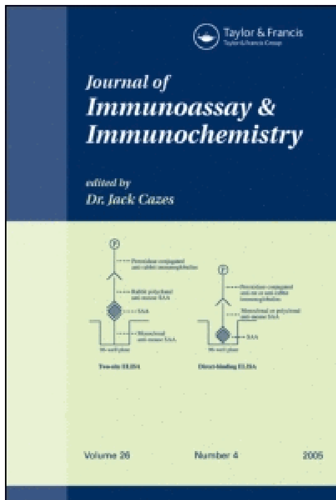
This article was downloaded by:
On: *16 January 2011*
Access details: *Access Details: Free Access*
Publisher *Taylor & Francis*
Informa Ltd Registered in England and Wales Registered Number: 1072954 Registered office: Mortimer House, 37-41 Mortimer Street, London W1T 3JH, UK



Journal of Immunoassay and Immunochemistry
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597271

Development of an Elisa Assay for *Clostridium Perfringens* Phospholipase C (Alpha Toxin)

R. J. Holdsworth[a]; D. Parratt[a]
[a] Department of Surgery and University Department of Microbiology, Ninewells Hospital and Medical School, Dundee, United Kingdom

To cite this Article Holdsworth, R. J. and Parratt, D.(1994) 'Development of an Elisa Assay for *Clostridium Perfringens* Phospholipase C (Alpha Toxin)', Journal of Immunoassay and Immunochemistry, 15: 3, 293 — 304
To link to this Article: DOI: 10.1080/15321819408009579
URL: http://dx.doi.org/10.1080/15321819408009579

PLEASE SCROLL DOWN FOR ARTICLE

Full terms and conditions of use: http://www.informaworld.com/terms-and-conditions-of-access.pdf

This article may be used for research, teaching and private study purposes. Any substantial or systematic reproduction, re-distribution, re-selling, loan or sub-licensing, systematic supply or distribution in any form to anyone is expressly forbidden.

The publisher does not give any warranty express or implied or make any representation that the contents will be complete or accurate or up to date. The accuracy of any instructions, formulae and drug doses should be independently verified with primary sources. The publisher shall not be liable for any loss, actions, claims, proceedings, demand or costs or damages whatsoever or howsoever caused arising directly or indirectly in connection with or arising out of the use of this material.

JOURNAL OF IMMUNOASSAY, 15(3), 293-304 (1994)

DEVELOPMENT OF AN ELISA ASSAY FOR *CLOSTRIDIUM PERFRINGENS* PHOSPHOLIPASE C (ALPHA TOXIN)

Richard J. Holdsworth and David Parratt
Department of Surgery and University Department of Microbiology, Ninewells Hospital and Medical School, Dundee. DD6 8HN. United Kingdom.

ABSTRACT
A new method for the assay of *Clostridium perfringens* alpha toxin (phospholipase C) is described using a sandwich ELISA. This assay has been shown to be quantitative, to have a high specificity for the toxin and is capable of detecting purified *Clostridium perfringens* phospholipase C at concentrations of as little as 0.005 units/ml in cooked meat culture medium.

KEY WORDS: CLOSTRIDIUM PERFRINGENS, ALPHA TOXIN, PHOSPHOLIPASE C

Downloaded At: 11:26 16 January 2011

INTRODUCTION

Many different methods have been described for the assay of *Clostridium perfringens* phospholipase C (1,2). All these methods rely indirectly on the measurement of the chemical properties of the enzyme. They are therefore dependent on both retained enzymatic activity and the purity of the enzyme and require careful interpretation (1). The sandwich ELISA described in this paper was developed using standard ELISA methods to directly detect the presence of toxin in culture media rather than to detect enzymatic activity.

Copyright © 1994 by Marcel Dekker, Inc.

MATERIALS

1) Antisera

Horse anti-*Clostridium perfringens* type A anti-toxin (type RP04) was obtained from Welcome Diagnostics, Dartford, UK. The World Health Organisation reference anti-*Clostridium perfringens* alpha toxin was obtained from Statens Seruminstitut, Copenhagen, Denmark. A monoclonal antibody against *Clostridium perfringens* alpha toxin was obtained from Cambio Ltd. 34 Millington Road, Cambridge, UK.

