Enterotoxigenic Clostridium perfringens Type A Isolated from Intestinal Contents of Cattle, Sheep and Chickens

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ABSTRACT

One hundred and fourteen strains of Clostridium perfringens, isolated from the intestinal contents of cattle, sheep, and chickens with enteritis or other disease conditions were studied for their ability to produce enterotoxin. Reversed passive hemagglutination, fluorescent antibody and immunodiffusion tests were used. On the basis of the reversed passive hemagglutination titres, supported by the other two tests, enterotoxigenicity of the strains was arbitrarily classified into two categories: highly enterotoxigenic and potentially enterotoxigenic, with 12% falling into each category. All the highly enterotoxigenic strains originated from cases of enteritis and included all three animal species. Apart from enterotoxigenicity, one C. perfringens strain produced beta toxin (type C) and 21 strains produced large amounts of alpha-toxin. The latter strains were predominantly associated with necrotic enteritis in chickens.

RÉSUMÉ

Cette expérience visait à vérifier si les 114 souches de Clostridium perfringens, isolées du contenu intestinal de bovins, de moutons et de poulets atteints d'entérite ou d'autres conditions pathologiques, pouvaient élaborer une entérotoxine. On utilisa à cette fin l'épreuve d'hémagglutination passive renversée, celle de l'immunofluorescence et celle de l'immunodiffusion. En se basant sur les résultats de l'épreuve d'hémagglutination passive renversée, en rapport avec ceux de deux autres épreuves, on classifia arbitrairement les souches en deux catégories: très entérotoxinogènes et potentiellement entérotoxinogènes; 12% des souches se classaient dans chacune de ces deux catégories. Toutes les souches très entérotoxinogènes provenaient de cas d'entérite affectant des sujets des trois espèces sur lesquelles portait l'expérience. Une souche produisait la toxine bêta (type C) et 21 souches produisaient une quantité considérable de toxine alpha, en plus d'être entérotoxinogènes. Ces 21 souches provenaient surtout de cas d'entérite nécrotique aviaire.

INTRODUCTION

Certain strains of *Clostridium perfringens* type A produce enterotoxin which causes food poisoning in man. The enterotoxin also causes experimental enteritis in lambs, calves, rabbits, and chickens (11, 12, 14, 18, 19). The role of this enterotoxin in natural cases of enteritis in animals is not known. Many cases of enteritis of uncertain etiology reveal an apparent increase in the population of *C. perfringens* in the gut flora suggesting that this enterotoxin might be involved in the enteric pathology.

Until recently, the lack of suitable laboratory methods may explain why this aspect has not been extensively investigated. The purpose of this study was to examine *C. perfringens* strains isolated from selected cases of enteritis in domestic animals at necropsy and to determine their ability to produce enterotoxin. It was hoped that the results obtained would provide information on the relationship between *C. perfringens* infection and incidence of enteritis in animals and, also, indicate the importance of enterotoxigenic *C. perfringens* in animals as a potential source for human food poisoning outbreaks.

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MATERIALS AND METHODS

SPECIMENS

Intestinal contents were selected from postmortem material of cattle, sheep, and chickens submitted to veterinary diagnostic laboratories. The institutions included Animal Diseases Research Institute (Western), as well as several other veterinary laboratories across Canada. The origin of the selected specimens is given in Table I. The specimens were divided into two categories: (i) cases showing gross signs of any type of enteritis as a predominant lesion and excluding demonstrated etiological agents such as salmonella and coccidia. In the case of chickens, these were predominantly cases of necrotic enteritis and (ii) those which did not reveal enteritis.

TABLE I. Origin of Specimens

Geographic Origin	Cattle	Sheep	Chickens
Alberta	76	9	4
British Columbia	—	—	5
Saskatchewan	2	3	—
Ontario	—	—	11
Maritimes	—	—	4
Total No.	78	12	24

CULTIVATION

C. perfringens strains were isolated from intestinal contents either by common anaerobic bacteriological methods or by preliminary overnight cultivation in CP-2V medium (10) followed by plating and isolation on blood agar and TSN agar.[1] The isolates were transferred to sporulation medium of Duncan and Strong (5) in 20 ml screw-cap tubes, heat shocked at 75°C for 20 minutes and grown for 13 to 17 hours at 37°C. Cultures which contained less than 10% sporulated cells were reprocessed up to three times. A few cultures which, on reprocessing, contained less than 5% of sporulated cells were discarded.

