Identification of Isolates of Clostridium perfringens Types C and D by Agglutination and Fluorescent-Antibody Methods

TAKAYOSHI YAMAGISHI, JUN YOSHIZAWA, MITSUTERU KAWAI, NAGAKI SEO, AND SHOKI NISHIDA

Department of Bacteriology, School of Medicine, Kanazawa University, Kanazawa, Japan

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Agglutination and fluorescent-antibody methods were employed for screening Clostridium perfringens types C and D from 393 isolates of this organism. All of 50 strains which were isolated in Japan and were agglutinable with an antiserum prepared against a stock strain of type C no. 3182 toxigenically belonged to type C, but the antiserum showed no cross-agglutination with any of type C strains isolated in Denmark. All of the latter strains, however, were agglutinated by an antiserum prepared against a Danish strain, CWC11. Of 64 strains, showing heat-labile agglutinability by type D antiserum L9, 22 strains were toxigenically identified as type D strains which can be divided into three groups by the heat-stable antigens; no strains which were L-agglutination-positive but O-agglutination-negative were epsilon-toxicogenic. All of 13 strains, the heat-stable antigen of which was agglutinable by a type D antiserum VX81, were toxigenically type D strains. The results of fluorescent-antibody tests were almost in agreement with those of agglutination test with type C strains and completely with those of the O-agglutination test with type D strains. No beta-, epsilon- or delta-toxicogenicity could be demonstrated in strains which were not agglutinated by our test sera for types C and D strains. Further examination of cultural properties of Japanese and Danish type C strains revealed that the two groups were considerably different in urease production, capsule formation, and delta- and alpha-toxicogenicities.

The typing of Clostridium perfringens at present is not based on serological methods but on the test of the main toxins produced by this organism (15-17). The test antiserum for agglutination of C. perfringens type A usually react fully with the homologous strains and with only a few heterologous strains (19). Although Henderson (7) and Bergmann and Rapp (2) demonstrated that all types, except type A strains, possessed type-specific antigens, no investigator has attempted to utilize the type-specific antigens for identification and isolation of C. perfringens strains other than type A. The present investigation was undertaken to evaluate the use of the agglutination and fluorescent antibody (FA) methods for isolation and identification of C. perfringens types C and D and to examine the cultural properties of the isolates so identified.

MATERIALS AND METHODS

Isolation of C. perfringens. Samples of animal feces about the size of peas were suspended in 8-ml portions of saline and emulsified. Each emulsion was heated in a bath at 60 C for 10 min, and 0.5-ml portions of heated suspensions were transferred immediately into chopped meat broth media containing lactose to 0.5%.

After 18 hr of incubation at 37 C, a loop of each culture was plated onto blood-agar containing glucose to 1% and kanamycin (Takeda Co., Osaka, Japan) to 0.01 or 0.02% and cultured anaerobically for 24 hr. Suspected colonies were fished from the agar plate and committed for further serial subcultures to avoid the contamination which occurs frequently during isolation of clostridia. The isolates were identified as C. perfringens by the methods of Willis and Hobbs (18). They were stored in liver broth.

Preparation of immune sera. Strains used for immunization were as follows; 4969 for type B, 3182 and CWC11 for type C, and L9 and VX81 for type D, L10 for type E, and a nontoxicogenic strain, AA220. The strains 4969, 3182, L9, and L10 were brought from the Department of Bacteriology, Univ. of Leeds, England, by one of the authors; CWC11 and AA220 were sent by P. Høgh, the State Veterinary Serum Laboratory, Copenhagen, Denmark, who had isolated a number of type C strains from swine enterotoxemia (8, 9); and VX81 was isolated from a fecal sample of healthy sheep by one of the authors. The antigens were pre-
pared from 18-hr cultures of *C. perfringens* grown in medium containing 2% (w/v) polypeptone (Daigo, Osaka, Japan), 1% (w/v) glucose, and 0.5% NaCl (pH 7.2). Two kinds of antigens, those suspended in 0.5% Formol saline and those heated at 100 C for 60 min, were employed. Immunization was carried out by injection of 0.5, 1.0, 1.0, 1.5, 2.0, 2.0, 3.0, 3.0, and 3.0 ml of the suspension, respectively, into the ear vein of a rabbit at 3-day intervals.

