EFFECT OF USING DIFFERENT ANTIFOAMS ON TOXIN PRODUCTION OF *CLOSTRIDIUM PERFRINGENS* TYPE A.

Elham F. El-Sergany, Taha, M.M., El-Helw, H.A., Hala El-Sawy
Anaerobic bacterial Vaccine Research Department, VSVRI, Abbassia, Cairo

A B S T R A C T

In this study, three types of anti-foaming agent (Polyethylene glycol, simethicone, and charcoal) were used in the process of preparation of *C. perfringens* type A toxoid. It was found that simethicone and polyethylene glycol when added to toxin producing medium gave a high yield of alpha toxin (α toxin) of *C. perfringens* type A (120, and 90 Minimum Lethal Dose (MLD) respectively) in comparison to that of medium without anti-foaming agent, which gave alpha toxin with 60 MLD. Medium with added charcoal gave least amount of α toxin (30 MLD). Two formula of *C. perfringens* type A vaccines were prepared with Simethicone and polyethylene glycol as anti-foams, and the third one was prepared without anti-foam as control one. Testing of these vaccines in rabbits revealed that simethicone as anti-foam in toxin producing medium of *C. perfringens* type A produced high level of toxin production and reduced the dose of vaccine used

Key words: *C. perfringens* type A, Anti-foam, Vaccine.

(BVMJ-27(1):1-7, 2014)

1. INTRODUCTION:

Clostridium perfringens *a*-toxin, which possesses lethal, hemolytic, dermonecrotic and phospholipase C (PLC) activities, has been reported to be a major pathogenic factor in the development of gas gangrene caused by the microorganism (Titball, 1993; Awad *et al*., 1995; Sakurai, 1995; Titball, 1997a, b; Bryant *et al*., 2000a, b). The production of Alpha toxin from *C. perfringens* type A, requires culturing of cells either in large flasks or in continuously stirred tank fermentors. Foaming can lead to reduced yield of toxins since it bursting bubbles can damage proteins (Holmes *et al*., 2006) and can also result in a loss of sterility if the foam escapes (Varley *et al*., 2004). In bioreactors, foaming can lead to over-pressure if a foam-out blocks an exit filter. To prevent the formation of foam, mechanical foam breakers, ultrasound or, most often, the addition of chemical antifoaming agents (or "antifoams") are routinely employed (Varley *et al*., 2004). In this study, three antifoams (Poly ethylene Glycol, Simethicone, Charcoal) that are widely used in controlling the foaming (Jahic *et al*., 2003; Charoenrat *et al*., 2005; Jungo *et al*., 2007) were chosen to analyze effect of their de-foaming action in toxin production medium and testing its effect on toxin yield and further more in preparation of vaccine.

2. MATERIAL AND METHODS:

2.1. Strains:

C. perfringens type A isolate was previously isolated from rabbits and identified in Anaerobic bacterial vaccine research department, Veterinary Serum and Vaccine Research Institute was used in preparation of α toxin. Robertson's Cooked meat medium (Oxoid) Smith and Holdman (1968) was used for rehydration and culturing of lyophilized strain of *C.*

perfringens type A Production Medium (Roberts *et al*., 1970). It was used for production of *C. Perfringens* exotoxins.

2.2. Antifoams:

1-Polyethylene Glycol 6000 (Titan Biotech Limited, 1013), it was added to toxin production medium at concentration of 1% (w/v) according to Wiebe *et al*., (2001).

2-Simethicone it was supplied from Pharco pharmaceutical Co., it is derivatives of silicone polymers, it was added to toxin production medium at concentration of 0.05% (v/v) according to Keill (1976).

3-Vegetable charcoal class as organic anti-foam it was supplied from South Egypt Drug Industries Co. (SEDICO), it was added to toxin production medium at concentration of 0.05% (w/v) according to Dworschack *et al*., (1954).