2) Bacteria

All bacteria used in cultures were obtained from stock cultures kept at Ninewells Hospital. Ten enteropathic strains of *E.coli* were kindly donated by Dr D.C.Old. Having grown pure cultures under standard laboratory conditions all sub-cultures were grown at 37oC in tubes of Robertson's cooked meat medium (Bacto cooked meat medium, Difco laboratories, Detroit, USA). After overnight culture, tubes were centrifuged and the supernatent assayed for toxin.

Downloaded At: 11:26 16 January 2011

3) Reagents

PBS (Phosphate buffered saline); PBST (PBS with triton); Carbonate/bicarbonate coating buffer; and p-nitrophenyl phosphate in glycine buffer alkaline phosphatase substrate have all been previously described (3). Type VII-T bovine alkaline phosphatase (cat.no. P6774); Type XIV *Clostridium perfringens* phospholipase C (cat.no. P4039); Type III *Bacillus cereus* phospholipase C (Cat.No. P6135); grade 1, 25% aqueous gluteraldehyde (Cat.No. G5882) and bovine serum albumin were all purchased from Sigma, UK.

METHODS

1) Conjugation of Anti-Toxin with Alkaline Phosphatase

The Welcome RP04 anti-toxin was conjugated with alkaline phosphatase using a method previously described (3). The pure anti-toxin (100μl) was combined with 1000 units of alkaline phosphatase and 10μl of 25% aqueous gluteraldehyde, left at room temperature for 2 hours and then dialysed against PBS for 48 hours with three changes of buffer. The prepared alkaline phosphatase conjugate was stored at 4oC until used.

2) Basic Assay Procedure

Flat-bottom, 96-well, micro-titre plates were used in all assays (Nunc type F, Gibco Life Technologies, Paisley, UK). Plates were initially prepared by coating the Welcome RP04 anti-toxin onto the surface. The anti-toxin was diluted 1:500 in PBS and coated onto the wells (100μl) using carbonate/bicarbonate coating buffer in an antibody:buffer ratio of 9:1. The plate was incubated at 37oC for 4 hours and stored at 4oC overnight or until used. On the day of assay, the plates were washed three times with PBST and any unoccupied binding sites blanked by incubating with a 1% w/v bovine serum albumin (BSA) solution for 45 minutes at 37oC followed by a further three washes with PBST.

The solutions containing toxin to be assayed (100μl) were placed in the wells, incubated for 45 minutes at 37oC, and the plate washed three times with PBST. The alkaline phosphatase linked anti-serum was then put on the plates (100μl), incubated for a further 45 minutes at 37oC and then cold stored at 4oC overnight.

The following day, the plate was washed three times with PBST and the alkaline phosphatase substrate (100μl) added. Plates were left at room

Downloaded At: 11:26 16 January 2011

temperature and read in an automated plate reader when the colour developed (EL312 microplate reader, Bio-Tek Instruments Inc. Winooski, VT., USA).

All wells were run in triplicate and the average optical density of three wells taken as the assay value. Controls within the assay depended on the solution under test. The assay was zeroed using either a control serum, PBS or cooked meat medium depending on the circumstances.

RESULTS

1) Choice of Antigens

It was found that the Welcome RP04 anti-toxin was satisfactory for both binding and conjugating the assay. The WHO reference anti-toxin gave an identical performance when used as an alternative binding antibody. This is also a horse anti-toxin and although the source is unknown we suspect that this is probably the same product as the Welcome RP04 anti-toxin. We attempted to use a monoclonal antibody with a claimed specificity for the alpha toxin in both binding and detecting. We were unable to show that this monoclonal antibody had any affinity for phospholipase C and we stopped using it at an early stage of the assay development.

2) Binding and Detecting Concentrations

A number of dilutions of the binding antibody were tried (figure 1). Increasing the concentration of the antibody for initial coating did not alter the sensitivity of the assay but it did alter the speed at which it could be read. At the higher coating concentration (1:250 dilution) it also increased the range of optical densities over which concentrated toxin solutions were measured. We accepted a coating dilution of 1:500 as a compromise between excessive use of antibody and increasing the speed of the assay.

