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VIRULENCE EXALTATION OF *Clostridium perfringens* STRAINS FROM BOVINES.

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ABSTRACT. Ten out of eighty-nine strains biochemically identified as *Clostridium perfringens*, isolated from bovine organs, were selected by their different results showed in toxigenicity test on mice. Those and the standard strains, ATCC types A, B, C, and D, had their virulence exalted through serial intramuscular inoculation into guinea pigs.

Results showed that, for toxigenic strains (6), one or two passages were enough to cause exaltation, while for the atoxigenic (4), five or six inoculations were needed. Esterase electrophoresis of standard and isolated strains, with and without exaltation, was performed. Electrophoresis analysis permits the following conclusions: strains that do not show any clinical symptoms in mice, when exalted demonstrate decreased esterase activity; on the contrary, it is increased when correlated with animal symptoms.

KEY WORDS: *Clostridium perfringens*, bovine, electrophoresis, virulence exaltation.

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INTRODUCTION

Clostridia have little low invasive ability, and the pathogenicity of these bacteria is mainly determined by toxin production. From the species of this genus, *C. perfringens* is the most widely spread (13,15,30). According to Willis (38), the predominance of the several types (A, B, C, and D) is not uniform, and type A is the most prevalent one; soil is believed to be its natural environment (33,35,38). *C. perfringens* is also found in the feces of some animal species (35). The other types are predominantly related to the intestinal tract of animals, and their presence in soil is due to fecal contamination (33,38).

C. perfringens produces several soluble antigens (toxins), and most of the studies focuses on the pathogenic effects they produce. However, the role each of the toxins has in the production of lesions and symptoms caused by this agent is not totally established, neither in men nor in animals (24,25).

Different cases of acute enteritis or fatal enterotoxaemia have been reported in several animal species, such as sheep, cattle (20,21,31), pigs (12), dogs (9), and horses (26). Sudden death in ovine, bovines and caprines has also been attributed to *C. perfringens* (3,27,34).

In the present trial, standard and isolated *C. perfringens* strains, from samples obtained in *post-mortem* examination of bovines, were submitted to virulence exaltation. Esterase-electrophoretic profile of strains with and without exalted virulence was compared.

MATERIAL AND METHODS

Sampling

Ten from eighty-nine strains biochemically identified as *C. perfringens*, isolated from *post-mortem* examination of 71 bovines, were selected by their different results showed in the toxigenicity test on mice (4).

As a positive control, four other *C. perfringens* strains from *American Type Culture Collection* (ATCC), ATCC 3624 (type A), ATCC 3626 (type B), ATCC 3628 (type C), and ATCC 3629 (type D), were used.

Isolation and biochemical identification of C. perfringens in field samples

Isolated and standard strains were cultured in Cooked Meat Medium (CMM) at 37°C for 18-24 hours and then kept at 4°C. Three µl of this culture was streaked in a plate containing Brain Heart Infusion (BHI) agar, with 5% of defibrinated sheep blood and incubated in anaerobiosis in McIntosh and Fields jars at 37°C for 18-24 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 18-24 hours. These cultures were submitted to the following biochemical tests for species identification: production of catalase, lecithinase and gelatinase, fermentation of glucose, lactose and skim milk. Interpretation was performed according to Holdeman and Moore (16); Mahony and Swantee (22); Mahony *et al*. (23).

All the strains of the present trial were incubated in CMM, and after 18-24 hours of incubation at 37°C, cultures were stored at 4°C.

Toxigenicity of isolated strains

Each bacterial isolate and standard strain was cultured in plates containing Brain Heart Infusion Blood (BHIB) agar, incubated in MacIntosh and Fields jars at 37°C for 18-24 hours. Five colonies were transferred from these cultures (23) to tubes containing 10 mL of Tryptose Yeast Extract (TYE) broth, followed by incubation in the same conditions as described above. After incubation, all the contents of each tube was transferred to a flask containing 90 mL of the same medium, and incubated the same way (29). Culture morphology and purity was assessed in each step of the procedure, by Gram staining.

