Veterinary Medical Journal –Giza (VMJG), Vol. 60(2)B - , December 2014
http://www.vetmedjcu.com/
ISSN 1110-1423

Using cell culture in evaluation of *clostridium perfringens* type A vaccines

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Abstract

Cell culture assays are possible alternatives to replace *in-vivo* neutralization tests currently required for potency testing of clostridial vaccines. So, the objective of this work was to titrate *C. perfringens* type A alpha vaccine in Vero (African Green Monkey Kidney) cells and standardize an *in-vitro* seroneutralization test to evaluate the potency of the alpha toxoid by comparison with the animal bioassay. The present work revealed that alpha toxin of *C. perfringens* type A, has a characteristic cytopathic effect (CPE) on African green monkey kidney cell line (Vero) represented by retraction and cell rounding. Vero cells can be used for titration of such toxin where the CPE of a prepared toxin was determined up to a dilution of 1:800 (using 50ul for tissue culture inoculation) while the minimum lethal dilution of the same toxin in mice was found to be 1:40 (using 100 ul for mice inoculation) revealing the high sensitivity of Vero cells. The Testing potency of a prepared hyperimmune serum and nine *C. perfringens* type A vaccines through application of serum neutralization test (SNT) in Vero cells and mice showed that there was no significant difference between the two tests. Therefore, these cell culture assays could be suitable *in-vitro* alternatives to *in-vivo* mouse neutralization experiments required for potency tests of *C. perfringens* type A vaccines to save time, cost, effort and avoid the use of live animals.
Keywords: *C. perfringens* type A, Evaluation, In-vitro, In-vivo, Vero.

Introduction

Clostridum perfringens (*C. perfringens*) type A is a bacterial strain that can rapidly produce potent toxins, primarily alpha toxin. Alpha toxin is thought to be associated with a number of potentially fatal gastrointestinal diseases, such as hemorrhagic bowel syndrome (HBS) or "bloody gut, Enterotoxaemia in lambs, (McGowan et al., 1958 and Fleming, 1985). A similar condition has been reported in goats (Russell, 1970) and calves (Ginter et al., 1995). Necrotic enteritis is a disease of domestic chickens worldwide is usually caused by *C. perfringens* type A (Al-Sheikhly and Truscott, 1977, Balauca 1978, Niilo 1978 and El-Meneisy et al., 2007). The disease is also occurs in at least some species of captive wild birds (Stuve et al., 1992) and in turkeys (Gazdzinski and Julian 1992 and El-Helw et al., 2014). *C. perfringens* type A was isolated from caecum of affected rabbits which died suddenly after short illness with severe diarrhea (Kunstyr et al., 1975, Versuchstierkd et al., 1975 and Diab et al., 2003).

Control of *C. perfringens* type A infection is based on preventive measures and systematic vaccination of the herd. Vaccination with alpha toxoid vaccine is carried out using monovalent or polyvalent clostridium vaccines. The potency testing of *C. perfringens* containing vaccines requires vaccination of laboratory

rabbits followed by quantitative determination of the induced antibodies in the rabbit sera using the toxin neutralization test (TNT) in mice. The antitoxin levels are titrated *in-vivo* using lethality in the mouse as an indicator of non neutralized toxin. This is a methodology relatively costly and time consuming representing some inaccurate results due to variations in individual sensitivity and cause pain suffering to a large number of animals, generating ethical questions (Wood, 1991 and Ebert et al., 1999). Therefore, there is a need to develop and standardize low cost *in-vitro* method that provide quick results of high sensitivity and specificity with reduction in the variability of results, testing costs, and reduce substantially the number of animals used.

The use of cell lines has become a viable and efficient option to replace animal models (Metz et al., 2002). Cell cultures can be effective indicators of free toxin in toxin neutralization tests. Compared with other *in-vitro* methods, the use of this model indicator allows evaluating *in-vitro* cytotoxicity and studying the biological activity of alpha toxin, which is not possible with other techniques such as ELISA and agar gel immunodiffusion (Borrmann et al., 2001). Toxin neutralization tests using cell cultures are available for potency testing for *Clostridium perfringens* type C and D, *Clostridium septicum* and *Clostridium novyi* type B vaccines (European Pharmacopoeia, 2008). So far, there have been no reports of studies investigating the suitability of cell cultures for the titration of *C. perfringens* alpha toxin. By comparison of 10 permanent cell lines, the Vero cells (African Green Monkey Kidney) proved to be the most suitable cell system for the detection of the *Clostridium novyi* type B alpha toxin (Borrmann and Schulze, 1999). Also, Vero cell cultures appear to be the most suitable to use for this purpose due to its sensitivity and relatively easy to grow and maintain (Knight et al., 1986, Bette et al., 1989 and Knight et al., 1990).