The sporulated cultures were centrifuged at 12,000 x g for ten minutes. The clear supernatant fluids were collected for toxicity and immunological tests and the sedimented cells (resuspended in a small amount of saline) were smeared on microscope slides for fluorescent antibody tests.

Toxicity of the supernatant fluids and *C. perfringens* type was determined by intravenous inoculation of mice with 0.3 ml aliquots of mixture of the fluid and *C. perfringens* diagnostic antisera[2] used according to the recommendations supplied by the manufacturer. If confirmation of a positive reaction (presence of lethal factors) was required, the typing procedure was repeated by intradermal inoculation in guinea pigs using supernatant from cultures grown in CP-2V medium.

A total of 114 strains of *C. perfringens* were chosen for examination for enterotoxin. Six additional isolates which did not produce spores or sporulated very poorly were discarded. One of the 114 strains was *C. perfringens* type C, the remainder were classified as type A.

REVERSED PASSIVE HEMAGGLUTINATION (RPHA) TEST

The method was a modification of the reversed passive hemagglutination test described by Uemura *et al* (21). Sheep erythrocytes were treated with glutaraldehyde by a modified (4) technique of Bing *et al* (2) and stored in 0.85% saline with 0.1% sodium azide at 5°C. The antibody was a fractionated antiglobulin of sheep origin against *C. perfringens* enterotoxin, described previously (16). Bis-diazo-benzidine, for conjugation of the antiglobulin to the treated erythrocytes, was prepared according to the description of Gordon *et al* (9), using benzidine dihydrochloride.[3] This reagent was dispensed into vials in 1 ml amounts, quick-frozen in a dry ice-acetone bath and stored at –40°C until used.

For sensitization the stored glutaraldehyde-fixed sheep erythrocytes (RBC) were washed twice with 0.01 M phosphate buffered saline containing 0.15 M NaCl, pH 7.0 (PBS) and suspended as a 20% concentration in PBS. One milliliter of *C. perfringens* enterotoxin antiglobulin, at a protein concentration of 5 mg/ml, was added to 3 ml of the 20% suspension of glutaraldehyde-fixed sheep RBC (protein

[1]Baltimore Biological Laboratories, Cockeysville, Maryland.

[2]Wellcome Research Laboratories, Beckenham, England.

[3]Sigma Chemical Co., St. Louis, Missouri.

concentration of 10 mg/ml caused partial nonspecific agglutination of RBC). To this mixture was added 4 ml of freshly thawed bis-diazo-benzidine diluted 1:10 with PBS. The resultant reaction mixture was stirred gently for 20 minutes at room temperature and then the cells washed twice with PBS. The sensitized erythrocytes for immediate use were suspended in 1% concentration in PBS containing 0.2% bovine serum albumen (BSA) and 0.1% sodium azide. These cells proved satisfactory for up to four days when stored refrigerated.

The reversed passive hemagglutination test was performed in U-bottom MicroTiter plates.[4] Serial twofold dilutions of culture supernatant fluids, beginning at 1:10 were prepared in 0.050 ml amounts in PBS containing 0.2% BSA. The 1% sensitized erythrocyte suspension was added to each well (0.025 ml) and the plates immediately sealed and shaken to mix the contents. The test was read after incubation at room temperature for two to four hours. Each batch of tests included three sets of serial dilutions of purified enterotoxin of known concentration beginning with 1:10, 1:100 and 1:1000 dilutions as positive controls and a nonenterotoxic culture filtrate as a negative control. The endpoint (titre) at which hemagglutination could be clearly read was equivalent to enterotoxin concentration of approximately 1 ng/ml.

Fluorescent Antibody (FA) Test

Duplicate smears of sporulated cells, along with known controls, were air-dried and fixed in acetone for ten minutes. The staining procedure has been described previously (16).