**Agglutination test.** Formolized or heated antigens and the corresponding antisera were employed. The definition for L- and O-agglutinations were made according to Henderson (7), depending on the character of floccule in the agglutination test and on the different ways of assessment no matter what antisera and antigens might be used; the end point of L-agglutination was read by observing for agglutinated floccules at the bottom of test tubes (12 by 105 mm). In contrast to the floccules in L-agglutination, those in O-agglutination were so firm that they could not be dispersed easily by gentle shaking, and the end point was sharply distinct.

**FA test.** The preparation of (FA) sera and staining by the FA solution were carried out by the method of Batty and Walker (1). FA test sera against two toxigenic strains of type C (no. 3182 and CWC11), a nontoxigenic strain AA220, and two toxigenic strains of type D (VX81 and L9) were prepared.

**Estimation of toxigenicities.** Alpha-toxigenicity was examined by the method of Nishida et al. (11). Beta- and epsilon-toxigenicities were investigated as follows. Cooked meat broth (pH 6.8 to 7.0) containing 20% (v/v) chopped meat particles, 2% proteose peptone (w/v; Difco), and 0.5% NaCl was used for toxin production. To each 10-ml portion of this broth, fructose was added to make a final concentration of 1% (w/v) for beta-toxin and 0.5% for epsilon-toxin production. The incubation length for beta-toxin production was 9 hr and that for epsilon-toxin production was 24 hr. Estimation of beta- and epsilon-toxigenicities was carried out by the method of Oakley and Warrack (12). Delta-toxigenicity was examined by using the above-mentioned 20% (v/v) chopped meat broth without sugar added. The incubation length was 5 to 6 hr. Estimation of delta-toxigenicity was performed as follows: to 0.5 ml of the culture filtrate, 0.1 ml of alpha-antitoxin (100 units/ml) and 0.4 ml of saline were added. After the mixture was held at 37 C for 20 min, it was diluted by twofold dilutions. To 1 ml of each dilution, 0.2 ml of 2% (v/v) sheep red blood cell suspension was added. The mixtures were held at 37 C for another hour, and the end point of complete hemolysis was noted. The final reading was made after the mixtures had been left at room temperature overnight.

**Capsule staining.** This was performed according to the method of Keppie and Robertson (10).

**Urease test.** The method of Brooks (3) was adopted.

**RESULTS**

A preliminary test was undertaken to evaluate the type specificity of agglutination between Formol-treated or heated antigen and the antisera prepared for these two kinds of antigens (Table 1). The agglutination test with the Formol-treated antigens of type D strains showed extremely high L-agglutination titers of 104 level, but that with the heated antigens of these strains showed considerably lower O-agglutination titers, such as 50 or 100. Table 1 indicates also that the antiserum prepared against heated bacterial suspension of type D contains as much L antibody as the antiserum prepared against Formol-treated antigen, although the heated cells of type D completely lost the L-agglutinability by heating. Antigens of type B treated with Formol exhibited a feeble L-agglutination with the two type D antisera, but none of the other type antigens reacted with these antisera.

Heat-labile antigen could not be found in type C strains examined. None of the other type antigens reacted with type C antisera.

Based on these findings, further investigations were undertaken to isolate type C or D strains from the natural environment by the use of these antisera. Formol-treated antigen versus their antisera were employed for the identification of type C strains. Both Formol-treated and heated antigens versus antisera against Formol-treated antigens were employed for the identification of type D strains; the agglutination test was performed by using Formol-treated antigen first and then using heated antigen.