These research works could be considered the first work that evaluates an alternative *in-vitro* for titration of anti alpha-toxin of *C. perfringens* type A in sera of rabbits and chicken.

The objectives of this work include titration of *C. perfringens* type A vaccines in Vero cells instead of mice as indicators for the presence of active toxin and standardize it as *in-vitro* seroneutralization test to evaluate the potency of the alpha toxoid in comparison with the animal bioassay.

Material and Methods

1- Laboratory animals

All laboratory animals were kindly obtained from Central Laboratory for Evaluation of Veterinary Biologics and maintained under standard conditions of nutrition and management.

1.1 Rabbits

One hundred rabbits proved to be free from *C. perfringens* type A antibody of either sex weighing not less than 2 kg were used. Five of them were used for preparation of hyperimmune serum, ninety rabbits were used for experimental vaccination with commercial *C. perfringens* type A vaccines and five were kept without vaccination as control negative.

1.2 Chickens

Twenty chickens four weeks old were used for experimental vaccination with commercial *C. perfringens* type A vaccine (necrotic vaccine) while five chickens were kept without vaccination as control negative.

1.3 Mice

Clinically healthy Swiss albino mice of either sex weighing not less than 18-20g were used for toxin neutralization test.

2- Vaccines

Nine clostridial vaccines containing *C. perfringens* type A include local polyvalent vaccine (one batch), local Necrotic Enteritis vaccine (one batch), local rabbit enterotoxaemia vaccine (one batch), Covexin 10 (two batch, Intervet/Schering-Plough), Coglavax (two batch, Ceva) and Cubolac (two batch, CZ Veterinaria). Local and imported vaccines were supplied by Central Laboratory for Evaluation of Veterinary Biologics.

3- Toxin

Alpha toxin of *C. perfringens* type A was prepared according to Chou, (1971) from a locally isolated strain of *C. perfringens* type A.

4- Antisera

4.1 Standard antisera:

C. perfringens type A alpha antitoxin was obtained from (Central Veterinary Laboratory, Weybridge, UK). It contains 270 alpha antitoxin international units per ml. It was used for both standardization of alpha toxin and for control the titration of the tested sera.

4.2 Preparation of hyperimmune serum:

It was prepared to avoid the presence of preservative in standard antisera which have cytopathic effect on Vero cells. The hyperimmune serum was prepared in rabbits (Yamagishi et al., 1971 and El-Jakee et al., 2010) then calibrated against international standard toxin using serum neutralization in mice.

5- Cell lines

African Green Monkey Kidney (Vero) continuous cell line was supplied by Veterinary Serum and Vaccine Research Institute and cultivated in minimum essential medium (MEM – Gibco Laboratories – Massachusetts, USA) supplemented with 5% fetal bovine serum (Difco Laboratories – New Jersey, USA), 40,000 IU/ml of penicillin and 20,000IU/ml of streptomycin (Hang'Ombe et al., 2004). Cells were harvested by trypsin-EDTA treatment and counted using a haemocytometer cell counting chamber.

6- Diphenyl tetrazolium bromide (MTT)

Evaluation of viable cells was carried out using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT): 5 mg/ml MTT (Sigma-Aldrich). Phosphate-buffered saline (PBS).

7- Standardization of alpha toxin in-vivo and in-vitro:

7.1 Determination of minimal lethal dose in mice:

The minimal lethal dose per ml (MLD/ml) was determined according to the British Pharmacopoeia (2013).

