obtained in *post-mortem* examination of 23 bovines – 60.86% presented only sudden death (14/23), 21.74% of which were preceded by enteric disorders (5/23), 13.05% by neurological symptons (3/23) and 4.35% by both enteric and neurological disorders (1/23) – were submitted to toxin-gene typing by

polymerase chain reaction (PCR). Macroscopically, hemorrhagic small intestines were reported in 39.13% of cases (9/23), hemorrhage in other tissues such as heart, kidney or liver in 21.74% (5/23), while icteric liver was observed in 17.4% (4/23) and pethechiae in 21.74% (5/23). Macroscopic pathologies were absent in six animals (26%). The samples were collected in five Brazilian states (São Paulo, Minas Gerais, Mato Grosso do Sul, Goiás and Paraíba) from 2005 to 2007, and the age of the animals varied from 2 to 10 years.

MATERIAL AND METHODS

Isolation and Biochemical Identification of *C. perfringens*

The samples were cultured in Cooked Meat Medium (CMM) at 37°C for 18 to 24 hours and 10 µL of this culture was streaked in a plate containing Mueller-Hinton agar with 5% defibrinated sheep blood and incubated under anaerobic conditions in McIntosh and Fields jars submitted to vacuum conditions before hydrogen inoculation at 37°C for 18 to 24 hours.

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 to 24 hours. 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. Interpretation was performed according to Cowan (9).

All strains were incubated in CMM, and after 18 to 24 hours of incubation at 37°C, cultures were stored at room temperature

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Polymerase Chain Reaction (PCR)

The strains biochemically identified as *C. perfringens* isolates were typed by PCR with toxin-specific primers. DNA extraction was performed for each strain kept in CMM by the guanidine isothyocianate methodology adapted from Boom *et al.* (10). The extracted DNA was used for multiplex PCR detection of alpha, beta, epsilon and iota toxin genes with specific primers described by Meer and Songer (11) (Table 1) which amplifies 324 bp, 196 bp, 655 bp and 446 bp fragments, respectively, in a 30-cycle amplification protocol as follows: denaturation at 94°C for 30 seconds,

hybridization at 53°C for 30 seconds and extension at 72°C for 40 seconds. As positive control, three other *C. perfringens* strains from the Biological Institute (types A, C and E) were used.

Amplification reactions were carried out in a Peltier Thermal Cycler-200 (MJ Research) and the analysis of the amplified products was performed by means of electrophoresis in 1.3% agarose gel with TBE 0.5 X running buffer (0.045 M TRIS-Borate and 1 mM of EDTA ph 8.0). Gel was stained with ethidium bromide, visualized with a UV transiluminator (300-320 nm) and photographed by a photodocumentation system (Kodak Digital Camera DC/120® Zoom, Brazil) and analyzed with the software 1D Image Analysis® (Kodak Digital Science, Brazil).

Table 1. Primer sequences

Primer	Sequence	Reference
cpa F	5´ GCT AAT GTT ACT GCC GTT GAC C 3´	Meer and Songer (11)
cpa R	5´TCT GAT ACA TCG TGT AAG 3´	Meer and Songer (11)
cpb F	5´GCA GGA ACA TTA GTA TAT CTT C 3´	Meer and Songer (11)
cpb R	5´ GCG AAT ATG CTG AAT CAT CTA 3´	Meer and Songer (11)
etx F	5´ CCA CTT ACT TGT CCT ACT AAC 3´	Meer and Songer (11)
etx R	5´ GCG GTG ATA TCC ATC TAT TC 3´	Meer and Songer (11)
ia F	5´ ACT ACT CTC AGA CAA GAC AG 3´	Meer and Songer (11)
ia R	5´ CTT TCC TTC TAT TAC TAT ACG 3´	Meer and Songer (11)

RESULTS

Biochemical Characterization of the Strains

Twenty-three strains were biochemically identified as *C. perfringens*, due to the production of catalase, lecithinase and gelatinase, and to fermentation of glucose, lactose and tumultuous fermentation of skim milk.

Polymerase Chain Reaction (PCR) for *C. perfringens* Strain Typing

Among the 23 *C. perfringens* strains isolated, nineteen (82.6%) were differentiated as type A with presence of only alpha toxin gene, two strains (8.7%) as type E that besides alpha toxin presented an iota toxin gene, and two strains (8.7%) as type D that displayed both alpha and epsilon toxin genes (Table 2).

Table 2. Results of *C. perfringens* strain typing by PCR

Sample number	Locality (state)	Type of *C. perfringens*
1	Paraíba	E
2	Minas Gerais	E
3	São Paulo	D
4	Minas Gerais	D
5	São Paulo	A
6	São Paulo	A
7	São Paulo	A
8	São Paulo	A
9	São Paulo	A
10	São Paulo	A
11	São Paulo	A
12	São Paulo	A
13	São Paulo	A
14	São Paulo	A
15	São Paulo	A
16	São Paulo	A
17	Goiás	A
18	Minas Gerais	A
19	Minas Gerais	A
20	Minas Gerais	A
21	Minas Gerais	A
22	Mato Grosso do Sul	A
23	Mato Grosso do Sul	A

DISCUSSION

The samples submitted to the Biological Institute (São Paulo, Brazil) for diagnostic screening are not necessarily representative; nevertheless, there have been no reports regarding *C. perfringens* typing in Brazil.

Most strains were identified as type A, which is reported to be easily isolated from tissues, effusions and intestinal tract of cadavers within a few hours after death; furthermore, it grows rapidly in culture and may mask other organism growth. Also, prevention of infections or intoxications caused by type A is complicated for at least two reasons: firstly, the organism is so ubiquitous that it is impractical to resort to mass immunization and, secondly, the enzymatic nature of the majority of the antigens given that enzymes are generally considered weak antigens in comparison

to other proteins (3). However, recently a *C. perfringens* type A toxoid for cattle in the US was developed since standard clostridial vaccines do not offer protection against the alpha toxin of *C. perfringens* type A, nor there is any cross-protection.

The amplification of toxin genes from different types of *C. perfringens* is specific when compared with the *in vivo* methodology by toxin seroneutralization tests as described by Kadra *et al*. (12), and is also shown to be more rapid and reliable.

It has been reported that *C. perfringens* type E is an apparently uncommon cause of enterotoxemia in calves (1), and most reports describe other types of the microrganism involved in these cases (13,14). In the present trial, most strains (82.6%) were found to be *C. perfringens* type A, similarly to a recent report by Manteca *et al*. (15). In Brazil, Baldassi *et al.* (16) reported the typing of 89 *C. perfringens* strains from bovines by ELISA test and, besides cross-reactivity among samples, the presence of *C. perfringens* type E was not evaluated.

The fact that all these strains had originated from a single host type and sudden death condition is notable, and this report provides some information about which types of *C. perfringens* are causing enterotoxaemia in Brazilian herds, thus alerting investigators to the occurrence of type E. More rigorous epidemiological and diagnostic pursuit of similar cases may be warranted.

It is also important to note that currently available commercial toxoids in Brazil will likely offer little or no protection against type E infections.

The inclusion of genotyping as part of the diagnostic approach will provide more information on the true importance of the occurrence herein reported.

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