

TOXIGENICITY CHARACTERIZATION OF *Clostridium perfringens* FROM BOVINE ISOLATES

L. BALDASSI¹✉, M. L. BARBOSA², E. E. BACH³, S. T. IARIA⁴

1 Laboratório de Bacteriologia Geral e Micobacterioses, Instituto Biológico; 2 Instituto Adolfo Lutz; 3 Departamento de Química UNICASTELO/UNESP; 4 Departamento de Microbiologia, Instituto de Ciências Biomedicina, USP.

ABSTRACT: Clinical samples from 71 bovine from different Brazilian states were processed for the analysis of anaerobe organisms with emphasis on the isolation and characterization of *Clostridium* spp. From these, eighty-nine *Clostridium perfringens* strains were recovered: 32 from liver, 19 from intestinal contents, 14 from kidney, 6 from rumen, 5 from nervous system, 4 from bone marrow, 2 from udder tract, blood, spleen and lung, and one from muscle. Four reference *Clostridium perfringens* types A, B, C, and D were used as controls in this study. All isolates were cultivated in appropriate media, and after centrifugation the supernatant and sediment were separated. From pure supernatant post exopolysaccharide (EPS) extraction, mouse toxigenicity tests were performed, determining protein and protein plus carbohydrate, respectively. ELISA was performed from sediments. The results showed that 51 (57.3%) of the isolates were toxigenic to mice when inoculated by intraperitoneal route; bacteria from different organs had variable patterns of toxigenicity. Toxigenicity of EPS extracts was only expressed when protein concentration was 0.04 mg/mL and between 0.31 and 0.5 mg/mL for carbohydrate. Isolates were characterized as toxigenic when showing optimum protein and carbohydrate concentrations.

KEY WORDS: *Clostridium perfringens*, toxigenicity, bovine isolates, ELISA.

INTRODUCTION

Clostridium perfringens belongs to the *Clostridium* genus that includes many gram-positive anaerobe bacteria, a group that is sensitive to oxygen and has the ability to form heat-resistant endospores.

This organism is characterized by the ability to produce numerous extracellular toxins and is classified into five types (A to E). Bacteria are notoriously prolific toxin producers; there are at least 17 different reported bacteria (31,32) associated with pathogenicity (21,25,26). *Clostridium perfringens* is responsible for many different extra- and intraintestinal diseases, such as gas gangrene, necrotic enteritis, animal enterotoxemia, and human food poisoning (21,25,29).

Although enterotoxemia diagnosis is usually based on history, clinical signs, and necropsy findings, laboratory analysis is essential to confirm the presence of toxins and to properly identify the pathogen (31,34).

Characterization of *C. perfringens* and its toxins is well established, although few data are available in Brazilian literature about its prevalence related to human and animal diseases.

Several laboratory methods have been used to characterize *C. perfringens*, such as enzyme linked immunosorbent assay (ELISA), bacteriocin production, and toxigenicity for Vero cells, but none is sufficiently adequate to identify all *C. perfringens* strains (27).

This study was performed to investigate isolates by using different tests in order to obtain a better method to substitute the *in vivo* test.

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MATERIALS AND METHODS

Strain selection

Samples were collected from 71 bovine with clinical manifestation of from many Brazilian states between 1996 and 1998.

These samples were initially cultivated in Cooked Meat Broth (CMB) and incubated at 37°C for 48 hours. From these cultures, 0.1mL loop aliquot was streaked on 5% sheep blood agar and incubated in anaerobic conditions at 37°C for 24 hours. Colonies showing related characteristics of *C. perfringens* form, aspect, color, and hemolysis were submitted to Gram stain; colonies corresponding to Gram-positive bacilli were cultivated in CMB in the same above conditions. After the incubation period, cultures were submitted to additional tests including catalase, lecithinase and gelatinase production, and glucose, lactose, and skimmed milk fermentation used to species identification. Strains identified as *C. perfringens* were then sub cultivated in CMB and stored at 4°C.

We used 89 isolates of *C. perfringens* (32 recovered from liver, 19 from intestinal contents, 14 from kidney, 6 from rumen, 5 from nervous system, 4 from bone marrow, 2 from udder tract, blood, spleen, lung, and one from muscle) were tested. Four reference strains from American Type Culture Collection (ATCC) numbers 3624 (type A), 3626 (type B), 3628 (type C), and 3629 (type D) were used as controls.

The 89 isolates were used in toxigenicity determination, immunoenzymatic assay (ELISA), and exopolysaccharide (EPS) analysis.