Downloaded At: 11:26 16 January 2011

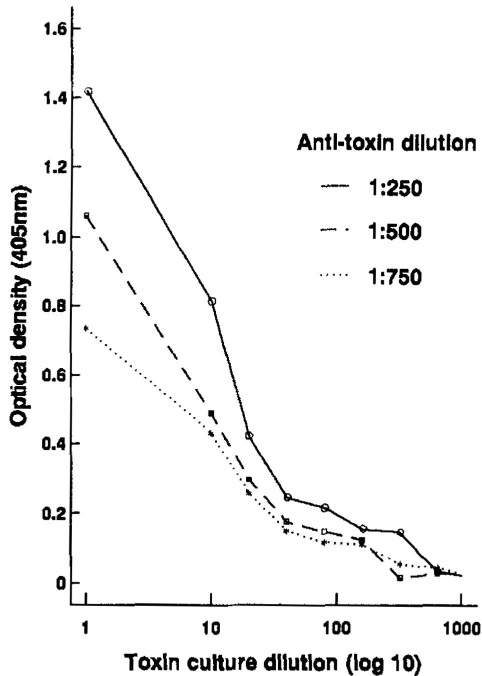


FIGURE 1: Plot of behaviour of assay when altering the concentration of binding antibody placed on plate. Assay performed on toxin culture in cooked meat medium. Effect of coating dilutions of 1:250, 1:500 and 1:750 are shown.

Downloaded At: 11:26 16 January 2011

Similarly, increasing the concentration of the conjugating antibody also had the dual effect of increasing the speed at which the assay could be read and the range of optical densities over which it worked (figure 2). However, the overall sensitivity was not increased. A compromise dilution of 1:500 was used for detection to conserve reagents at the expense of a slower assay.

3) Effect of BSA Blanking

The one part of the assay where performance was improved was blanking the assay with 1% BSA after the coating with the binding antibody (figure 3).

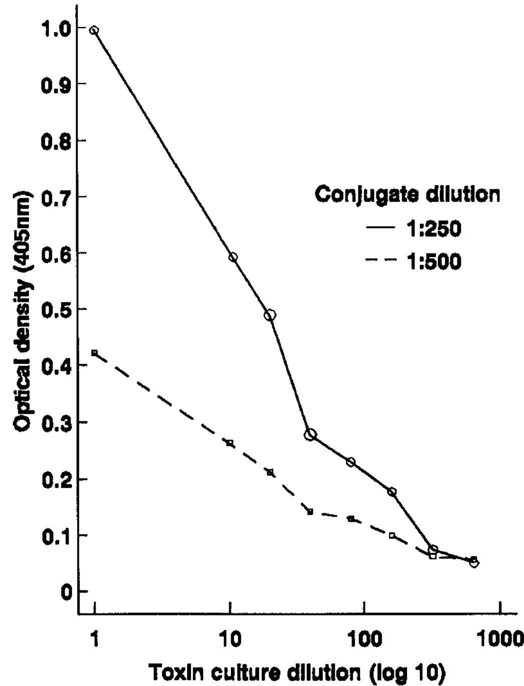


FIGURE 2: Plot of behaviour of assay when altering the concentration of conjugating antibody placed on plate. Assay performed on toxin culture in cooked meat medium. The comparison of conjugating with alkaline phosphatase linked antitoxin at dilutions of 1:250 and 1:500 is shown.

Downloaded At: 11:26 16 January 2011

Although, this reduced the peak optical density within the assay it also removed a considerable amount of background activity, presumably due to the binding of other proteins later in the assay.

4) Overnight Cold Incubation

Figure 4 shows the effect of leaving the conjugate on the plate overnight at 4oC. The assay behaves in an identical manner whether read the same day or left overnight. However, reading a plate the same day typically took three hours and

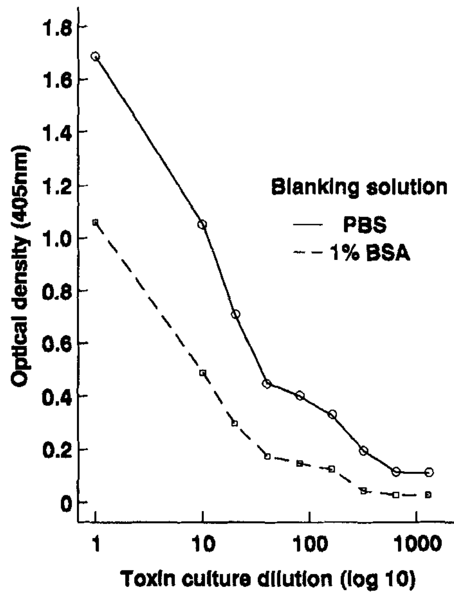


FIGURE 3: Plot to show effect of blanking the plate with 1% bovine serum albumin (BSA). Assay performed on toxin culture in cooked meat medium. The blanking of unoccupied binding sites with 1% BSA is compared with an identical assay where phosphate buffered saline (PBS) was used instead.