Immunodiffusion (ID) Test

Micro-slide double diffusion test was conducted according to the method of Casman *et al* (3). The agar concentration used in the test was 1.2%. Sheep antiserum against purified *C. perfringens* enterotoxin was used in two concentrations: undiluted and 1:20. The diffusion time was 48 hours at approximately 24°C. The reactions were judged qualitatively. This test was sensitive to detect a minimum enterotoxin concentration of 0.5 μg/ml.

[4]Cooke Laboratory Products, Alexandria, Virginia.

RESULTS

Results of the RPHA test, showing distribution of the titres, are listed in Table II. The highest titre obtained with a culture supernatant fluid was 5120, equivalent to an enterotoxin concentration of 5.1 μg/ml. This strain originated from a chicken. Eighteen strains were completely negative in this test (titre < 10).

Table III lists correlation of the RPHA titres with FA reactions. The FA reactions were judged qualitatively; the presence of any degree of characteristic fluorescence in the smears was termed positive. However, the number of fluorescing cells or the intensity of fluorescence were uniformly minimal in the 14 strains which had RPHA titres of 20 to 40. On the basis of distribution of the RPHA titres among the FA-positive strains and the lack of correlation between the low titres and ID test it was decided to establish a significant titre level for practical purposes. Consequently, the RPHA titre of 80 (enterotoxin concentration of 0.08 μg/ml) was arbitrarily chosen as the significant reaction. Thus, RPHA titres of 80 or more were termed positive while lower values were considered negative. Using the above criteria, results of the three tests were grouped and allotted to the source of the 114 *C. perfringens* strains (Table IV).

Twenty-two strains revealed strong dermonecrotic reactions in the typing process. These were considered to be due to alpha-toxin of type A and one of beta-toxin of type C. These strains are listed in Table VI to demonstrate their relationship to RPHA and FA reactions and their source. All these strains were negative in ID test.

DISCUSSION

The results of the quantitative RPHA test in this study revealed a wide range of titres, the majority of which were 40 or less. Only 16% of the strains tested in this system were completely negative. It appears that the determination of enterotoxigenicity in field strains of *C. perfringens* may not be a simple matter. With sensitive tests such as RPHA and FA, the interpretation

TABLE II. Distribution of Reversed Passive Hemagglutination Titres of 114 *C. perfringens* Strains Isolated from Intestinal Contents of Cattle, Sheep and Chickens

RPHA Titre[a]	Source						Total No.
	Cattle		Sheep		Chickens		
	Enteritis	Other[b]	Enteritis	Other[b]	Enteritis	Other[b]	
0(<10)	4	8	—	1	5	—	18
10	6	3	1	2	3	—	15
20	12	10	3	—	2	—	27
40	9	8	1	2	7	—	27
80	9	1	—	—	1	—	11
160	5	1	1	—	2	1	10
320	1	—	—	—	1	—	2
640	1	—	—	—	1	—	2
1280	—	—	1	—	—	—	1
2560	—	—	—	—	—	—	—
5120	—	—	—	—	1	—	1
Total No.	47	31	7	5	23	1	114

[a]Reciprocal of dilution of culture supernatant fluid. One RPHA titre is equivalent to 1 ng of *C. perfringens* enterotoxin per ml
[b]Conditions other than enteritis

TABLE III. Correlation of RPHA Titres with FA Reactions on 114 *C. perfringens* Strains Isolated from Intestinal Contents of Cattle, Sheep and Chickens

RPHA Titre	No.	FA+ No. (%)
0-10	33	0(0)
20	27	5(18)
40	27	9(33)
80	11	9(82)
>160	16	16(100)
Total	114	39

RPHA = Reversed passive hemagglutination test
FA = Fluorescent antibody test
\+ = Positive reaction
Percentages rounded to full figures