Fecal samples were collected mainly from sheep and guinea pigs but a few of them were collected from oxen. All except a few oxen with a slight diarrhea were healthy. Three to five strains of *C. perfringens* were isolated from each sample, and a total of 393 isolates were tested for their agglutinability with types C and D antiserum. Fifty of these strains were agglutinated by type C antiserum 3182 and 49 by type D antiserum L9. None of the 50 strains agglutinable by the type C antiserum was agglutinated by the type D antiserum and vice versa. The above-mentioned 50 type C-agglutinable strains were classified into two groups on beta- and delta-toxigenicities; a group consisting of 23 strains produced both beta and delta toxins and another group consisting of 27 strains produced delta toxin only. Considering that delta toxin is formed only by type C strains, we regarded these strains as aberrant strains of type C. Of the 50 strains agglutinated by type C antiserum 3182, 48 strains were quantitatively tested for agglutination and toxigenicity (Table 2). Furthermore, out of the remaining 294 strains which were not agglutinated either by type C or type D antiserum, 122 strains were randomly selected and examined for beta- and delta-toxigenicities. Table 2 indicates that all strains which were agglutinated by the type C
Table 1. Type specificity of agglutinations of Clostridium perfringens types C and D

| Antigen | Antiserum |
|---------|-----------|
| Type    | No. of strains used | Treatment | 3182 (Type C) | L9 (Type D) |
|         |            |           | F | H | F | H |
| B       | 4           | F          | - | - | (+) | (-) |
| C       | 6           | F          | + | - | - | - |
| D       | 11          | F          | - | - | (++) | (++) |
| E       | 2           | F          | - | - | + | + |
| F       | 3           | F          | - | - | - | - |

* F, Formol-treated antigen and the corresponding antiserum. H, heated antigen and the corresponding antiserum.
* Provisional grades of agglutination. -, No agglutination; ±, agglutination is positive but the titer is negligible; +, positive agglutination; ++, strongly positive agglutination. The positive results in parentheses were read by L-agglutination and those with no parentheses were read by O-agglutination.

Antiserum 3182 could be identified as type C strains by their toxigenicity, whereas the 122 strains mentioned above which were not agglutinable with the type C antiserum were neither beta- nor delta-toxigenic.

In the course of this study, 49 strains of C. perfringens isolated from swine enterotoxemia were sent to us by P. Høgh of Denmark to examine for their agglutinability with our type C antiserum. However, all of these strains were nonagglutinable with the antiserum 3182 which has been used for identification of type C strains in Japan. On the contrary, Danish strains exhibited a high ratio of agglutinability when tested with antisera prepared from one toxigenic and one nontoxigenic Danish strain (Table 3). Of the 49 Danish strains examined, 43 exhibited agglutinability with antiserum CWC11 at agglutination titers higher than 200. Of these 43 strains, 39 were beta-toxigenic although not delta-toxigenic and four were neither beta- nor delta-toxigenic. Also, the remaining six strains which were inagglutinable were neither betanor delta-toxigenic. When examined, the 49 Danish strains against antiserum AA220 showed a slightly lower ratio of agglutinability; of 38 strains which were agglutinable, 35 were beta-toxigenic.

Table 2. Toxigenicity of strains agglutinable by type C antiserum 3182

| Determination | Agglutination titer |
|--------------|---------------------|
|              | 800^a  | 400 | 200 | 0 |
| No. of strains at each agglutination titer | 3 | 40 | 5 | 122 |
| No. of strains producing beta and delta toxins | 2 | 18 | 1 | 0 |
| No. of strains producing only delta toxin | 1 | 22 | 4 | 0 |
| No. of strains producing epsilon toxin | 0 | 0 | 0 | 0 |
| Agreement ratio | 100 | 100 | 100 |

^a Maximum dilution of antiserum showing agglutination.
^b Agreement ratio between toxigenic and serological typing.