Downloaded At: 11:26 16 January 2011

may take up to five hours. Leaving the same plate overnight meant that development the following day was much quicker and the plate could be read in under one hour. Although the whole assay could theoretically be run in one day, to do this, both the initial binding concentration and the conjugating concentration required increasing.

5) Assay Specificity

The assay was developed using *Clostridium perfringens* type A grown in cooked meat medium. The assay was tested with a number of other common organisms grown under identical circumstances. Results of the effect of other

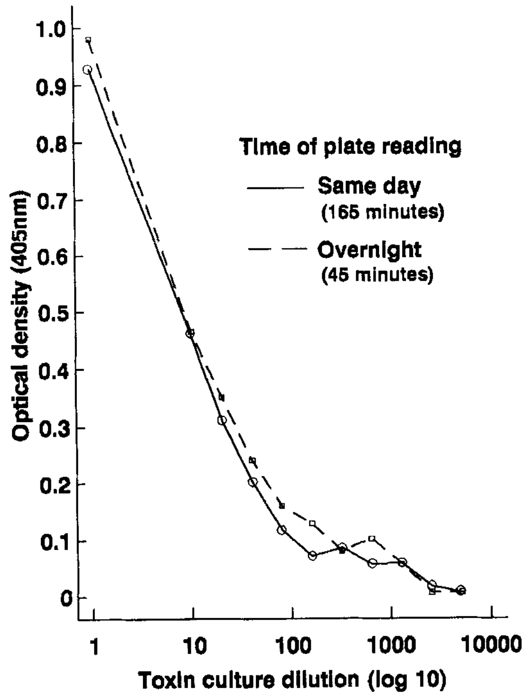


FIGURE 4: Plot to show the comparison of leaving a plate overnight at 4°C before adding alkaline phosphatase substrate. Both assays were set up with identical solutions. One plate was read the same day at 165 minutes and the other plate was left overnight at 4°C and read the following day at 45 minutes.

Downloaded At: 11:26 16 January 2011

organisms in the same medium are summarised in Table 1. The only organism that showed any degree of cross-reactivity was *Clostridium sporogenes*. None of the 10 enteropathic strains of *E.coli* produced any reaction in the assay.

The specificity of the assay for *Clostridium perfringens* phospholipase C was also tested by comparing the effect of *Bacillus cereus* phospholipase C in the assay (Table 2). This alternative source of the enzyme was not detected by the assay. The assay could detect as little as 0.005 units of pure *Clostridium perfringens* phospholipase C when diluted in PBS.

TABLE 1

Results of assay of supernatents from cultures of different organisms. Optical density (OD) measured at 405nm. Assay zeroed against normal cooked meat medium.

ORGANISM	OPTICAL DENSITY
Clostridium perfringens Type A	1.743
Clostridium tetanii	0.080
Clostridium bifermentans	0.038
Clostridium sporogenes	0.123
Bacteroides fragillis	0.086
Streptococcus pyogenes	0.015
Staphylococcus aureus	0.003
E.Coli 10961	0.010
E.Coli 9064	0.004
E.Coli 9046	0.051
E.Coli 9966	0.033
E.Coli 9121	0.043
E.Coli 8203	0.004
E.Coli 9016	0.015
E.Coli 17043	0.001
E.Coli 66643	0.031
E.Coli 9094	0.001

Downloaded At: 11:26 16 January 2011

TABLE 2

Results of assay of dilutions of pure phospholipase C from either *Clostridium perfringens* or *Bacillus cereus* diluted in PBS. Optical density (OD) measured at 405nm. Assay zeroed against PBS.

PHOSPHOLIPASE C units/ml	Clostridium perfringens	Bacillus cereus
10	1.193	0.053
2	0.942	-0.003
1	0.911	-0.015
0.5	0.715	0.013
0.025	0.646	-0.009
0.0625	0.505	-0.002
0.03125	0.336	-0.010

TABLE 3

Results of assay of dilutions of pure phospholipase C in either PBS or 1:10 diluted human serum. Optical density (OD) measured at 405nm. Assay zeroed against either PBS or 1:10 human serum.