The final culture was then centrifuged at 7,500xg at 4ºC for 15 minutes for cell debris removal. Supernatant was withdrawn and used in the determination of toxigenicity on mice.

The supernatant of each isolate or standard strain culture was intraperitonially inoculated into ten albino mice, weighting from 20 to 25 g, in a dose of 0.5 mL. Pure supernatant was injected into five mice, and the remaining animals received trypsin-treated supernatant, in a final concentration of 1%, after incubation at 37ºC for 30 minutes (2). Animals were observed for up to 96 hours in order to detect clinical signs or death.

Virulence exaltation

Four isolates and ten standard strains presenting toxigenicity variations were submitted to virulence exaltation. They were cultured in CMM, and after 18-24 hours at 37°C, intramuscularly inoculated into the internal part of the hind legs of guinea pigs. By an incision in the previously wounded muscle, a fragment was collected and cultured in CMM. Successive passages were performed, until the administration of 0.1 mL inoculum of a 6-hour culture killed a guinea pig, weighting from 200 to 250 g, in 24 hours (19).

Sediments of exalted and non-exalted strains were washed in 100 mL of buffered tris-glycine solution 60 mM, pH 8.7, followed by centrifugation at 10,000x*g* at 4°C for 10 minutes. Cell mass of each isolate was suspended in 1.0 mL of the same buffer, grinded in a mortar containing liquid nitrogen, and centrifuged once more at 10,000x*g* at 4ºC for 30 minutes (29). From the sediment obtained in the last centrifugation, aliquots of about 1 mL were withdrawn and kept at -20ºC until submitted to electrophoresis.

Electrophoretic analysis

Estimated protein concentration was determined by the Bradford method (6), using bovine albumin V as standard protein. Readings were performed in a microplate reader (BIO-RAD, model 3550-UV) connected to a computer with Microplate Manager software.

Sediments were applied to a polyacrylamide gel, in 18 μL of 7% acrylamide/bis-acrylamide, corresponding to 250 μg of proteins. Tris-glycine buffer pH 8.2 – 0.1M was used both in the preparation of the gel and in the analysis (5).

The gel was stained with a solution containing Fast Blue substrate and α-naftil acetate for 60 minutes, kept in distilled water for complete removal of the substrate, and dehydrated in a drier (BIO-RAD, model 583). Reading was performed in a densitometer (BIO-RAD, model GS-700) connected to a microcomputer and analysed by Molecular Analyst software.

RESULTS

Biochemical characterization of the strains

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

Toxigenicity determination

From the 89 strains tested, 51 (57.3%) have shown to be toxin-producers. Fourteen of them were toxigenic (15.7%) only when the supernatant was in the untreated form, 8 (9.0%) only after trypsin treatment, and 29 (32.6%) in both forms. Thirty-eight (42.7%) strains did not kill any mice and were considered to be atoxigenic (Table 1).

Characterization and analysis of esterase electrophoretic profile

In the densitometry of electrophoretic mobility (Rm), standard strains A, B and D presented only one band each, between 1.7 and 3.2 cm; 3.0 and 4.8 cm; and 2.5 and 6.0 cm, respectively. Standard strain C presented two bands, one between 1.5 and 3.5 cm, and the other between 3.5 and 5.4 cm. The 89 strains selected were classified using these Rm as parameters (5).

Virulence exaltation

The 10 strains selected in the toxigenicity assay presented the following variation: two of them were toxigenic before and after trypsin treatment; two were atoxigenic only without treatment; two were toxigenic only after treatment, and four did not show to be toxigenic in neither forms (Table 1). The inoculation of these strains into guinea pigs showed that for strains that were already toxigenic, one or two passages were enough to cause virulence exaltation, while the atoxigenic ones only showed pathogenicity after five or even six passages.

Electrophoresis of the standard strains type A and D produced bands similar in the original form and with exalted virulence. Strain type B presented three bands and type C only one band, while the original form presented two bands (Figure 1). The bands volume densitograms percentiles, bands areas, mean absorbance, and relationship absorbance X area

of strains in their original form and exalted are presented in Figure 2.