The correlation between L- and O-agglutinability and epsilon-toxigenicity was investigated. In addition to the abovementioned 49 strains which were L-agglutinable with the type D antiserum L9, 15 strains which were isolated by F. Kondo, School of Agriculture, Tokyo Univ., Japan, and have been demonstrated by him to exhibit L-agglutination were employed for this test. All of these 64 strains exhibited extremely high titers of L-agglutination ranging between 10^4 and 10^5. Table 4 indicates that these strains which possessed common heat-labile antigen could be divided into three groups by the heat-stable antigens. Ratios of toxigenic strains in each group were considerably different; all of the VX81 group strains were epsilon-toxigenic, whereas only 9 of 40 L9 group strains were epsilon-toxigenic. Epsilon-toxigenicity could not be demonstrated in any of the 11 strains which were L-agglutination-positive but O-agglutination-negative. Also, none of the above-mentioned randomly selected 122 strains which were L-agglutination-negative was epsilon-toxigenic.

Further serological studies were carried out by the use of FA test. The strains examined were as follows: a total of 59 Danish type C strains consisting of the above-mentioned 49 strains plus a further 10 strains lately sent from P. Høgh and a total of 75 reference strains consisting of 53 strains of type A, 3 strains of type B, 7 strains of type D, 4 strains of type E, and 8 strains of type F (Table 5).

Of the 59 Danish strains, 48 were beta-toxigenic and were not delta-toxigenic and 11 were neither beta- nor delta-toxigenic. Of the 48 beta-toxigenic strains, 45 were positive in FA test by the use of
TABLE 3. Agglutinabilities and beta-toxigenicities of 49 Danish strains

| Antisera* | No. of agglutination-positive or -negative strains | No. of beta-toxigenic-positive or -negative strains | Remarks |
|-----------|---------------------------------------------------|--------------------------------------------------|---------|
| CWC 11    | + 43                                              | + 39                                             | Agglutination titers: 800, 400, 200, 200 |
|           | - 6                                               | - 4                                              |         |
| AA220     | + 38                                              | + 35                                             | Agglutination titers: 800, 400, 400 |
|           | - 11                                              | - 3                                              | Minimal lethal doses: 640, 320, 320, 80 |
| 3182      | + 0                                               | + 0                                              |         |
|           | - 49                                              | - 10                                             |         |

* None of 55 reference strains of type A (20 strains of human origin and 35 strains of animal origin) were agglutinated against these sera.

TABLE 4. O agglutination and epsilon-toxigenicity of L-agglutination-positive strains of Clostridium perfringens

| O antigen | O-agglutination titers against the antiserum | Epsilon-toxigenicity ratio* |
|-----------|---------------------------------------------|-----------------------------|
|           | VX81                                        | L9                          |                     |
| I. VX81 group | 100-200                                      | 0                           | 13/13b (100)       |
| II. L9 group  | 0                                           | 50-100                      | 9/40 (22.5)        |
| III.        | 0                                           | 0                           | 0/11 (0)           |

* Values in parentheses indicate percentages.

b Denominator indicates a total number of strains. Numerator indicates a total number of epsilon-toxigenic strains.

the FA serum CWC11, whereas none of them was positive with the FA serum 3182. On the contrary, 36 type C strains consisting of 3 stock strains and 33 strains isolated by us in Japan were positive with the FA serum 3182 but negative with the FA serum CWC11. All 75 reference strains examined were negative with both FA sera.

Considering the difference of the serological finding between the two groups of type C strains, Danish and Japanese, we examined their cultural properties and found distinctive differences in potency of alpha-toxigenicity, urease production, and capsule formation between them (Table 6). Most of the strains belonging to no. 3182 group possessed distinctively higher alpha-toxigenicity than did the Danish strains. All of the 44 Danish strains examined were negative in delta-toxigenicity. On the contrary, all of the 20 strains of the 3182 group were delta-toxigenic. No distinction was found between them about the potency of beta-toxigenicity. Of the 44 Danish strains, only two were urease-positive, whereas 19 out of 20 Japanese strains were urease-positive. Furthermore, 39 out of 44 Danish strains formed capsules, but only 1 of 20 Japanese type C strains produced a capsule. To confirm the finding that Japanese type C strains could not form capsules in the capsule medium (10), a further experiment was performed by using 157 strains of types A, B, C, D, E, and F. Table 7 discloses that not only our type C strains but also type D strains did not produce capsule in the medium although 66 of 67 type A strains examined produced capsule. However, our type C strains produced capsule in the peritoneal cavity of mice; for instance, four strains each of types C and D randomly selected from our collection of strains were injected into peritoneal cavities of mice, and capsulated strains were seen in the fluid withdrawn. These capsulated type C strains, when examined by FA serum 3182, were still stainable by the serum but were untestable by FA serum CWC11. Further FA testing was carried out for 45 strains
which were demonstrated to be L-agglutination-positive against type D antiserum L9. Table 8 reveals that the results of FA tests were completely in agreement with those of O-agglutination; of the 45 strains, 37 were positive both for O-agglutination and for the FA test, whereas the remaining 8 strains were negative for both tests. None of the other type strains were stainable by either of the two FA sera, VX81 and L9.