Protein extraction and toxigenicity test

After incubation under anaerobic conditions on plates with 5% sheep blood agar, 5 colonies of each plate were transferred to Tryptose Yeast Extract Broth (TYB) and further incubated in anaerobic conditions. The whole volume (10mL) of TYB was transferred to a fresh TYB (90mL) and incubated according to Pons *et al.* (24). The purity of each culture was analyzed by Gram stain method. Cultures were then centrifuged at 7.500 xg for 15 minutes at 4°C, and supernatants were submitted to protein - Bradford (4), adapted for microtechnique quantification (30,33). A standard curve was based on concentrations of 20, 40, 60, 80, 100, and 120 mg/mL bovine serum albumin (BSA). The OD value at 500nm was read in a microplate reader (BIO-RAD, model 3550-UV) coupled to a computer using Microplate Manager Program Protein extracts were tested for toxigenicity.

Toxigenicity was determined by the IP inoculation of each protein extract supernatant into 10 mice, as follows: five animals with 0.5mL of pure extract and the other five with 0.5 mL of extract treated with 1% trypsin (final concentration) after incubation at 37°C for 30 minutes.

Exopolysaccharide extraction and toxigenicity determination

Fifty milliliters from each TYB culture were freeze-dried using a lyophilizator (CHRISS, model Delta 1A), diluted 1:4 with 95% ethanol, and maintained at 4°C for 24 hours. Sediments obtained after centrifugation (3.000xg for 10 minutes) and total ethanol evaporation were re-suspended in sterilized distilled water. Evaluation of carbohydrate concentration was performed according to Dische (11) and read by spectrophotometer (Pye Unicam) through 620 nm-filter. The standard curve was prepared with a glucose solution at concentrations of 63, 125, and 250 mg/mL. Protein concentration was determined using these extracts.

Toxigenicity was determined by the IP inoculation of each EPS extract into 5 mice. All inoculated animals were observed over 96 hours for clinical manifestations or death (3).

Enzyme-linked immunosorbent assay (ELISA)

Antibody was obtained from New Zealand rabbits (protein extraction culture of each strain). Rabbits were immunized with 0.5 mL of antigen-sediment re-suspended in 0.5mL of 0.85% saline solution and homogenized with 0.5mL of Freund complete adjuvant in lymphonods (1). The animals were bled after 30 days.

Sediments were washed twice with tris-glycin buffer, 60mM, pH 8.7 (24), and suspended in 1mL of the same buffer, triturated on liquid nitrogen, and stored at -20°C until ELISA was performed.

Each triturated and washed sediment was diluted 1:10 in carbonate buffer solution, pH 9.6, distributed in four wells of polystyrene microplates, and incubated at 4°C for 24 hours as per Bach *et al.* (1). The plates were washed three times with 200 µL of phosphate buffer solution (PBS), pH 7.4, with 0.05% of Tween 20. Then, 200 µL of diluted antibody (1:5 in PBS) of each type (A, B, C, and D) was added to each corresponding well, and the plates incubated at 37°C for 4 hours. The plates were washed again and re-incubated with 200 mL of sheep anti-rabbit IgG conjugate marked with phosphatase (Sigma). After another wash, the wells received 200 mL of p-nitrophenyl phosphate buffer and were incubated at 37°C for 30 minutes. The reaction was interrupted by the addition of 200 µL of NaOH 3M, and absorbency was measured at A405 nm using ELISA reader (BIO-RAD, model 3550-UV) coupled with a computer with Microplate Manager Program.

For test optimization, antigens were analyzed at different concentrations (600, 300, and 150µg BSA/mL) and conjugated in 1:5 and 1:10 dilutions. These results were replicated three times and compared with negative controls (reagent and normal serum) (28,37).

RESULTS

Protein toxigenicity

From the 89 *C. perfringens* isolates, 14 (15.7%) were not toxic when treated with trypsin, 8 (9.0%) were toxic only after trypsinization, 29 (32.6%) with both treatments, and 38 (42.7%) were not toxic.

Exopolysaccharide toxigenicity

EPS extracts inoculated into mice showed 12 (13.5%) deaths, 9 with treated or non-treated treated EPS

extracts, and 3 with non-treated extracts. Quantification of carbohydrate extracts of these strains varied from 0.31 to 4.74 mg/mL, and protein concentration from 0.04 to 0.36 mg/mL.