Exalted strains that were totally atoxigenic or did not show any clinical symptom demonstrated decrease in the esterase activity when compared to not exalted strains. On the contrary, when clinical symptoms are present, the activity of esterase was higher in exalted strains (Figure 3).

DISCUSSION

Disease and death in herds pose potential risks to public health. Economic losses are estimated in 17% of the production costs in developed countries, and more than twice as this for developing ones. Diseases caused by clostridia are, up until today, a great economic problem in Brazil. When vaccination schemes are strictly followed, these diseases may be adequately controlled. However, deaths caused by *C. perfringens*, mainly in bovine herds, are responsible for great losses by cattle producers, decreasing meat exports and protein availability to the population. Most of the times, the cause of these deaths is not determined, or when it is, diagnosis is restricted to bacterial genus and species, without type identification.

In veterinary practice, diagnosis of enterotoxaemia is based on history, clinical signs, and findings in the *post-mortem* examination. However, laboratory analyses are essential to confirm the presence of toxins (34). In some cases, toxigenic strains found in bacterial cultures of recently collected clinical samples are confirmatory proofs.

In general, detection of *C. perfringens* toxins in feces samples by the assay in mice is the standard confirmatory diagnostic test. However, tests performed in sheep detected low levels of ε toxin in healthy and immunized animals (8). Both enterotoxin and ε toxin have their toxic activity increased when treated with trypsin (14,28,37), while β toxin is inactivated by the same treatment (11). This may explain the finding in the present study of 9% toxigenic strains only after trypsin treatment and 15.7% strains that lost their toxigenicity after the treatment, as observed in Table 1.

The inoculation of supernatants from cultures of isolated strains into mice (Table 1) showed the presence of toxins; however, their types were not identified. From the 89 strains isolated,

51 (57.3%) were toxin producers and, therefore, potentially pathogenic. This result is in accordance with those obtained by Tsai *et* al., (36), who observed, in Canada, 60% of toxigenicity in *C. perfringens* strains isolated from samples of bovines from slaughterhouse.

According to Holliday (17), the degree of encapsulation is associated with pathogenicity, since stronger reactions were observed with clinical isolates than with fecal or environmental strains. Strains kept for long periods in laboratory or those that have undergone many passages in artificial media lost their capsule and became uneven (18). These observations, in the present trial, justify the occurrence of toxigenic and atoxigenic strains in different organs of the same animal and the fact that atoxigenic strains started to present toxigenicity after their virulence exaltation. In relation to enterotoxin production, it was demonstrated that the strain ability of producing toxin *in vitro* does not reflect its ability to produce it *in vivo*. However, cultures kept under the same conditions may determine the strain toxigenic potential (23).

The existence of atoxigenic variants or variants that lose their toxicity should be considered (7,10), as well as the fact that serological tests for toxins do not show a clear separation between the several types of *C. perfringens* (32).

Analysis of proteins and enzymatic standards by electrophoresis is a valuable method for the determination of genetic and physiological compounds in the host, and in vegetal or animal pathogens (1,29). In this case, electrophoresis was done after exaltation to verify if the increasing toxigenicity of esterase activity would be altered. The results obtained in this work permit the following conclusion: strains that do not show any clinical symptom in mice, when exalted, have their esterase activity decreased; on the contrary, this activity is increased when correlated with animal symptoms. It must be pointed out, however, that the results of this study should be carefully considered due to the small number of strains tested.

Table 1. Tripsinizated and non-tripsinizated supernatans toxigenicity of 89 isolated *C.perfringens* strains from bovine origin.