PHOSPHOLIPASE C units/ml	PBS diluted	1:10 SERUM diluted
10	0.838	0.087
1	0.649	0.063
0.1	0.426	0.065
0.01	0.171	0.029
0.005	0.090	0.004
0.0025	0.037	0.007
0.00125	0.033	0.006

Downloaded At: 11:26 16 January 2011

6) Use of Assay on Serum Samples

When pure phospholipase C was diluted in human serum all activity disappeared (Table 3). Caution in interpretation of the results of serum assays is therefore merited due to the possibility of anti-phospholipase C antibodies.

DISCUSSION

To date we have found the assay method described in this paper to be effective in detecting the phospholipase C (alpha toxin) of *Clostridium perfringens* in bacterial culture medium. The assay has been shown to be reliable, reproducible and with a high specificity for the phospholipase C of *Clostridium perfringens* rather than the phospholipase C of *Bacillus cereus*. In addition, this assay is not only qualitative but it is also able to quantitate the amount of toxin present.

Although the method described may appear complex, it is a simple ELISA and requires no specific reagents other than the antitoxin. This antitoxin is

present in most laboratories and is used in a standard Nagler reaction. In addition, the assay is capable of detecting very low levels of toxin in pure culture media.

The principal drawback of the assay is speed. Using the concentrations of binding and detecting antibody described, the assay is slow, and cannot be carried out completely in one day. However, increasing the concentration of the solutions can accelerate the assay and it is likely that the incubation times at each step could be reduced.

It was disappointing that the only monoclonal anti-toxin we could obtain appeared to have activity against neither the toxin in culture nor the purified phospholipase C. Other monoclonal antibodies to the toxin have been produced although we have had no opportunity to test these in our assay (4).

It was also disappointing to find that the assay behaved poorly when used on serum. In particular, when we attempted to dilute purified phospholipase C in serum we failed to detect this at concentrations that gave very high readings when diluted in PBS or culture medium. We suspect there may be some naturally occuring anti-toxin in serum that is responsible for this neutralisation effect. To date, we have not accurately calibrated the assay in serum and we are currently investigating the antibody theory.

Although this assay is not recommended for general usage, we feel that with further refinement, including the use of specific monoclonal antibodies this could become a valuable tool for measuring *Clostridium perfringens* alpha toxin in a number of laboratory situations including mixed bacterial cultures and in tissue culture media (5,6).

Downloaded At: 11:26 16 January 2011

ACKNOWLEDGEMENTS

The authors would like to thank Dr. David C. Old, Department of Microbiology, Ninewells Hospital for providing ten enteropathic strains of *E.coli*. This development work was funded with a grant provided by Tayside Health Board Research Committee. Requests for reprints to Mr R.Holdsworth, Department of Surgery, Ninewells Hospital and Medical School, Dundee. DD1 9SY, United Kingdom.

REFERENCES

1 Jolivet-Reynaud,C. Moreau,H. and Alouf,J.E. Assay methods for alpha toxin from *Clostridium perfringens*: phospholipase C. Methods Enzymol 1988;165:293-7.

2 Kurioka,S. and Matsuda,M. Phospholipase C assay using p-nitrophenylphosphorylcholine together with sorbitol and its application to studying the metal and detergent requirement of the enzyme. Anal Biochem 1976;75:281-9.

3 Holdsworth,R.J. McKenzie,H. Parratt,D. Irving,A.D. and Cuschieri,A. The role of the spleen in the immune response following naturally acquired exposure to encapsulated bacteria. Int J Exp Path 1990;71:835-43.

4 Sato,H. Chiba,J. and Sato,Y. Monoclonal antibodies against alpha toxin of *Clostridium perfringens*. FEMS Microbiol Lett 1989;59:173-6.

5 Gustafson,C. and Tagesson,C. Phospholipase C from *Clostridium perfringens* stimulates phospholipase A2-mediated arachidonic acid release in cultured intestinal epithelial cells (INT 407). Scand J Gastroenterol 1990;25:363-71.

6 Gustafason,C. Kald,B. Sjodahal,R. and Tagesson,C. Phospholipase C from *Clostridium perfringens* stimulates formation and release of platelet-activating factor (PAF-acether) in cultured intestinal epithelial cells (INT 407). Scand J Gastroenterol 1991;26:1000-6.

Downloaded At: 11:26 16 January 2011