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USE OF AN INDIRECT HAEMAGGLUTINATION TEST FOR THE DETECTION OF *CLOSTRIDIUM PERFRINGENS* TYPE A ENTEROTOXIN

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An indirect haemagglutination (IH) test is described for the detection of Clostridium perfringens *type A enterotoxin, produced by strains isolated from human cases of food poisoning and from contaminated food. Though no strict relationship could be observed between titers in the IH test and the time it took mice to die from the intravenous inoculation of mice (IIM test), results of the supernatants examined by both methods demonstrated that the IH test was more sensitive than the IIM one. No unspecific reaction was obtained in the IH with a negative control and the inhibitions of the IH and IIM tests by specific antiserum against* C. perfringens *enterotoxin showed that the IH test is very specific. The IH assay is recommended for its sensitivity and easy performance by less-equipped laboratories, by these and other data.*

Key words: *Clostridium perfringens* – enterotoxin – indirect – haemagglutination

Clostridium perfringens type A is a very common cause of human food poisoning, when the bacteria are ingested in large numbers (Hobbs et al., 1953; Hobbs, 1965; Walker, 1975). In Brazil studies have implicated *C. perfringens* as pathogens in food-borne intoxications (Serrano, 1976; Salzberg et al., 1982; Schocken-Iturrino et al., 1986; Tortora & Zebral, 1988). A 34.000 MW enterotoxin has been shown to be responsible for the typical symptoms of the clostridial food poisoning (Stark & Duncan, 1972; Granum & Skjelkvale, 1977). This enterotoxin has been detected by several animal assays, such as erythemal activity (Hauschild, 1970; Stark & Duncan, 1971), mouse lethality (Hauschild & Hilsheimer, 1971; Sakaguchi et al., 1973), rabbit ligated ileal loop (Duncan & Strong, 1969) and suckling mice assay (Torres-Angel et al., 1975b). Tissue culture assays in VERO cells have also been described (Mc Clane & Mc Donel, 1979; Uemura et al., 1984).

Since *C. perfringens* enterotoxin has been purified (Hauschild & Hilsheimer, 1971; Stark & Duncan, 1972; Sakaguchi et al., 1973), serological methods were developed for its detection (Casman et al., 1969; Stark & Duncan, 1971; Ducan & Somers, 1972; Uemura et al., 1973; Torres-Anjel et al., 1975a). Among the serological methods, the reverse passive haemagglutination (RPH) has been reported as very sensitive (Uemura et al., 1973; Genigeorgis et al., 1973). High sensitivity has also been claimed for the ELISA and radioimmunoassay (RIA) (Mc Clane & Mc Donel, 1979; Skjelkvale, 1980; Olsvik et al., 1982). The animal assays, besides being laborious, are neither very sensitive nor specific (Genigeorgis et al., 1973) whereas the VERO assay needs special facilities not available in small or less-equipped laboratories. Some of the serological tests, such as ELISA, RIA and RPH cannot be carried out routinely in small labs.

Therefore, a sensitive and easy-performing serological test for detection of *C. perfringens* enterotoxin is still needed.

The purpose of this investigation is to report an indirect haemagglutination (IH) test for the detection of *C. perfringens* enterotoxin.

MATERIALS AND METHODS

Strains – Fourteen strains of *C. perfringens* type A were isolated in our laboratory from human cases of diarrhea. Sixteen other type A

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strains, isolated from contaminated food, were kindly provided by Antonio M. Serrano (Faculdade de Engenharia de Alimentos, UNICAMP, Brazil). All strains were maintained in Brain Heart Infusion (BHI, Difco) and Cooked Meat Medium (BBL) in the dark at room temperature and at $-30\,^{o}C$. Subcultures were made each two months. A non-enterotoxigenic strain (V) of *C. perfringens* was used as a negative control for enterotoxin production. Purified *C. perfringens* enterotoxin was kindly provided by Gengi Sakaguchi, College of Agriculture, University of Osaka, Japan.

Production of crude enterotoxin – For this purpose the strains of *C. perfringens* were subcultured 4-5 times in the liquid medium of Duncan & Strong (1968) until the production of free spores reached about 50%. Cultures were then centrifuged (3000 rpm for 20 min), supernatants recovered and filtered through membrane filters (pore size 0.22 m, Millipore Corp., Bedford, Ma, USA). Aliquots of 0.5 ml of each crude enterotoxin preparation were stored at $-70\,^{o}C$ until examination.