of the results in practical terms requires the setting of limits for sensitivity and specificity. When the RPHA and ID tests are compared there is a calculated 50-fold difference in the minimum sensitivity to detect *C. perfringens* enterotoxin. There are two possible factors which may contribute to nonspecificity of low-titre RPHA reactions: (i) impurities present in the enterotoxin preparation used for antiserum production and (ii) enterotoxin-like antigenic proteins in spore or cellular components of nonenterotoxigenic strains. Although the enterotoxin used for antiserum production in this study was at least 98% pure (15), the remaining contaminant of cellular origin (nonenterotoxic) could interfere with subsequent immune reactions at low test dilutions. Attempts to eliminate such possible residual nonspecificity by absorption of the antiserum with completely enterotoxin-negative cells were largely unsuccessful, although a few reactions at RPHA titres of 10 and 20 were eliminated. Since the cellular antigens of *C. perfringens* are very heterogeneous (6, 13), the variable results obtained are not unexpected. Enterotoxin-like proteins, although in comparatively small amounts, have been demonstrated by immunochemical means in structure of spore coats of enterotoxin positive as well as negative strains (7). Possibly, fractions of such antigenic substances could be released into the growth medium during cultivation of the organism and subsequently react in low dilutions of the RPHA test.

Although the FA test is generally more sensitive than RPHA, it is not directly comparable to the latter because the examination is based on intact cells before the release of enterotoxin into culture supernatant fluid. Therefore, a complete correlation between the results of these two tests conducted on 15-h cultures is not necessarily expected (16, 17). Also, in the case of weak reactions, the FA test could be subject to the same antigen-related discrepancies as the RPHA test.

So far there is no evidence that negative or weakly enterotoxigenic *C. perfringens* strains will readily become profuse enterotoxin producers. If such alteration did occur, the incidence of *C. perfringens* food poisoning would be much greater than presently found. Laboratory work with many

TABLE IV. Distribution of FA, ID and Significant RPHA Reactions of 114 *C. perfringens* Strains Isolated from Intestinal Contents of Cattle, Sheep and Chickens

Source	FA + RPHA + ID +	FA − RPHA − ID −	FA + RPHA − ID −	FA − RPHA + ID −	FA + RPHA + ID −	Total No.
Cattle						
Enteritis	7	25	9	1	5	47
Other[a]	0	25	2	1	3	31
Sheep						
Enteritis	1	5	0	0	1	7
Other[a]	0	4	1	0	0	5
Chickens						
Enteritis	5	14	2	0	2	23
Other[a]	1	0	0	0	0	1
Total No. (%)	14(12)	73(64)	14(12)	2(2)	11(10)	114(100)

[a]Conditions other than enteritis
FA = Fluorescent antibody test
RPHA = Reversed passive hemagglutination test at a titre of 80 or greater
ID = Immunodiffusion test
\+ = Positive reaction
− = Negative reaction
Percentages rounded to full figures

TABLE V. Distribution of *C. perfringens* Strains from Table IV Evaluated According to an Arbitrary Scheme

Source	*C. perfringens* Strains Isolated			
		Graded Enterotoxigenicity[a]		
	Total No.	High No. (%)	Potential No. (%)	Total No.
Cattle				
Enteritis	47	7(15)	6(13)	13
Other[b]	31	0(0)	4(13)	4
Sheep				
Enteritis	7	1(14)	1(14)	2
Other[b]	5	0(0)	0(0)	0
Chickens				
Enteritis	23	5(22)	2(9)	7
Other[b]	1	1—[c]	0—	1
Total No. (%)	114(100)	14(12)	13(12)	27(24)

[a]Graded according to amount of enterotoxin produced (see text)
[b]Conditions other than enteritis
[c]Percentage not calculated because of small number
Percentages rounded to full figures

strains isolated from cases of food poisoning has shown that such strains produce large amounts of enterotoxin and readily cause pathogenic and biological effects (17). The biologically (and ID) negative strains have consistently failed to synthesize significant amounts of enterotoxin even if they sporulated adequately.

Experiments with ligated intestine in sheep, cattle, and chickens have shown that at least 20 to 40 μg of enterotoxin per 5 to 20 cm of intestine are required to produce a positive reaction (15). This is more than 100 times the amount produced in 1 ml of a culture having a RPHA titre of 80. Enterotoxigenic strains (with demonstrated biological activities) used for experimental work under similar growth conditions have produced enterotoxin RPHA titres of 640 to 10,240 (equivalent to 0.6 to 10 μg/ml) (17).