2.3. Preparation of vaccines:

Briefly *C. perfringens* type A was cultivated in cooked meat broth and incubated anaerobically at 37°C for 24 hrs. and then inoculated into toxin production medium (Roberts *et al.,* 1970) which added to it the different anti-foams and incubated at 37˚C for 5 hrs. The minimum lethal dose of produced toxin was determined according to Fu *et al*., (2004) by injecting mice weighted between (14-16 g) intravenously with 0.1 mL aliquots of tenfold serial dilutions of toxin samples and observing the mice 24 h for toxicity. Two mice were used for each amount of toxin dilutions.

The prepared toxin was inactivated by adding of 37% formalin at 0.5% (v/v) to inoculated medium and left at 37˚C for 7 days until complete inactivation of toxin and convert to toxoid. The produced toxoids were Centrifuged and the supernatant were taken where 2% Aluminum Hydroxide Gel (Suprex, Copenhagen, Denmark), it was added to each toxoid at concentration of 20% (v/v). Safety and sterility tests were applied on prepared vaccines according to OIE (2008).

Three types of vaccines were formulated according to addition of antifoams to toxin production medium (Vaccine #1 Polyethylene Glycol); (Vaccine #2 Simethicone); and (Vaccine #3 Control without antifoam).

2.4. Vaccination:

Fifteen rabbits were assigned into three groups, each group (n=5). Groups (1, 2, 3) vaccinated with (vaccine #1, #2, #3 respectively). Each rabbit in each group vaccinated subcutaneously with two doses of 2 ml, 21 day apart containing 60 MLD. Blood samples obtained from ear vein of rabbits after 14 days from second dose, sera were separated and stored at -20˚C until determination of antitoxin titers in them.

Antitoxin Titration:

2.5. Antiserum:

Diagnostic antiserum for α toxin of *C. perfringens* type A, was obtained from Welcome, Diagnostics Dart ford, England. It was used as control positive among the used toxin in toxin neutralization test and ELISA for evaluation of produced vaccine.

a. Toxin Neutralization Test:

It was done according to British Veterinary Pharmacopoeia (2010), briefly L+ dose of α toxin of *C. perfringens* type A firstly determined, then equal amount from serial diluted tested sera and L+ dose of α toxin were mix then incubated at 37 ˚C/1hour. From each mixed dilution, 2 mice were injected with 0.2 ml intravenously, and then observed for 24 hours.

b. Enzyme Linked Immunosorbent Assay (ELISA):

Indirect ELISA was used to detect the antibodies against toxin of *C. perfringens* type A, according to Harlow and Lane (1988). The antibody titers were determined by a least squares-weighted modification of the parallel line model according to Grabowska *et al.,* (2002).

Table 1: Effect of using different anti foam agents in toxin production medium of *C. Perfringens* type A

Anti-Foaming Agent	Minimum Lethal Dose (MLD)/ml
Polyethylene Glycol 6000	90
Simethicone	120
Charcoal	30
Control group	60

MLD: Minimum Lethal Dose

Table 2: Vaccines composition for 2ml dose for rabbits

Types of vaccines	MLD/ml	Volume of toxoid	Volume of Gel	Volume of sterile distilled water
Vaccine #1 (Polyethylene glycol)	90	0.66 ml	0.4 ml	0.93 ml
Vaccine #2 (Simethicone antifoam)	120	0.5 ml	0.4 ml	1.1 ml
Vaccine #3 Control group	60	1ml	0.4 ml	0.6 ml

MLD: Minimum Lethal Dose

Table 3: Antibody titers measured by toxin neutralization test in sera of rabbits vaccinated with different types of vaccines

Types of vaccines	Antibody titers expressed as IU/ml
Vaccine #1(containing Polyethylene glycol)	5
Vaccine #2 (Simethicone antifoam)	5
Vaccine #3 Control group	5

Table 4: antibody titers measured by ELISA in sera of rabbits vaccinated with different types of vaccines

Types of vaccines	Antibody titers expressed as ELISA Units/ml
Vaccine#1(containing Polyethylene glycol)	5.9
Vaccine #2 (Simethicone antifoam)	5.7
Vaccine #3 Control group	5.5