These results indicated that the extracts with 0.04 mg/mL protein killed mice and were then considered toxigenic. In the same samples, carbohydrate concentration varied from 0.31 to 0.59 mg/mL. The relationship carbohydrate/protein varied from 7.75 to 14.75 mg/mL. This ratio, however, does not seem to be important because values ranging from 3.33 to 64.40 mg/mL were not responsible for death in those animals ([Table 1](#)).

Table 1. Carbohydrate (mg/mL) and protein (mg/mL) quantification and mouse toxicity of 89 *C. perfringens* EPS extracts.

Isolates	Carb.	Prot	Carb/Prot	Tox.	Isolates	Carb.	Prot	Carb/Prot	Tox.
1	0.53	0.04	13.25	+	46	3.64	0.07	52.00	-
2	0.42	0.04	10.50	+	47	3.11	0.06	52.83	-
3	1.91	0.34	5.62	-	48	2.74	0.10	27.40	-
4	2.38	0.36	6.61	-	49	4.35	0.20	21.75	-
5	1.72	0.30	5.73	-	50	2.90	0.18	16.11	-
6	1.85	0.13	12.69	-	51	3.08	0.13	29.23	-
7	2.74	0.28	9.78	-	52	1.35	0.20	6.75	-
8	3.10	0.14	22.14	-	53	1.98	0.08	24.75	-
9	2.80	0.13	21.54	-	54	2.05	0.11	18.63	-
10	4.46	0.09	52.77	-	55	3.10	0.13	23.84	-
11	4.95	0.30	13.50	-	56	1.00	0.30	3.33	-
12	3.48	0.18	19.30	-	57	1.60	0.08	20.00	-
13	1.83	0.06	30.50	-	58	1.85	0.10	18.50	-
14	4.10	0.21	19.52	-	59	0.45	0.04	11.25	+
15	1.15	0.18	6.38	-	60	3.10	0.15	20.66	-
16	1.14	0.30	3.80	-	61	4.60	0.17	27.06	-
17	4.16	0.10	41.60	-	62	2.80	0.16	17.50	-
18	0.31	0.04	7.75	+	63	2.94	0.18	16.33	-
19	0.59	0.04	14.75	+	64	0.48	0.04	12.00	+
20	2.54	0.13	19.53	-	65	1.39	0.28	4.96	-
21	2.21	0.06	36.83	-	66	1.86	0.00	0.00	-
22	3.53	0.26	13.57	-	67	2.90	0.10	29.00	-
23	2.66	0.10	26.60	-	68	1.30	0.09	14.44	-
24	0.39	0.04	9.75	+	69	1.68	0.10	16.80	-
25	1.96	0.09	21.77	-	70	1.84	0.26	7.07	-
26	1.20	0.10	12.00	-	71	1.95	0.11	17.72	-
27	3.20	0.10	32.00	-	72	3.10	0.22	14.09	-
28	0.93	0.17	5.47	-	73	3.28	0.10	32.80	-
29	0.58	0.04	14.50	+	74	0.45	0.04	11.25	+
30	3.51	0.05	50.20	-	75	0.48	0.04	12.00	+
31	3.22	0.05	64.40	-	76	0.55	0.04	13.75	+
32	1.00	0.11	9.09	-	77	4.15	0.08	51.87	-
33	1.54	0.06	25.66	-	78	4.40	0.08	55.00	-
34	3.20	0.17	18.82	-	79	3.99	0.14	28.50	-
35	0.93	0.10	9.30	-	80	0.95	0.18	5.27	-
36	0.66	0.09	7.33	-	81	1.58	0.22	7.18	-
37	4.23	0.21	20.14	-	82	2.01	0.09	22.00	-
38	4.82	0.10	48.20	-	83	1.28	0.22	5.81	-
39	4.67	0.08	58.37	-	84	3.32	0.23	14.43	-
40	3.64	0.22	16.54	-	85	1.48	0.30	4.93	-
41	1.96	0.06	32.36	-	86	1.03	0.25	4.12	-
42	1.79	0.06	29.83	-	87	0.40	0.04	10.00	+
43	1.47	0.18	8.16	-	88	1.70	0.29	5.86	-
44	4.67	0.10	46.70	-	89	1.90	0.31	6.13	-
45	2.86	0.22	13.00	-					

+ mouse death
- mouse survival

Immunoenzymatic assay

At first, ELISA was performed using as antigens (Ag) *C. perfringens* types A, B, C, and D, reference strains with 600 µg of proteins and goat anti-rabbit immunoglobulin G conjugated with alkaline phosphatase at a dilution 1:5. Antisera (Ab) were produced in rabbits against *C. perfringens* isolates (ATCC) types A, B, C, and D. ELISA demonstrated low specificity and crossreactivity against all antigens. Antiserum type D (Ab) recognized its own antigen, showing crossreactivity with the other antigens. No reaction was observed with normal serum nor with the reagents employed ([Table 2](#)).