Sample number	Supernatant without tripsinization	Supernatant tripsinizated	Sample number	Supernatant without tripsinization	Supernatant tripsinizated
1	+	+	46	-	-
2	+	+	47	-	-
3	-	-	48	+	-
4	-	-	49	-	-
5	+	+	50	-	-
6	-	-	51	-	-
7	+	-	52	-	-
8	-	-	53	-	-
9	-	-	54	-	-
10	+	+	55	-	-
11	-	+	56	-	-
12	-	-	57	-	-
13	+	+	58	-	-
14	+	+	59	+	-
15	+	+	60	+	+
16	+	+	61	-	-
17	+	-	62	-	-
18	+	-	63	+	-
19	+	+	64	+	-
20	-	-	65	-	+
21	-	-	66	-	-
22	+	+	67	+	-
23	-	-	68	+	-
24	+	+	69	+	-
25	+	+	70	-	+
26	+	-	71	+	+
27	-	-	72	-	-
28	+	+	73	-	-
29	+	+	74	+	+
30	+	+	75	+	+
31	+	+	76	+	+
32	-	-	77	-	-
33	-	-	78	-	-
34	+	+	79	-	-
35	-	+	80	+	+
36	+	+	81	-	-
37	-	+	82	-	-
38	+	+	83	-	-
39	+	-	84	+	+
40	+	-	85	-	-
41	-	-	86	-	-
42	+	+	87	+	+
43	-	+	88	-	+
44	+	-	89	-	+
45	+	+			

+ Induction of mice death (toxin producer)
- No induction of mice death (non-toxin producer)

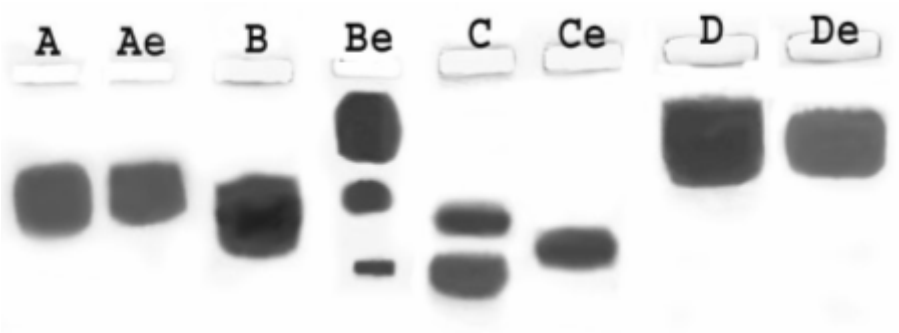

Figure 1. Polyacrilamide gel stained for esterase – electrophoretic analysis of ATCC *C. perfringens* strains 3624 type A; 3626 type B; 3628 type C, and 3629 type D, normal and exalted (Ae, Be, Ce, De).

