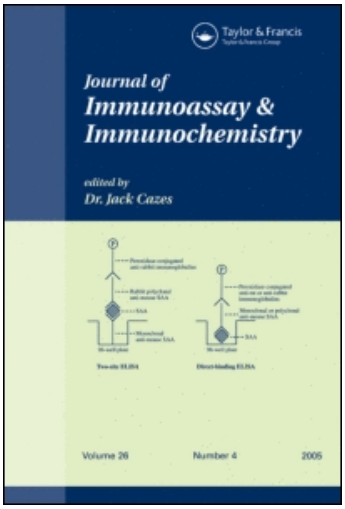

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COMPARISON OF SANDWICH-ELISA AND GM1-ELISA FOR THE DETECTION OF *ESCHERICHIA COLI* THERMOLABILE ENTEROTOXIN

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ABSTRACT

Two different microtiter plate ELISA tests were devised for the detection of *Escherichia coli* thermolabile toxin (LTh) either free or extracted from isolated colonies. Both tests used as detection systems purified anti-LTh rabbit immunoglobulins conjugated to biotin, streptavidin peroxidase and TMB. The tests differed by their capture phase which was the GM1-ganglioside for GM1-ELISA and purified anti-LTh rabbit immunoglobulins for sandwich ELISA. The two methods were rapid since they could be performed in less than 2 hours. The detection limits for purified LT were 50 pg/ml and 1.3 ng/ml for sandwich ELISA and GM1-ELISA respectively. For the detection of toxinogenic isolates the extraction buffer containing Triton X-100 was always superior to polymyxin buffer. Using the polymyxin extraction buffer the sandwich ELISA was again more sensitive than the GM1-ELISA since a lower number of isolated colonies could be used for the detection of positive strains. With the Triton X-100 buffer both ELISAs could detect positive strains using a single colony but the sandwich ELISA gave the highest ΔOD. We concluded that our sandwich ELISA can rapidly detect either the free *Escherichia coli* thermolabile toxin or LTh producing strains and could be applied routinely (KEY WORDS :).

INTRODUCTION

Enterotoxigenic *Escherichia coli* (ETEC) are an important world-wide cause of diarrhoea in all age groups (1). These strains can produce a heat-labile (LT) or a heat-stable enterotoxin (ST) or both.

Several groups have described tests for LT using immunological assays such as the biken test (2) or enzyme linked immunosorbent assays (3, 4, 5). Tests using the GM1-ganglioside (the cell target of the toxin) were described as very sensitive (6) but generally less than sandwich ELISA (4, 5).

A high sensitivity is needed for the direct detection of LT in stool or to detect a single colony of LT producing bacteria among many non-enterotoxigenic colonies in a stool isolate. Here we compared two sensitive ELISA methods for their ability to detect LT from a low number of ETEC or from weak producer strains.

MATERIALS AND METHODS

In sandwich ELISA rabbit immunoglobulin anti-LTh (7) was used as capture antibody, and the same antibody was used for detection after being biotinylated as previously described (8). The method was optimised with purified LTh as antigen prepared as described by TAKEDA et al. (9). Appropriate concentration of the reagents was determined by checkerboard titration.

Microtiter plates (NUNC, Maxisorp) were coated by incubating wells with 0.1 ml of 10 µg/ml of anti-LTh IgGs in 0.1 M carbonate/bicarbonate buffer (pH 9.6) at 37° C for 18 hours. After three washes with PBS-Tween (Behring, Marburg) the plates were used immediately or stored at -20° C for month. A 0.1 ml sample of LTh, or cholera toxin (CT, Sigma, St Louis), at different concentrations was added to the coated wells. After 30 min at 37° C, the plates were washed three times with PBS-Tween, and a 0.1 ml aliquot of biotinylated antibody was added. After another 30 min incubation at 37° C, the plates were

washed three times again with PBS-Tween, and 0.1 ml of 1:10 000 streptavidin-peroxidase (Calbiochem, La Jolla) solution was added. After 5 min incubation at room temperature the plates were again washed three times with PBS-Tween. The peroxidase activity was determined spectrophotometrically with a commercially available TMB substrate solution (Behring, Marburg). A 0.1 ml volume of solution was introduced into the well and the reaction was stopped after 30 min incubation at room temperature by adding 0.1 ml of H_2SO_4. The resulting yellow coloration was measured at 450 nm with a Behring ELISA processor. An absorbance of 0.1 above the background was considered positive since it corresponded to more than a 3 fold increase. The GM1-ELISA method was conducted as described above except that the GM1-ganglioside was used to immobilise LTh on the solid phase. For coating the plates the GM1-ganglioside (Supelco, Bellefonte) was diluted to 3 μM in PBS buffer (0.1 M phosphate, 0.15 NaCl, pH 7.2) and 0.1 ml of this solution was introduced in each well. After 18 h at 37° C the plates were washed three times with PBS-Tween, and used immediately or stored at -20° C for month. We tried to compare the two ELISA methods under field condition by screening strains of *Escherichia coli* for LT production. In addition we compared the efficiency of the non-ionic detergent Triton X-100, and polymyxin B sulfate to release the toxin from the cells. Thus two extraction buffers were made by adding 0.1 % Triton X-100 (TEB) or 4 000 IU/ml polymyxin (PEB) to the 0.05 M Tris-Cl, 0.01 M EDTA (pH 8) buffer.