Production of specific antitoxin – Specific antiserum against *C. perfringens* enterotoxin (AE) was prepared by inoculating subcutaneously adult New Zealand albino rabbits with 0.5 ml (100 μg) of purified enterotoxin emulsified in an equal volume of complete Freund's adjuvant. Further injections were performed 20, 40 and 60 days later using as inoculum the same amount of enterotoxin emulsified in incomplete Freund's adjuvant. Ten days after the last inoculation rabbits were bled and the antisera stored in small aliquots (0.5 ml) at $-25\,^{o}C$.

Detection of enterotoxin – The performance of the IH test was very similar to that described for the detection of thermolabile (LT) enterotoxin produced by *Escherichia coli* (Ricci & Castro, 1986). Briefly, using Microtiter trays, aliquots of 100 μl of sheep red blood cells (SRBC) suspension (10%) in PBS 0.05M, pH 7.4, were mixed with equal volumes of the crude enterotoxin preparations. Mixtures were incubated at $37\,^{o}C$ for 30 min with intermittent shaking. Thereafter, 20 μl of each mixture of sensitized SRBC was dropped on a clean microscopic slide and mixed with an equal volume of 1:10 diluted AE. Afterwards, 20 μl of sheep anti-rabbit IgG (diluted 1:2), prepared as described previously (Ricci & Castro, 1986) was added to SRBC-enterotoxin-AE complexes on the glass slide. Reagents were again mixed with a toothpick and readings were taken within 2 min. Any reactions showing clumping of SRBC were considered positive. In order to determine the titers of the enterotoxins under study these were successively diluted two-fold (starting with 1:2) and tested as described for crude enterotoxins. The reciprocal of the highest dilution of enterotoxin still able to cause SRBC agglutination in the IH test was considered as the titer of the respective enterotoxin or one haemagglutinating unit (1HU). With some enterotoxin preparations volumes of 0.5 ml, (containing 4 HU/ml) were mixed with equal volumes of specific AE, using as diluent triethanolamine buffer plus 0.05% of gelatin, Ca^{++} and Mg^{++} ions (Oliveira Lima & da Silva, 1970). These mixtures were incubated at $37\,^{o}C$ for 45 min and then examined by the IH test. When no haemagglutination was observed, as compared to the controls, the inhibition of the indirect haemagglutination (IH) test was considered positive.

A biological assay, the intravenous inoculation in mice (IIM), was used for comparative purposes. To carry out this test the recommendations of Sakaguchi et al. (1973) were followed. Briefly, sporulating cultures in Duncan & Strong's medium of the *C. perfringens* strains to be tested were centrifuged at 4.000 rpm for 30 min. The supernatants was saved and inoculated intravenously (0.5 ml), in duplicate into adult albino mice (15-22g). Death of both animals within 30 min were considered as a presumptive positive test. Results were recorded as follows: $^{+++}$ = death of both animals within 5 min after inoculation; $^{++}$ = death of both animals within 15 min after inoculation and $^{+}$ = death of both mice within 30 min after inoculation. Confirmation that the deaths were caused by enterotoxin activity was made by the neutralization of this lethality with specific AE(NIIM). To save AE this test was carried out with only 6 enterotoxigenic strains as detected by the IH and IIM tests. For this test (NIIM), supernatants of *C. perfringens* cultures were mixed with 0.5 ml of 1:2 diluted AE and then inoculated in adult mice as described for the IIM test. The animals were observed for 30 min and if they remained alive, as compared to the controls inoculated with enterotoxin alone, the NIIM test was considered positive, confirming therefore that the mice died due to of biologically active enterotoxin

TABLE

Comparison of the results obtained in the indirect haemagglutination (IH) test and intravenous inoculation in mice (IIM) for the detection of *Clostridium perfringens* enterotoxin