It is impossible to relate the degree of enterotoxin production in a culture to the potential enterotoxigenesis in the intestinal

tract because of many unknown variables. For practical guidelines an arbitrary scheme was adopted to grade the reacting isolates into two categories according to their capability to produce enterotoxin: (i) high enterotoxigenicity, where the RPHA titre was 80 or more and the other two tests gave positive reactions and (ii) potential enterotoxigenicity in strains giving the FA and RPHA reactions as above, but not reacting in the ID test. The remainder, including those with RPHA titre below 80 and FA positive, were classified as negative. This evaluation is made on the assumption that the culture in question produced a minimum of 5% sporulated cells when grown for 15 ± 2 hours and that the RPHA and ID tests were conducted on the culture supernatant fluid. The sensitive FA test, performed on cell smears, would then serve as an auxiliary supportive test (17).

On the basis of the above scheme applied to the results of this study (Table V) 24% of the isolates would be classified in the two categories, with equal proportions in each (12%).

In California, Tsai *et al* (20) studied the prevalence of enterotoxigenic *C. perfringens* in cattle and chickens and found that 60 and 68% respectively, of the strains isolated, were positive. These figures were based on RPHA, ID, mouse inoculation, erythema reaction and ligated intestinal loop response. If positive reactions in all of these tests are considered quantitatively equivalent to the "highly enterotoxigenic" category of the present study, then the prevalence of these strains in Canada from livestock is considerably less than in California. On the other hand, without critical evaluation, and based solely on the minimal RPHA titres found in this study, overall prevalence becomes 84%. It is assumed that in the California study, normal healthy animals were chosen (8). The present investigation involved only diseased animals. *C. perfringens* isolates from normal, healthy animals have not been studied in Canada.

Considering the correlation between enterotoxigenic *C. perfringens* and disease all the "highly enterotoxigenic" strains originated from cases of enteritis (Table V). Although this is a significant finding, it does not indicate whether the *C. perfringens* strains contributed to the pathogenesis of the enteritis or became associated with the intestine because of an enteric disease. If the former conjecture is true, the role of *C. perfringens* in animal diseases in general may be more significant than has been assumed.

The selection of enteritis cases from cattle and sheep was based primarily on postmortem findings of nonspecific etiology, but this was not the case with chickens. All but three of the disease conditions in this species were diagnosed as necrotic enteritis. The small number of specimens from nonenteric disease in chickens precluded meaningful conclusions with respect to this group of *C. perfringens* isolates. However, the relatively large number of strong alpha-toxin producers among *C. perfringens* strains isolated from cases of necrotic enteritis in chickens may be sig-

TABLE VI. Distribution of Strong Dermonecrotic Reactions of 22 *C. perfringens* Strains Isolated from Intestinal Contents of Cattle, Sheep and Chickens

	Strong alpha-toxin				Type C
Source	RPHA − FA −	RPHA − FA +	RPHA + FA +	RPHA + FA −	RPHA − FA −
Cattle					
Enteritis	2	1	1	1	1
Other[a]	3	−	−	−	−
Sheep					
Other[a]	2	−	−	−	−
Chickens					
Enteritis	11	−	−	−	−
Total No.	18	1	1	1	1

[a]Conditions other than enteritis
RPHA = Reversed passive hemagglutination test at titre of 80 or greater
FA = Fluorescent antibody test
\+ = Positive reaction
− = Negative reaction

nificant (Table VI). This finding may be important in confirming that "classical" *C. perfringens* strains are involved in the pathogenesis of necrotic enteritis in chickens (1). Also, there is generally an inverse relationship between alpha-toxin and enterotoxin production.

From the public health standpoint it is premature to state whether the 12% prevalence of "highly enterotoxigenic" strains found in animals in this study constitutes a cause for concern with respect to food poisoning caused by *C. perfringens*. These strains were selected from disease conditions and there is no information available on their prevalence in normal animals in this geographical area. Animals, rather than environmental sources such as soil and water, would appear to be important as potential sources for food poisoning strains of *C. perfringens*. In a recent study conducted in Japan (22) only 0.8% of the *C. perfringens* strains isolated from the natural environment (soil, water, feces) were found to be enterotoxigenic, but 77% of strains from food poisoning cases were positive. As enterotoxigenic *C. perfringens* strains appear to be widely distributed among food animals eradication does not seem feasible and prevention of food poisoning should be based on sound meat hygiene, preservation and food preparation practices.

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