Strains 19, 27, 41, 51, 60, 63, 68, 144 and 162 isolated from patients were provided by Pr. JOLY (Clermont-Ferrand, France). Among

these strains 8 were previously screened by the VERO cells assay in the laboratory of Pr. JOLY for their ability to produce LT and 3 were found negative. Reference strains H 10407, WHO 1, WHO 15 and WHO 18 provided by Pr. MEGRAUD (Bordeaux, France) were used as positive controls, and C600 as negative control. The strains were plated on CAYE agar (10) and incubated at 37° C for 18 h. In a classical experiment 10 colonies removed with a loop were suspended in 1 ml of extraction buffer. Suspensions were shaken (100 rpm) 1 h at room temperature, and then centrifuged for 2 min at 10 000 g (MLW TH 21 microfuge). The presence of LT in supernatant was assayed by sandwich ELISA and GM1-ELISA. Each assay was made in duplicate.

In another experiment we tried to determine the minimal number of colonies needed for a positive assay. Then 1 to 10 colonies of a strong or weak producer ETEC were extracted in TEB instead of the 10 used in the standard method. The remaining procedure was as described above.

The enterotoxin production of some strains was assayed on CHO-K1 cells as previously described (11).

RESULTS

When rabbit IgG anti LT were used as capture antibodies, the toxin could be detected at concentrations down to about 50 pg/ml (table 1), and CT at 1 ng/ml. In the GM1-ELISA, the detection level of LT was about 1 ng/ml.

The sandwich and GM1 ELISAs were compared for their ability to detect LT content of bacterial cells extracts from 18 h old *Escherichia*

TABLE I

COMPARISON OF ΔOD 450 nm OF FREE LT AT DIFFERENT CONCENTRATIONS IN SANDWICH AND GM1 ELISAS

ELISA format	LT concentration (ng/ml)								
	10	5	2.5	1.25	0.6	0.3	0.15	0.075	0.038
Sandwich ELISA	> 2.2	> 2.2	> 2.2	> 2.2	1.310	0.669	0.390	0.174	0.076
GM1 ELISA	1.178	0.533	0.234	0.091	0.040	ND	ND	ND	ND

ND : Not determined

TABLE 2

LT TITERS OF DIFFERENT STRAINS OF *E. COLI*

Strains	IT	IP	GT	GP	VERO cells assay
19	32	8	8	1	+
27	0	0	0	0	-
41	64	8	8	0	+
51	0	0	0	0	+
60	0	0	0	0	+
63	128	32	32	8	+
68	0	0	0	0	ND
144	0	0	0	0	-
162	0	0	0	0	-
H10407	64	8	8	2	+
WHO1	32	8	8	1	ND
WHO15	128	64	32	8	ND
WHO18	128	64	32	16	ND

ND : not determined
Titers were determined by sandwich ELISA in strains extracted with Triton X-100 (**IT**) or polymyxin (**IP**) or by GM1-ELISA in strains extracted with Triton (**GT**) or polymyxin (**GP**). Results of VERO cells assay were kindly provided by Pr. JOLY (Clermont-Ferrand, France).

coli cultures. Each strain was extracted by the use of TEB or PEB. Table 2 shows the LT titer, the last dilution of extract which gave a positive result, obtained for each strain which the different methods of extraction and assay. In addition results of VERO cells assay kindly provided by Pr. JOLY were shown. In all cases the complete lysis of bacteria with Triton gave a higher titer in toxin than polymyxin did especially in the case of a weak producer like 19, WHO1, 41, H10407. Furthermore the background obtained with TEB was lower than those with PEB which made the assay easier to read. Of the strains

TABLE 3

OD 450 nm IN LT-SANDWICH OR LT-GM1-ELISA BY DIFFERENT NUMBER OF COLONIES EXTRACTED IN 1 ml OF TRITON X-100 CONTAINING BUFFER

Strain method	nb of colonies/ml					
	1	2	4	6	8	10
WHO 18 sandwich ELISA	> 2.2	> 2.2	> 2.2	> 2.2	> 2.2	> 2.2
WHO 18 GM1-ELISA	2.1	> 2.2	> 2.2	> 2.2	> 2.2	> 2.2
19 sandwich ELISA	> 2.2	> 2.2	> 2.2	> 2.2	> 2.2	> 2.2
19 GM1-ELISA	1.450	> 2.2	> 2.2	> 2.2	> 2.2	> 2.2

previously found positive by VERO cell assay 2 were negative for LT production in both ELISAs (strains 51 and 60). A subsequent CHO-K1 cell performed for these strains also gave negative results. In all other enterotoxin producing strains the sandwich ELISA showed LT titers 4 to 8 fold higher than the GM1 ELISA.