**DISCUSSION**

Antigenic analysis by agglutination of *Clostridium perfringens* types other than type A have been carried out by a few workers (2, 5, 7). All of them seemed to agree that types C and D possessed type-specific antigens. However, they mentioned nothing about the false-positive cases (i.e., agglutination-positive but toxigenicity-negative) or false-negative cases (i.e., agglutination-negative) but toxigenicity-positive. Since understanding the reason for these discrepant cases is necessary to allow the agglutination method to be used for practical diagnosis, we attempted to study these cases in the present investigation. Our findings indicate that false-negative cases were not found when both of antisera 3182 and CWC11 were used for typing type C strains and when both of antisera VX81 and L9 were used for typing type D strains. On the other hand, a few false-positive cases were seen. The definition of false-positive, however, seems to be controversial. It is extremely illogical that in identifying a toxigenic species of clostridia, a nontoxigenic variety of the species can not be identified with the present means of identification (15). During an enzootic of enterotoxemia due to type C, Høgh isolated atoxic strains of *Clostridium perfringens* as well. One of these strains, AA220, was highly agglutinated by antiserum CWC11. When this strain was injected into rabbits, it gave rise to an antiserum (AA220) which agglutinated only type C strains.

For the identification of type D strains, we employed Formol-treated antigen which seems to be common to all our type D strains. Henderson, however, demonstrated that there existed more than three heat-labile antigenic groups and that the grouping made according to the specificity of the heat-labile antigen coincided with the grouping made according to the country of origin of the strains. The common L-agglutinability of type D strains examined by us might be due to their similar origin.

Furthermore, Henderson demonstrated a considerable multiplicity of the heat-stable antigen, not less than 9 heat-stable antigenic types for the 13 type D strains examined by him. On the contrary, we could find that only two antigenic types were significant. This inconsistency is left unexplained in the present investigation although

**Table 5. Fluorescent-antibody (FA) test for identification of Clostridium perfringens type C**

| Toxigenic types | No. of strains tested | No. of strains agglutinable with FA serum CWC11 | No. of strains agglutinable with FA serum 3182 |
|-----------------|-----------------------|-----------------------------------------------|-----------------------------------------------|
| Strains isolated in Denmark |                       |                                               |                                               |
| β(+) δ(−)       | 48                    | 45                                            | 0                                             |
| β(−) δ(−)       | 11                    | 4                                             | 0                                             |
| Strains isolated in Japan |                      |                                               |                                               |
| β(+) δ(+) e(−)  | 20                    | 0                                             | 20                                            |
| β(−) δ(+) e(−)  | 16                    | 0                                             | 16                                            |
| Reference strains |                      |                                               |                                               |
| Type A          | 53                    | 0                                             | 0                                             |
| Type B          | 3                     | 0                                             | 0                                             |
| Type D          | 7                     | 0                                             | 0                                             |
| Type E          | 4                     | 0                                             | 0                                             |
| Type F          | 8                     | 0                                             | 0                                             |