3. RESULTS

There was double increase in Minimum lethal dose of α toxin of *C. perfringens* type A (120 MLD/ml) when simethicone was used as anti-foam in toxin production medium in comparison with control medium that gave (60 MLD/ml), followed by Polyethylene glycol gave higher MLD (90 MLD/ml) than control one. While charcoal gave minimum MLD (30 MLD/ml) also lower than control group, (table 1). Preparation of vaccines against *C. perfringens* type A using different anti-foam agents in medium with fixed dose of 60 MLD/ml, accordingly using 0.66ml; 0.5ml, and 1.0ml from *C. perfringens* type A toxoid with polyethylene glycol 6000; simethicone, and control group respectively. As showed in table (2). Tables (3 & 4) illustrated antibody titers measured by toxin neutralization test and ELISA in sera of vaccinated rabbits with *C. perfringens* type A toxoid. Antibody titers were 5 IU/ml in all 3 groups of vaccinated rabbits measured by toxin neutralization test, and antibody titers were 5.9; 5.7, and 5.5 IU/ml in groups (1, 2, and 3) respectively measured by ELISA.

4. DISCUSSION

Foam is the dispersion of a gas in a continuous liquid phase, and thus foam dispersions possess bulk densities closer to that of a gas rather than a liquid (Vardar-Sukan, 1991). A general definition of foam, applicable to bioreactors, determines foam to occur when gas holdup in a gas-liquid dispersion is greater than 90% (Schubert *et al*., 1993). Other authors have quantified the gas content of foam to be in the range of 60-90% (Van't Riet and Tramper, 1991). Anti-foam are defined as strongly surface active substances, which replace foam forming components and lower surface tension of liquids. Anti-foams are dispersed by stirring and foam is destroyed by bubble coalescence, which decrease the available surface area for gas liquid mass transfer (de Haut, 2001). Anti-foams typically are added to medium or broth before foaming occurs, it was used to knock down foam after it has formed (Ghildyal *et al*., 1988). As showed in table (1) there was double increase in minimum lethal dose of α toxin of *C. perfringens* type A (120 MLD/ml) when simethicone was used as anti-foam in toxin production medium in comparison with control medium that gave (60 MLD/ml), followed by Polyethylene glycol gave higher MLD (90 MLD/ml) than control one. Preferred anti-foaming agents are Dimethicone and simethicone. Simethicone is described in the USP dictionary of USAN and International Drug Names, US Pharmacopeia as a mixture of poly (dimethylsiloxane) and silicone dioxide, the calculated average of dimethylsiloxane units is 200 to 350, and also non-irritating and biocompatible, so that is meant non-damaging to tissue (Carter *et al*., 2001). From the above results shown in table (1) that using of charcoal as antifoam had negative effect on toxin production. This may be due to adsorption of protein content in medium resulting in precipitation of protein and also insolubility in medium. Therefore preparation of vaccine from it must be excluded. Preparation of vaccines from toxin production media containing antifoams (Polyethylene glycol and Simethicone) and control medium devoid of antifoam shown that in table (2) so using fixed dose of 60 MLD/ml for each vaccine. Results illustrated in tables (2 & 3& 4) revealed that using of specific dose of 60 MLD/ dose gave antibody titers of 5 IU/ml and this titer is over the permissible limits (4 IU/ml) according to United States Department of Agriculture (USDA) USDA (2002), which surpass requirements to receive a conditional license pass standardized test by the development of a serum antibody concentration of at least 4 international antitoxin units per ml in at least 80 % of vaccinated animals that were seronegative prior to vaccination. So that using *C. perfringens* type A toxoid for vaccination

of rabbits and poultry was allowed, and also from the results that using of half the dose of control group when using Simethicone as antifoam in toxin producing medium gave also 5 IU/ml, which meet requirements of vaccine potency. Therefore, reduce the cost of vaccine into the half. There was great correlation between results obtained from toxin neutralization test and ELISA for measuring antibody titers and this come with accordance with Grabowska *et al.*, (2002) who found that the calculation of ELISA titer by weighted parallel line model is preferable for the evaluation of antibody level for ELISA data, particularly for samples with medium and low antibody levels. They suggested using of ELISA as replacement test for toxin neutralization test as a precise, accurate and not require the use of laboratory animals, which is a global trend for reducing using lab animals.