Table 3 shows the result of an experiment made to determine the minimal number of colonies giving a positive test. It appeared that a single colony is needed for the two ELISAs, even in the case of a weak producer, when Triton X-100 containing buffer was used. When the colonies were treated with PEB, only the sandwich ELISA was able to detect a single colony of LT producer. The GM1 ELISA needed at least

TABLE 4

OD 450 nm IN LT-SANDWICH OR LT-GM1-ELISA BY DIFFERENT NUMBER OF COLONIES EXTRACTED IN 1 ml OF POLYMYXIN CONTAINING BUFFER

Strain method	nb of colonies/ml					
	1	2	4	6	8	10
WHO 18 sandwich ELISA	0.942	1.390	> 2.2	> 2.2	> 2.2	> 2.2
WHO 18 GM1-ELISA	0.058	0.106	0.152	0.275	0.291	0.312
19 sandwich ELISA	0.600	0.423	0.435	1.154	1.152	1.370
19 GM1-ELISA	0.040	0.024	0.020	0.005	0.030	0.010

2 colonies of a strong LT producer to give a positive result, and was negative with a weak LT producer even when 10 colonies were used (table 4).

DISCUSSION

Many ELISA procedures have been based upon the cross reactivity between *Escherichia coli* (LT) and *Vibrio cholerae* (CT) heat labile enterotoxins, and used anti-CT antibodies to detect LT (6, 12, 13, 14). Here we compared two ELISA methods using specific anti-LT antibodies for detection and the sensitivity of the test was increased since our detection limits were lower than in the previous reports using anti-CT antibodies (6, 12, 13,14). Furthermore this sensitivity was enhanced by

the use of a biotin-streptavidin peroxidase system using TMB as the chromogen. Thus our GM1-ELISA was able to detect LT at concentration as low as 1 ng/ml, which is a good detection limit when compared to 7 to 4 ng/ml previously published (3, 12, 15). But when the rabbit IgG anti-LT was used to immobilise the toxin, instead of GM1, the sensitivity of the test was raised about 20 times.

In order to compare these two methods in their ability to detect LT in bacterial extracts, we determined the LT titer of strains present in our laboratory. In addition we tried two methods of extraction of the toxin from colonies grown on agar plates. Although polymyxin B was used with success by many authors (3, 4, 12), the complete lysis of bacterial cells with Triton X-100 gave better results, especially for strains producing low amount of toxin. Furthermore, the presence of polymyxin in extraction buffer increased noticeably the background in sandwich ELISA. Therefore the use of Triton X-100 renders the test more efficient and easier to read. Of the 13 strains tested we noticed no false positives, which confirms the specificity of the test. In 2 cases ELISAs showed results in contradiction with previous VERO cell assays. But these ELISA results were confirmed by the CHO-K1 assay which is as sensitive as VERO cell and more commonly used. In these cases the negative results may probably be due to the loss of the LT producing character or to verocytotoxin producing strains and could not be considered as false negative results for both ELISAs. However the specificity of the test must be confirm with more strains. On the other hand, the sandwich ELISA gave always higher LT titers, than the GM1-ELISA did. Then the advantages noticed for sandwich ELISA when purified LT was

used as antigen were confirmed with bacterial extracts. The sandwich ELISA appeared as the best method for detecting enterotoxigenic *Escherichia coli* especially in the case of weak producer.

An additional experiment was made to determine how many colonies of LT producing bacteria were needed for a positive test. It appeared that the LT content of a single colony is sufficient to give a positive signal when sandwich ELISA was used with TEB extracted bacteria. This number is sufficiently low to allow the detection of ETEC in a mixture of colonies picked up randomly from a stool isolate.

The comparison between sandwich and GM1-ELISA established the superiority of the first in the level of LT detection. Then this ELISA procedure, in combination with Triton X-100 extraction method represents a sensitive and rapid test which can detect ETEC in stool isolates within 3 hours. This method seems to be a good alternative to the GM1-ELISA, and also the commercially available coagglutination test which was recently reported equally or less efficient than the GM1-ELISA (16, 17).

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REQUESTS FOR REPRINTS

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