**Table 6. Differences between Danish and Japanese type C strains**

| Strains                      | No. of strains tested | No. of strains positive for | Alpha-toxigenicity (Lb) |
|------------------------------|-----------------------|-----------------------------|-------------------------|
|                              |                       | Agglutinability by antiserum | Urease | Capsule | Range | Avg. |
|                              |                       | CWC11 | 3182                |            |         |      |      |
| Danish strains (CWC11 group) | 44a                   | 44   | 0                   | 2           | 39      | <0.05-0.6 | 0.21 |
| Japanese strains (3182 group)| 20b                   | 0    | 20                  | 19          | 1       | 0.4-1.5c | 1.15c |

* All of these 44 strains were beta-toxigenic, and none was delta-toxigenic.
* All of these 20 strains were beta- and delta-toxigenic.
* Two out of the 20 strains were not included in the test for alpha-toxigenicity because of the breakage of culture tubes.
TABLE 7. Capsule formation of each type of Clostridium perfringens

| Types | No. of strains tested | Capsule-positive strains |
|-------|-----------------------|--------------------------|
|       |                       | No. | Per cent   |
| A     | 67                    | 66  | 98.2       |
| B     | 13                    | 10  | 76.9       |
| C     | 34                    | 1   | 2.9        |
| D     | 32                    | 2   | 6.3        |
| E     | 5                     | 4   | 80.0       |
| F     | 6                     | 4   | 66.7       |

a All except three strains were isolated in Japan.

TABLE 8. Identification of Clostridium perfringens type D by fluorescent-antibody (FA) serum

| Types | No. of strains tested | No. of strains stainable by FA serum |
|-------|-----------------------|-------------------------------------|
|       |                       | VX81 | L9   |
| A     | 20                    | 0    | 0    |
| B     | 4                     | 0    | 0    |
| C     | 54                    | 0    | 0    |
| D-I²  | 22                    | 0    | 0    |
| D-II² | 15                    | 0    | 15   |
| D-III²| 8                     | 0    | 0    |
| E     | 5                     | 0    | 0    |
| F     | 8                     | 0    | 0    |

a Subtypes of heat-stable antigen of strains showing L agglutinability. Refer to Table 4; D-I—VX81 group, II—L9 group, III—the remaining group.

the simplicity of the structure of heat-stable antigen might be explained by the same reason as seen in that of heat-labile antigen of these strains.

Henderson stated that type C strains possessed only one common heat-stable antigen, but we demonstrated the existence of two. Henderson’s result seems to be due to the limited number of strains he used in his experiment. It has already been shown by Brooks et al. (4) that there existed two kinds of type C strains, the delta toxin-producing and non-delta toxin-producing strains. They, however, did not mention any difference of serological and biological properties. The striking difference of these properties between Danish and Japanese strains does not seem to be caused by the difference of the countries where these strains were isolated, because Henderson previously has demonstrated a common heat-stable antigen for the type C strains collected from different countries. It should be noted that almost all of our strains were isolated from sheep but not from swine and that all of the strains isolated by P. Høgh were from swine.

Our findings revealed that none of eight type F strains examined reacted with either anti-type C serum CWC11 or 3182, although Sterne and Warrack (16) recently claimed that type F strains should be grouped into type C. All of the type F strains examined by us toxigenically belonged to type C because they produced beta toxin and extremely weak alpha toxin, but no epsilon and iota toxins. They, however, differed from type C strains by their colonial and microscopical findings as suggested by Zeislsler and Rassfeld-Sternberg (20).

Bergmann and Rapp (2) were concerned with cross-agglutinability among different types of C. perfringens. The preparation of type-specific antigens seems to be influenced by the culture media from which antigens were prepared. We have examined different kinds of peptone water and tryptic digest media and found that 2% polypeptone medium containing 1% glucose was most suitable for the preparation of type-specific antigens. Although further antigenic analysis of C. perfringens has been carried out with the use of complement fixation (2, 5) and precipitation reaction (2, 5, 14) and with agar gel diffusion test (5, 6, 13) and some difference has been found among each type with these methods, these reactions are not practical for identifying C. perfringens strains into serological types.

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