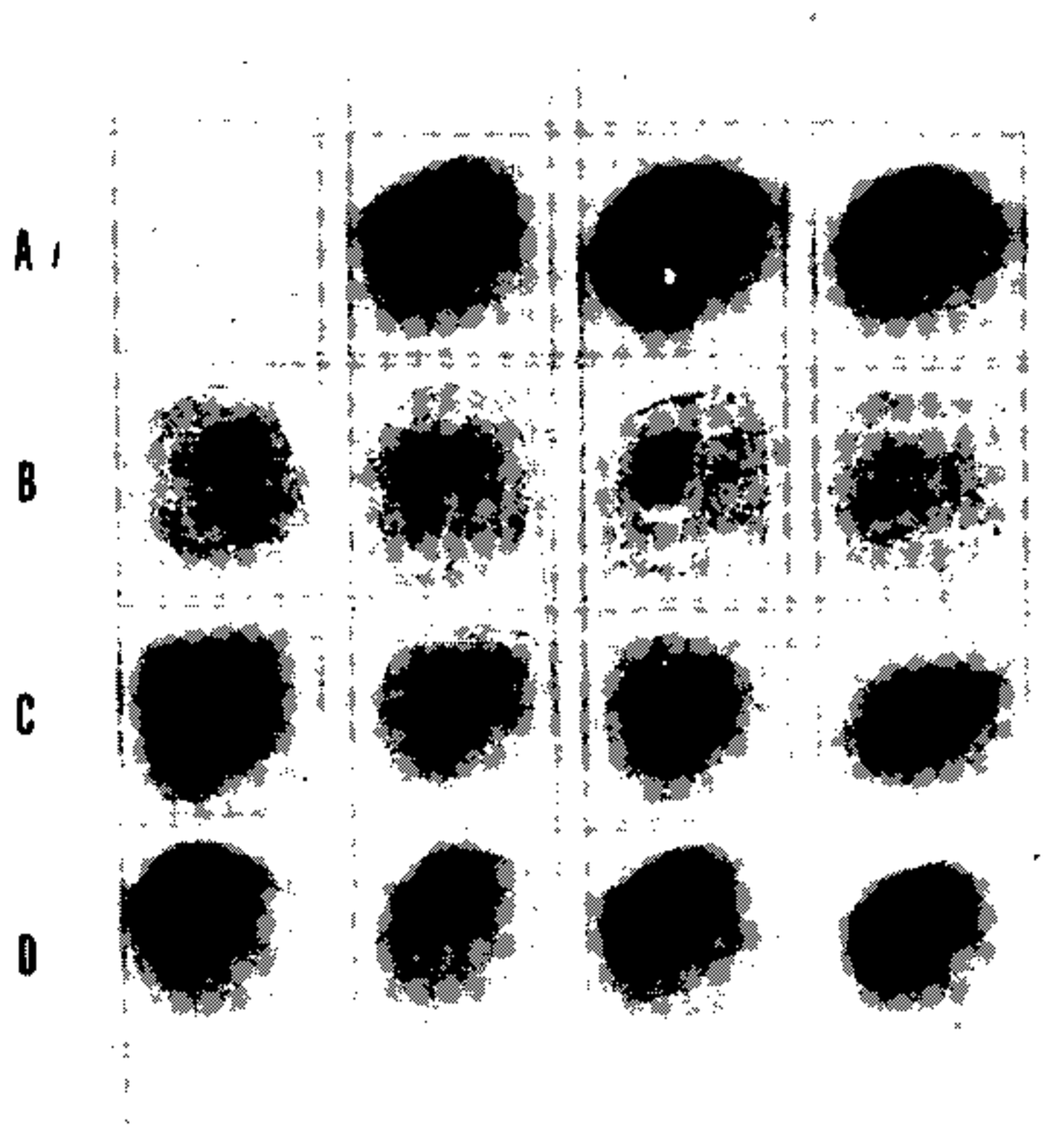
Identification of the *C. perfringens* strains (supernatants)	Tests			
	IH[a]	IIM[b]	IIH[c]	NIIM[d]
1H	32[e]	–	+[f]	ND
2H	256	+++	+	+[g]
4H	16	ND	ND	ND
9H	32	–	ND	ND
19H	ND[h]	ND	ND	ND
35H	8	ND	ND	ND
63H	64	+++	+	+
67H	8	ND	ND	ND
68H	8	ND	ND	ND
73H	4	ND	ND	ND
78H	128	+++	+	ND
79H	16	ND	ND	ND
102H	crude	+++	+	ND
103H	crude	+++	ND	ND
A	4	ND	ND	ND
B	4	ND	ND	ND
C	8	++	+	ND
D	32	–	+	+
4	16	+	+	ND
53	128	++	+	+
64	4	+	ND	ND
81	256[i]	+	ND	ND
97	256	+++	ND	+
143	8	+	ND	ND
208	8	+	ND	ND
210	16	++	ND	ND
218	16	++	+	ND
223	4	ND	ND	ND
246	16	+++	+	+
III	32	+	+	ND
V (negative control)	–	–	–	ND