It could be concluded from the above results that, using of simethicone as anti-foam in toxin producing medium of *C. perfringens* type A is recommended as it produce high level of toxin production and reduced the dose of vaccine used.

5. REFERENCES

Awad, M.M.; Bryant, A. E.; Stevens, D. L., and Rood, J. I. (1995): Virulence studies on chromosomal alpha-toxin and theta-toxin mutants constructed by allelic exchange provide genetic evidence for the essential role of alpha-toxin in *Clostridium perfringens* mediated gas gangrene. *Mol Microbiol,* 5:191-202.

British Veterinary Pharmacopoeia, (2010): Veterinary supplement, ISBN 9780113228287.

Bryant, A.E.; Chen, R.Y.; Nagata, Y.; Wang, Y.; Lee, C.H.; Finegold, S.; Guth, P.H., and Stevens, D.L. (2000a): Clostridial gas gangrene. I. Cellular and molecular mechanisms of microvascular dysfunction induced by exotoxins of *Clostridium perfringens*. *J. Infect. Dis.,* 182:799-807.

Bryant, A.E.; Chen, R.Y.; Nagata, Y.; Wang, Y.; Lee, C.H.; Finegold, S.; Guth, P.H., and Stevens, D.L. (2000b): Clostridial gas gangrene. II. Phospholipase C-induced activation of platelet gpIIbIIIa mediates vascular occlusion and myonecrosis in *Clostridium perfringens* gas gangrene. *J Infect Dis,* 182:808-815.

Carter, F.C.; Jackson, F.W., and Whalen, R.G. (2001): Endoscopy tissue stain. United States Patent No. 6,280,720 B1.

Charoenrat, T.; Ketudat-Cairns, M.; Stendahl-Andersen, H.; Jahic, M., and Enfors, S. (2005): Oxygen-limited fed-batch process: an alternative control for *Pichia pastoris* recombinant protein processes. *Bioprocess Biosyst Eng*, 27:309-406.

De Haut, C. (2001): Foam control solutions in fermentation. Cognis literature; 1-13.

Dworschack, R.G.; Lagoda, A.A., and Jackson, R.W. (1954): Fermentor for small scale submerged fermentations. *Appl. Microbiol.*, 2:190-197.

Fu, S.W.; Xue, J.; Zhang, Y.L., and Zhou, D.Y. (2004): Simplified purification method for *Clostridium difficile* toxin A. *World J Gastroenterol*., 10(18):2756-2758. ISSN 1007-9327.

Ghildyal, N.P.; Lonsane, B.K., and Karanth, N.G. (1988): Foam control in submerged fermentation: *State of the art. AdV. Appl. Microbiol*. 33: 173-222.

Grabowska, K.; Wang, X.; Jacobsson, A., and Dillner, J. (2002): Evaluation of cost-precision rations of different strategies for ELISA measurement of serum antibody levels. *J. Immun. Meth.* 271: 1-15.

Harlow, Z., and Lane D. (1988): Antibodies A Laboratory Manual, Cold Spring Harbor Laboratory.

Holmes, W.; Smith, R, and Bill, R. (2006): Evaluation of antifoams in the expression of a recombinant F_c fusion protein in shake flask cultures of *Saccharomyces cerevisiae*. *Microb Cell Fact*., 5:30.

Jahic, M.; Wallberg, F.; Bollok, M.; Garcia, P., and Enfors, S. (2003):Temperature limited fed-batch technique for control of proteolysis in *Pichia pastoris* bioreactor cultures. *Microb Cell Fact.*, 2.

Jungo, C.; Marison, I., and Stockar, U.V. (2007): Regulation of alcohol oxidase of a recombinant *Pichia pastoris* Mut^+ strain in transient continuous cultures. *J. Biotechnol.*, 130:236-246.