7.2. Determination of minimal cytopathic effect (CPE) of alpha toxin in Vero cell:

Serial two-fold dilutions of the toxin were prepared in a 96-well plate (Sarstedt – North Carolina, USA) with 50µl/well and four repetitions. Later, another 50µl of MEM and 50µl of cellular suspension containing $2.5x10^4$ cells were added to total volume of 150µl per well. The positive control contained 100µl of the toxin plus 50µl of cells, while the negative control included 100µl of MEM and 50µl of cells. The plate was incubated at 37°C in a humid chamber (Sheldon Manufacturin Inc – Oregon, USA) with a controlled atmosphere (5% CO_2 and 95% O_2) for 24 hours. The supernatant was removed after this period. The evaluation of viable cells was carried out using the 3-(4,5-dimethylthiazo-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) test (Mosmann, 1983) and microscopic examination. The MTT colorimetric assay determines the ability of viable cells to convert the yellow tetrazolium salt into the dark blue formazane precipitate. After dissolving in an organic solvent, the concentration of the formazane can be spectrophotometrically determined by means of optical density (OD). Metabolically active cells result in a high optical density; dead cells show a low optical density. The OD was measured at 550 nm in an ELISA-Reader. CPE was determined by visualization of the cytopathic effect on 80-90% of the cellular monolayer.

7.3 Determination of test dose in mice:

The dose of the toxin was determined in mice according to the procedures described by British Pharmacopoeia (2013).

7.4 Determination of test dose of cytopathic effect (CPE) of alpha toxin in Vero cell:

Determination of test dose of cytopathic effect (CPE) of alpha toxin in Vero cell was carried according to Borrmann et al. (2006) using calibrated hyper-immune serum containing one international unit against alpha toxin.

8- In-vivo and in-vitro titration of tested sera

Each tested vaccine was inoculated in ten rabbits (Except in Necrotic Enteritis vaccine, 20 chicken were used instead) subcutaneously using the recommended dose of the manufacturer. The rabbits received booster vaccination after three weeks then bled two weeks after the booster vaccination (British Pharmacopoeia, 2013).

Before titration in cell culture all test and hyperimmune sera were decomplemented by heating at 56°C for 30 min and sterilized by filtration through a 0.22 µm filter membrane. All sera were tested and found to be free from cytopathic activity before assay.

8.1 Seroneutralization in mice:

For determination of the potency of tested vaccines, different dilutions of the pooled serum were homogenized with the same volume of α-toxin previously standardized in mice. This mixture was kept at 37°C for one hour and each dilution was injected in three mice (British Pharmacopoeia, 2013). A back-titration toxin using standard antitoxin was performed as a control test.

9- Cell culture assay

The cell suspension was prepared by harvesting of a 4-day-old cell monolayer from the cell culture flasks using trypsin, followed by resuspension in the

respective media containing 5% fetal calf serum. The same medium was used for the dilution of the toxin and the sera. On each plate, one test antiserum was titrated by comparison to the laboratory standard antiserum and a negative control serum.

Two-fold serial dilutions of the test sera into MEM were prepared in the 96-well plate, with four repetitions for each serum dilution and a total volume of 50µl in each well. Another 50µl was then added to each well containing standardized alpha toxin. The plate were agitated for 30 seconds on a plate shaker and incubated at 37°C for one hour in a humid chamber with a controlled atmosphere. After this period, 50µl of a cellular suspension with $2.5x10^4$ cells were added. After the addition of cells the plate was sealed with film to ensure that each well was gas tight. The plate was then reincubated for another 24 hours. Positive control wells contained 50µl of standardized toxin, 50µl of MEM, and 50µl of cells. Negative control wells had 100µl of MEM and 50µl of cells. Control wells had 50µl of the tested serum, 50µl of MEM, and 50µl of cells. The plate was read as previously described. Retrotitration with standard anti-toxin was used to check the standardization of the toxin (Souza Júnior et al., 2010). Results were assessed after 24 hours using the 3-(4,5-dimethylthiazo-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) test (Mosmann, 1983) and microscopic examination. The mean values of the OD for the test sera and the laboratory standard antiserum were calculated using the ELISA-Reader relating to respective cell controls. The calculation of the antibody titer was done basing on a comparison of the test serum and the calibrated immune serum containing one international unit against alpha toxin.

10- Statistical analyses

Results of *in-vivo* and *in-vitro* SN were analyzed and compared with parametrical correlation using Student's t test (Sendecor, 1971).