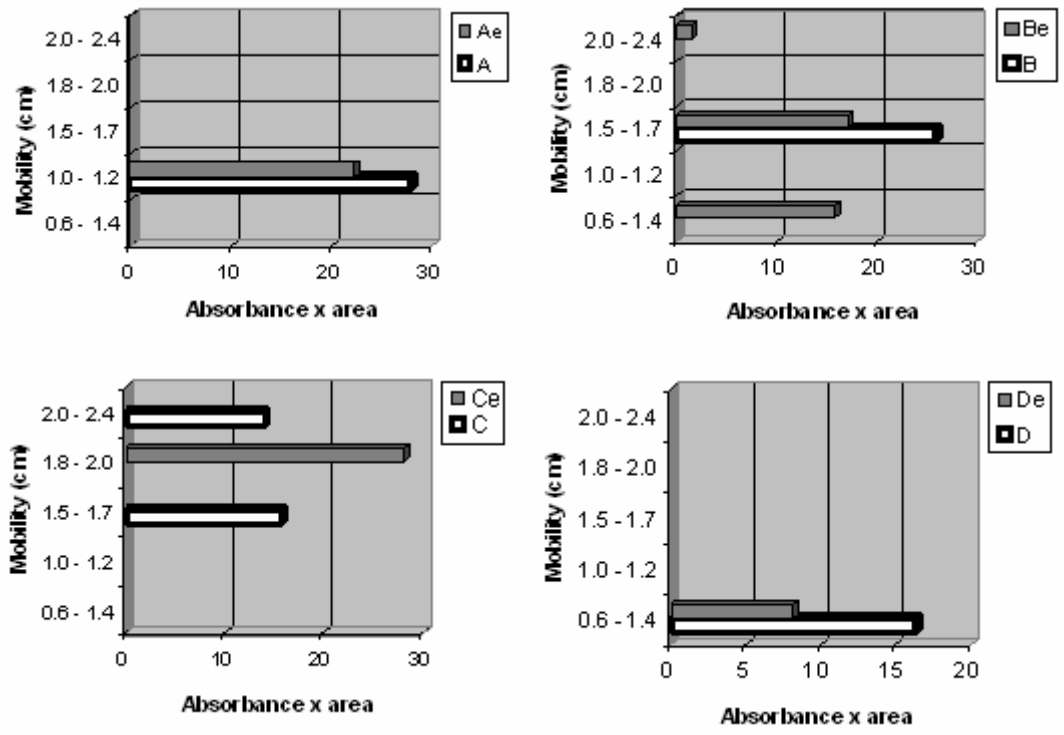

Figure 2. Mobility (cm) of esterase bands correlated with absorbance and area of ATCC *C. perfringens* strains 3624 type A; 3626 type B; 3628 type C, and 3629 type D, normal and exalted (Ae, Be, Ce, De).

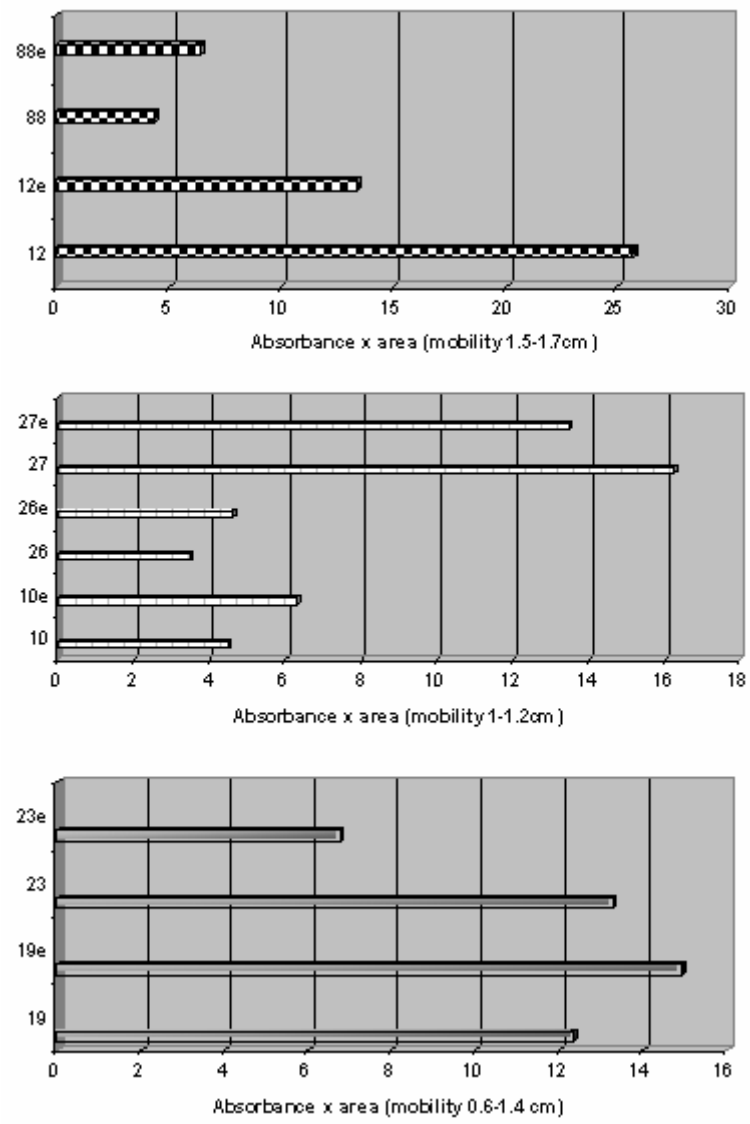

Figure 3. Absorbance x area correspondent to mobility (cm) of esterase bands of *C. perfringens* strain 10, 26, 27 type A; 88, 12 type B; 23, 19 type D, normal and exalted (10e, 26e, 27e, 88e, 12e, 23e, 19e).

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