Keill, J.W. (1976): Foam control composition. U.S. Patent 3,984,347.

OIE *Terrestrial Manual* (2008): Tests for sterility and freedom from contamination of biological materials, Chapter 1.1.9: 105-114.

Roberts, R.S.; Guven, S., and Narrat, E.F. (1970): Studies on *Clostridium oedematiens*. *J. Comp. Path*., 80: 9-18.

Sakurai, J. (1995): Major toxins of *Clostridium perfringens*. *Rev Med Microbiol,* 6:175-185.

Schubert, J.; Wan, L., and Lubbert, A. (1993): Foam suppression by bioreactor retrofitting. In Nienow, A. W. *Proceedings of 3rd International Conference on Bioreactor and Bioprocess*; MEP Ltd.: London.

Smith, L.D., and Holdman, L.V. (1968): The pathogenic anaerobic bacteria. Charles Thomas Publisher, USA.

Titball, R.W. (1993): Bacterial phospholipase C. *Microbiol Rev* 57, 347-366.

Titball, R.W. (1997a): Bacterial phosphorlipase. *Trends Microbiol,* 7: 265.

Titball, R.W. (1997b): Clostridial phospholipases. In *Clostridia: Molecular Biology and Pathogenesis*, London: Academic Press. pp. 223-242.

USDA (2002): Center for Veterinary Biologics Notice NO. 02-25, Conditional Licenses for Products Containing Clostridium *perfringens* Type A.

Van't Riet, K., and Tramper, J. (1991): Basic Bioreactor Design; Marcel Dekker, Inc.: New York, pp 274-292.

Vardar-Sukan, F. (1991): Effects of natural oils on foam collapse in bioprocesses. *Biotechnol. Lett.* 13(2): 107-112.

Varley, J.; Brown, A.; Boyd, R.; Dodd, P., and Gallagher, S. (2004): Dynamic multipoint measurement of foam behavior for a continuous fermentation over a range of key process variables. *Biochem. Eng. J.*, 20:61-72.

Wiebe, M.G.; Robson, G.D.; Shuster, J., and Trinci, A.P.J. (2001): Evolution of a recombinant (glucoamylase-producing) strain of *Fusarium* V*enenatum* A3/5 in chemostat culture. *Biotechnol. Bioeng. 73*:146- 156.


تأثير استخدام مضادات الرغوة على إنتاج السم المفرز من الكلوستريديم بيرفرينجينز نوع "أ"

إلهام فضل السرجاني–مدحت محمد طه –حامد عادل الحلو – هالة الصاوي أحمد

معهد بحوث الأمصال واللقاحات البيطرية – العباسية –القاهرة

الملخص العربي

في هذه الدراسة تم استخدام ثلاثة مواد من مضادات الرغوة (بولي إثيلين جليكول، سايمثيكون، فحم النباتي) في عملية تحضير توكسيد الألفا لعترة الكلوستريديم بيرفرينجينز نوع (أ). وجد عند إضافة سايمثيكون وبولي إثيلين جليكول إلى الوسط الغذائي المخصص لإنتاج السم أنها أعطت ناتج عالي من سم الألفا لعترة الكلوستريديم بيرفرينجينز نوع (أ) بالمقارنة بالوسط الغذائي المستخدم بدون مضادات الرغوة. ولكن عند استخدام الفحم النباتي أعطت اقل ناتج من سم الألفا. تم تحضير ثلاثة لقاحات لتوكسيد الألفا لعترة الكلوستريديم بيرفرينجينز نوع (أ) الأول باستخدام بولي إثيلين جليكول والثاني باستخدام سايمثيكون أما الثالث بدون مضاد رغوة (كمجموعة ضابطة) وباختبار اللقاحات الثلاث في الأرانب وجد أنها أعطت خمسة وحدات دولية /مللي في كل اللقاحات على الرغم من استخدام نصف الجرعة بالنسبة للقاح المحضر باستخدام سايمثيكون.

(مجلة بنها للعلوم الطبية البيطرية: عدد 27(1):1- 7, سبتمبر 2014)