Results

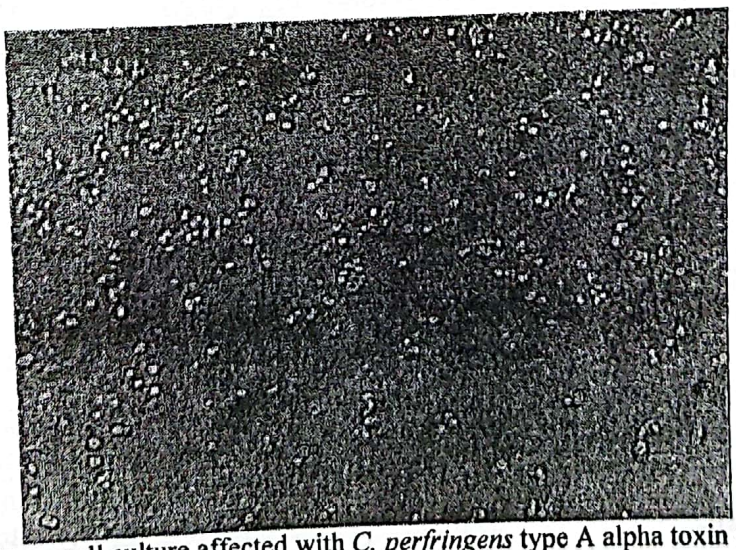

Photo (1): Vero cell culture affected with *C. perfringens* type A alpha toxin showing cell retraction and rounding (Light microscopy, magnification 100x)

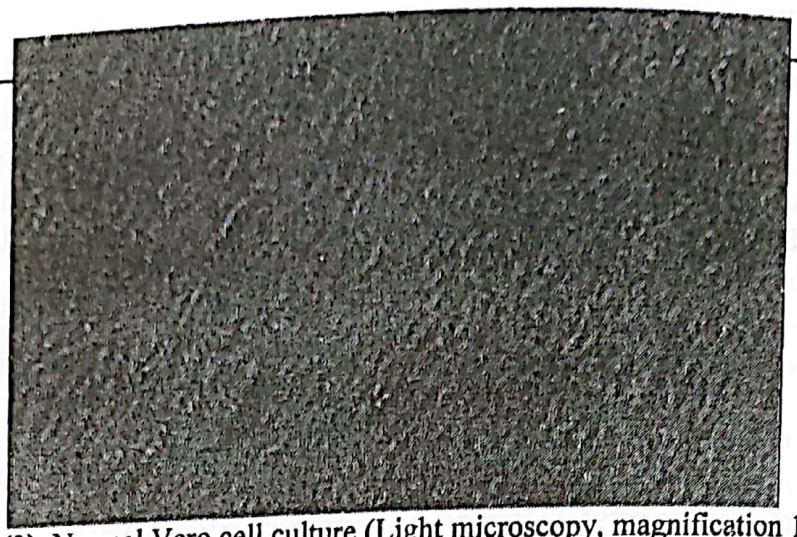

Photo (2): Normal Vero cell culture (Light microscopy, magnification 100x)

Table (1) Neutralizing antibody titers (IU/ml) against *C. perfringens type* A alpha toxin in pooled sera of immunized rabbits

Type of vaccine	Titre (IU/ml)	
	In mice	In Vero cells culture
Local polyvalent	84	85
Cubolac (I)	75	76
Cubolac (II)	1	1.5
Covexin 10 (I)	1	1.5
Covexin 10(II)	19	20
Coglavax (I)	64	64
Coglavax (II)	16	17
Rabbit enterotoxaeamia	18	18
Necrotic enteritis	6	5
Positive control	1	1
Negative control	0	0

Discussion

Vero cells were proved to be suitable biological test systems for the detection of cytotoxic effects of *C. novyi* alpha toxin (Borrmann et al., 2006 and Lima et al., 2011) and *C. septicum* alpha toxin (Salvarani et al., 2010).

The present study revealed that alpha toxin of *C. perfringens* type A caused cell shape alterations of Vero cells characterized by retraction and rounding. Using of antisera with high antibody titres resulted in an intact cell image under the microscope. Photo (1) shows the effect of *C. perfringens* type A alpha toxin on cultures of Vero cells.