Table 2. ELISA OD values for all extracts using antisera against ATCC isolates types A, B, C, and D.

Antigens (Ag)	Antiserum (Ab)			
	A	B	C	D
A	222	299	299	504
B	153	280	322	452
C	144	255	344	433
D	191	319	360	507

OD optical density

For the 89 isolates, ELISA was also performed the in the same optimization. [Table 3](#) shows that one isolate (1.1%) was classified as type C, 36 (40.4%) as A, 30 (33.7%) as D, and 9 (10.1%) as B. Thirteen isolates (14.6%) could not be classified.

Table 3. Classification of 89 *C. perfringens* isolates into different types by ELISA OD values.

Antigens (Ag)	Antiserum (Ab)				Type	Antigens (Ag)	Antiserum (Ab)				Type
	A	B	C	D			A	B	C	D	
1	284	389	319	415	D	46	591	1.097	352	824	B
2	197	915	342	880	B	47	246	349	368	428	OD
3	1.738	976	320	933	A	48	169	327	370	470	D
4	1.410	926	341	841	A	49	165	313	370	446	D
5	925	865	332	773	A	50	238	342	377	464	D
6	1.222	946	334	774	A	51	263	390	374	475	D
7	1.215	974	350	876	A	52	232	358	309	511	D
8	971	776	285	812	A	53	364	412	325	493	D
9	1.156	957	321	819	A	54	152	300	321	332	C/D
10	1.131	943	312	994	A	55	242	349	326	477	D
11	971	1.058	303	889	B	56	243	369	327	439	D
12	1.067	1.061	311	977	A/B	57	169	348	321	488	D
13	1.223	810	291	814	A	58	213	304	338	469	D
14	834	945	314	972	D	59	334	394	310	477	D
15	1.063	1.105	378	1.005	B	60	232	377	340	461	D
16	997	765	386	982	A/D	61	206	469	376	378	B
17	346	467	354	496	B/D	62	178	279	386	292	C
18	994	660	315	668	A	63	273	414	373	466	D
19	1.047	1.024	380	879	A/B	64	167	407	376	518	D
20	722	873	372	771	B	65	131	463	381	435	B/D
21	1.054	915	365	1.044	A/D	66	934	893	414	699	A
22	1.099	961	357	777	A	67	244	477	366	508	D
23	1.174	967	360	660	A	68	259	352	359	386	D
24	1.126	971	371	649	A	69	227	348	348	438	D
25	1.232	1.035	375	1.049	A	70	187	258	361	529	D
26	1.335	946	374	1.053	A	71	232	351	384	419	C/D
27	716	716	363	1.025	D	72	170	340	362	462	D
28	824	383	367	781	A	73	187	384	381	500	D
29	948	749	348	877	A	74	694	667	356	740	D
30	193	519	361	541	B/D	75	165	416	372	479	D
31	173	990	346	989	B/D	76	426	405	279	245	A/B
32	1.434	1.075	359	1.102	A	77	462	327	312	294	A
33	496	604	386	857	D	78	224	297	307	468	D
34	323	387	337	466	D	79	239	394	290	446	D
35	319	387	397	418	C/D	80	968	924	315	914	A
36	902	607	368	739	A	81	1.044	979	342	555	A
37	846	609	393	513	A	82	1.075	947	309	876	A
38	576	654	353	399	B	83	963	965	301	769	B
39	716	557	374	626	A	84	1.205	883	318	879	A
40	885	491	394	553	A	85	1.217	1.019	221	800	A
41	715	594	379	541	A	86	1.244	851	320	997	A
42	636	678	375	430	B	87	1.224	914	294	747	A
43	852	784	378	660	A	88	1.098	958	314	898	A
44	1.098	760	355	682	A	89	1.024	973	337	1.036	A/D
45	929	686	362	882	A						

DISCUSSION

Diagnosis of enterotoxemia in animals depends on historical data, clinical signs, and necropsy findings. Laboratory confirmation is essential for agent determination and toxin detection (38). Characteristics such as gelatinase production, nitrate reduction, motility absence, lactose and stormy skimmed milk fermentation can identify *C. perfringens* species (19,20,38), while toxin determination can indicate the circulating types (6,35).