a: indirect hemagglutination.
b: intravenous inoculation in mice.
c: inhibition of indirect hemagglutination.
d: neutralization of intravenous inoculation in mice.
e: reciprocal of the highest dilution of the *C. perfringens* supernatants which still cause haemagglutination.
f: total inhibition of indirect hemagglutination as described in Material and Methods.
g: inhibition of mice lethality caused by crude supernatant neutralized by *C. perfringens* anti-enterotoxin.
h: not done.
i: no hemaglutination was observed in the controls carried out with sensitized SRBC plus PBS.

present in the supernatants of the strains examined.

RESULTS

Comparative results of the IH and IIM tests and the respective inhibition of IH, as well as the neutralization of IIM by specific AE are shown in the Table, though the IIM, IIH and NIIM tests were not performed with all strains of *C. perfringens* under study. So, with regard to the IH test, 12 of the 14 strains isolated in our laboratory from human cases of diarrhea were positive with titers ranging from 4 to 256, with two strains giving positive results only with undiluted preparations. Some of these reactions can be seen in the Figure. As to the strains isolated from contaminated food were all also positive in this test, with titers ranging from 4 to 256.



Positive and negative reactions in the indirect hemagglutination (IH) test. Row A: unsensitized sheep red blood cells (SRBC) plus PBS. Row B: sensitized SRBC plus enterotoxin and anti-rabbit IgG, showing strong haemagglutination. Row C: the same as row B showing weak haemagglutination. Row D: sensitized SRBC plus anti-rabbit IgG.

A comparison between titers in the IH test and time elapsed for mortality in the IIM test shows no correlation (see for example, strains 102H, 103H, 81 and 246). Furthermore, strains 1H, 9H and D were positive in the IH test but negative in the IIM assay (Table). The inhibition of IH (IIH), in 12 strains, and the neutralization of IIM(NIIM) in 6 strains by specific AE, reinforced the reliability of the results of the tests we used in this investigation.

DISCUSSION

The results obtained in the detection of *C. perfringens* enterotoxin by the IH test recommend this easily-performed technique as useful for screening purposes, mainly for small laboratories lacking animal and equipment facilities.

Though we have not compared the IH with the RPH, considered as a very sensitive serological test (Genigeorgis et al., 1973) based upon our results we assume that the sensitivity of the IH test could be as high as that of the RPH test and better than that showed by the IIM test. On the other hand, the specificity of the IH test was demonstrated by our findings in the IIH test.

The lack of correlation between the IH and the IIM tests may be explained by a probable higher sensitivity of the IH test as compared to the IIM one. Thus, some strains, producing less enterotoxin, would not be detected by the IIM test. As a matter of fact, the titers in the IH test shown by these strains were not so high (1:32). Taking into consideration that the IH test is a serological method and the IIM is a biological assay one might also suppose that the biological activity of the enterotoxin in some supernatants could be lost sooner although maintaining its antigenic properties. Therefore in these conditions those strains would be positive in the IH test but negative in the IIM one. Nevertheless, this hypothesis cannot be easily accepted since strains 102H and 103H (Table), though weakly positive in the IH test, were able to kill mice very quickly when assayed by the IIM. We have no explanation for these results; as the supernatants of these strains were not examined by the NIIM test, we cannot exclude death caused by toxins other than the enterotoxin.

In conclusion, despite these comments and the lack of absolute correlation between the IH and IIM tests, we think that the former should be recommended for routine work in less equipped laboratories for several reasons such as: a) easy performance; b) realiable sensitivity and specificity; c) neither special equipments nor animal facilities are required and d) with a few exceptions the IH test seems more reliable than biological assays.

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