The minimum cytopathic effect of *C. perfringens* type A alpha toxin dilution determined in Vero cell was 1:800 (using 50ul for tissue culture inoculation) while the minimum lethal dilution was 1:40 (using 100ul for mice inoculation) in mice demonstrating the higher sensitivity of Vero cell than that of mice to detect alpha toxin of *C. perfringens* type A.

Toxin standardization by standard homologous anti-toxin is needed to use this toxin in the seroneutralization tests. The obtained titers in the standardization of *C. perfringens* alpha toxin in mice and Vero cells were 1:18 and 1:800 respectively.

Titration of the tested sera by *in-vivo* and *in-vitro* seroneutralization were carried out after standardization of the toxin. The titers of neutralizing antibodies against *C. perfringens* type A alpha toxin determined by neutralization in mice and in Vero cell culture are tabulated in Table (1).

Interpreting the obtained data in Table (1), the values obtained during seroneutralization in mouse and Vero cells revealed that there was no significant difference between the two methods by using Student's t test (at $P \leq 0.05$) (Sendecor, 1971). Cell culture presents a series of advantages over the animal bioassay in titration of antibodies against *C. perfringens* type A, specifically the drastic reduction in the number of animals used, higher sensitivity of the test, smaller variation in the individual responses, and decrease in the amount of standard reagents. In comparison to *in-vitro* method, the use of cell cultures has some advantages; the cell test required neither the use of highly purified toxins, in order to exclude unspecific reactions, nor the use of monoclonal antibodies, which is a prerequisite for several ELISA systems (Ebert et al., 1999; Rosskopf- Streicher et al., 2004). The animal experiment is considered to be the "golden standard" and the alternative method should lead to comparable results (Weißer and Hechler, 1997 and Hendriksen et al., 1998).

This study demonstrated that antibodies against *C. perfringens* type A can be detected in a precise, sensitive, practical, and relatively rapid way, with a significant reduction in the number of used animals. *In-vitro* seroneutralization in Vero cell culture, a method standardized in this experiment, was found to be a viable alternative to the current *in-vivo* model used for vaccine potency test of *C. perfringens* type A alpha toxoid.

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استخدام الزرع النسيجى لتقييم لقاحات الكلوستريديا بيرفرنجيز النوع (أ)

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تعتبر خلايا الزرع النسيجى من البدائل المتاحة والتى يمكن ان تحل محل استخدام حيوانات التجارب فى اختبارات التعادل المصلي المطلوبة لقياس كفاءة لقاحات الكلوستريديا وعليه فقد صممت هذه الدراسة لتقييم لقاح الكلوستريديا بيرفرنجيز النوع (أ) باستخدام خلايا الفيرو مع مقارنتها باستخدام حيوانات التجارب. أوضحت الدراسة الحالية أن لتوكسين الكلوستريديا بيرفرنجيز النوع (أ) ألفا توكسين تأثير سمى على خلايا الزرع النسيجى لكلى القرد الأخضر الأفريقى يتميز بإنكماش واستدارة الخلايا. وقد وجد أن هذه الخلايا صالحة لمعايرة هذا السم حيث أعطت نتائج إيجابية عند تخفيف ١: ٨٠٠ (باستخدام ٥٠ ميكروليتر فى حالة حقن الزرع النسيجى) مما يدل على الحساسية العالية لتلك الخلايا. بينما كانت ادنى جرعة مميتة للفئران كانت عند تخفيف ١ : ٤٠ (باستخدام ١٠٠ ميكروليتر فى حالة حقن الفئران). تم قياس القوة المناعية لمصل عالى العيارية محضر فى الأرانب وايضا امصال الارانب والدواجن المحصنة بعدد تسع لقاحات للكلوستريديا بيرفرنجيز النوع (أ) فى المزارع النسيجية المذكورة والفئران السويسرية باستخدام اختبار تعادل المصل حيث لم يثبت وجود إختلاف معنوى بين نتائج الطريقتين. ولذلك فان استخدام خلايا الزرع النسيجى تعتبر بديل معملى مناسب لحيوانات التجارب فى اختبارات التعادل المصلى المطلوبة لتقييم لقاحات الكلوستريديا بيرفرنجيز النوع (أ) بدلاً من الفئران لتوفير الوقت والجهد والمال مع تجنب استخدام حيوانات حية.