The ϵ toxin and enterotoxin have their toxic activity increased when treated with trypsin (13,23,39), while the β is inactivated by the same enzyme (12). These data are in agreement with our study, where 9% of the isolates showed toxigenicity after trypsin treatment, and 15.7% lost toxigenicity with the same treatment, although toxigenic isolates were found in all organs.

Of the 89 strains tested, 57.3% were toxin producers, therefore, potentially pathogenic; this is in agreement with Tsai *et al.* (34), who reported 60% of bovine *C. perfringens* isolated from slaughterhouses in Canada as toxigenic.

These observations are in agreement with Holliday (15), who associated the degree of encapsulation with pathogenicity, explained by the observation of higher toxigenic strains from clinical fecal isolates or from atmospheric origin. Bacteria can lose their capsules or become wrinkled (16) when maintained for a long time or submitted to many passages in artificial media. These observations justify the occurrence of a toxigenic strain in one organ and atoxigenic in another in the same animal. In relation to enterotoxin, Mahony *et al.* (20) demonstrated that the strain ability to produce toxin *in vitro* does not reflect its capacity to produce it *in vivo*. Therefore, toxin detection in laboratory cultures, maintained under the same conditions, can determine the toxigenic potential of a certain strain.

The scheme for serological typing of *C. perfringens* was initially developed in England, using 8 isolates from type A (14). Hughes *et al.* (16) using the same scheme same with 57 antisera obtained 65% of typing in food poisoning brotes and 59% in cases of gas gangrene and other infections caused by this agent. Mahony *et al.* (19,20), Klotz (18), and Willis (39) reported that the agglutination test demonstrated that there were non-typified isolates in four types. However, according to Watson (38), in epidemiological surveys it would be necessary to use 139 different antisera (75 English and 64 Japanese strains), not available commercially and still to have conditions for preparing it for other strains non-typable with this group of antisera. The International Serotyping Scheme describes 82% of success in the classification of *C. perfringens* strains with 143 antisera (17).

The existence of atoxigenic variants or those that have lost their toxigenicity besides the fact that the serological test related to the toxins are not clearly differentiated by serological tests for *C. perfringens* (5,9).

To perform this study, we used antisera produced against bacterial cells of the four ATCC pattern types of *C. perfringens* (20), as the use of non-commercially available antitoxins results in many difficulties in their production and purification; this led to trying a new ELISA technique to identify serological types of *C. perfringens*.

[Table 2](#) shows that ELISA demonstrated crossreactivity among samples that can be seen due to the presence of antigens with group-specific polysaccharides that are lost when isolates become wrinkled (8). The high level of crossreactivity can justify the use of test strains isolated at different times and artificially maintained.

According to Calvert *et al.* (7) and Dazzo (10), exopolysaccharides (EPS) are antigenic molecules and can be related to pathogenicity (22,36). As per Bach and Guzzo (2), EPS are found in cultures of phytopathogenic bacteria and are responsible for antigenicity. In this study, EPS seems to be influencing pathogenicity since the strains that caused death, when the inoculum was from protein extraction, were the same ones that caused death in EPS.

EPS extraction method was shown to be sensitive enough to detect considerable levels of carbohydrates in supernatants of *C. perfringens* strain cultures, although small protein levels were still present.

Toxicogenicity revealed by animal death should be related much more to protein rather than to carbohydrate levels. The relationship carbohydrate/protein does not seem to be important because in the samples that did not cause death, these values varied between zero and 64.40 mg/mL. The values 7.75 to 14.75 mg/mL that determined death were within this range. The values found for carbohydrate, 0.66 - 4.75 mg/mL are also inside of the parameters that caused death (0.31- 0.59 mg/mL). In relation to protein, the values found in EPS extraction were between 0.05 and 0.36 mg/mL for the strains whose extracts did not determine mouse death, and 0.04 mg/mL for those that caused death of the inoculated animals.

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Received March 5, 2001

Accepted June 21, 2001

CORRESPONDENCE TO:

L. Baldassi – Laboratório de Bacteriologia Geral e Micobacterioses, Instituto Biológico. Avenida Conselheiro Rodrigues Alves, 1252, 04014-002, São Paulo, SP, Brasil.
E-mail: baldassi@biologico.br



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Caixa Postal 577
18618-000 Botucatu SP Brazil
Tel. / Fax: +55 14 3814-5555 | 3814-5446 | 3811-7241



jvat@cevap.org.br