Veterinary Serum and Vaccine Research Institute,
Abbassia, Cairo.

IN VITRO ESTIMATION OF POTENCY OF SOME CLOSTRIDIAL TOXOIDS

(With 3 Tables)

By
M.M. FAYEZ; A.A. EL-MENISY and A.Z. HUSSEIN

(Received at 21/3/2005)

تحديد كفاءة توكسيدات بعض ميكروبات الكلوستريديا معمليا

محمود محمد فايز ، علاء عبد الفتاح المنيسي ، عبد السلام زكي حسين

في تلك الدراسة تم مقارنة اختبار الاتحاد السمي المثبط كاختبار معملي لقياس القوة المناعية للقاح الكلوستريديا الجامع باختبار السم المتعادل في الفئران. وكانت النتائج لكلا الاختبارين متوازية ومتساوية ولذلك يمكن استخدام ذلك الاختبار بدلا من استخدام اختبار السم المتعادل توفيرا لاستخدام حيوانات التجارب.

SUMMARY

The efficacy of the toxin binding inhibition test (ToBi) as an in vitro testing of potency for multicomponent clostridial vaccine was compared with the currently used toxin neutralization test (TN). It was found that the antitoxin titers obtained with ToBi were highly correlated to TN. So this test could be used as simple, rapid and non-expensive alternative to TN test.

Key words: *Clostridial toxoids, antitoxin.*

INTRODUCTION

The potency of multicomponent clostridial vaccines is currently tested by their ability to stimulate an antibody response in rabbits, and it is measured in vivo by using toxin neutralization test (TN) in mice (British Veterinary Pharmacopoeia, 1993). While the test is known to be sensitive and reliable, however a high costing and the long time taken to perform the assay is regarded as inherent disadvantage. Moreover, there is a growing concern about the extensive use of non-protected animals in which clinical manifestation due to clostridial toxins especially tetanus may appear. For ethical, economical and practical reasons, several in vitro immunoassay techniques have been suggested by a number of

authors as an alternative to in vivo TN test such as haemagglutination test (Peel, 1980), and ELISA (EL-Idrissi and Ward, 1992; Ebert *et al.*, 1999). These immunological techniques are not preferred because non-neutralizing antitoxins are commonly detected. Moreover, the results of these in vitro techniques and those of the in vivo assay are not highly correlated especially when sera with low antibody titers are titrated (Simonsen *et al.*, 1987; Hagenaars *et al.*, 1984).

Recently an antigen competition ELISA has been described which showed a good correlation between in vivo and in vitro titers even in low level of antitoxin (Simonsen *et al.*, 1987; Marcjanna *et al.*, 1989). In the present study, the efficacy of the toxin binding inhibition test (ToBi) as an alternative approach to the currently used toxin neutralization test for estimating the potency of clostridial vaccines was evaluated.

MATERIALS and METHODS

Clostridial toxoids:

Clostridial toxins (*C. perfringens* types B; D, and *C. novyi* type B) were prepared according to (Gadalla *et al.*, 1974), while *C. tetani* toxin was prepared according to (Rijks, 1980). After estimation of the minimum lethal dose (MLD) of each toxin, 0.5% formalin was added for toxoiding all the prepared toxins. A polyvalent clostridial vaccine containing the above-prepared toxoids was formulated according to the regulation of (British Veterinary Pharmacopoeia, 1993).

Experimental Animals:

A group of ten Boscat rabbits (2.3-3Kg) and six sheep (8-12 month old) were used for evaluation of the potency of the prepared polyvalent clostridial vaccine. Both rabbits and sheep were injected S/C with two doses of 5 ml and 3 ml at 3 weeks interval. Rabbits were bled two weeks after boostering and sera were pooled. Sheep sera samples were collected two weeks post the second dose and then every 3 weeks until the end of the experiment.

Toxin neutralization test:

Antibody titers of rabbit and sheep sera were estimated in mice using the standard method described by (Frerichs and Gray, 1973).

Toxin binding inhibition test: (ToBi)

The ToBi was carried out as described by (Hendriksen *et al.*, 1988) with some modification, keeping in consideration a fixed concentration of each toxin and antitoxin as illustrated in Table (1). In brief, a flat-bottomed polystyrene micro titer plates were coated with

250µl/well phosphate buffered saline (PBS), pH 7.2 containing 0.5% bovine serum albumin (BSA); 0.05% Tween 80. After incubating at 37°C for 2 hours, two fold dilution of each serum sample starting with 1:4 dilution were made in PBS in 100 µl volumes. Each serum dilution was mixed with 100µl of the toxin in PBS (A reference serum with known antitoxin concentration (one I.U. /ml) was titrated for comparison. The antitoxin concentration for this reference serum has been previously determined in vivo). The plates were gently shaked and incubated overnight at 37°C in humid atmosphere. Next day, 100µl of the serum toxin mixture was transferred from the micro titer plates to the corresponding wells of immunoassay micro titer plates coated with (one I.U./ml) antitoxin. All plates were incubated for 1.5 hours at 37°C, and then washed with tape water containing 0.05% tween 80. After washing, (one I.U./ml) antitoxins were added again each well in 100 µl quantities, and incubated at 37°C for 1.5 hours. Thereafter a diluted peroxidase labeled antispecies was added in 100 µl/well quantities, incubated at 37°C for 1.5 hours, followed by washing with tape water containing 0.05% tween 80. Finally, 100 µl/well of the substrate (34mg OPD dissolved in 100 ml phosphate citrate buffer, pH 5, and 15µl of 30% H_2O_2) was added to each well. After ten minutes the reaction was stopped by addition of 100 µl of 2M H_2SO_4 to each well. The absorbance was measured at 490nm.

Table 1: Concentration of toxins and antitoxins of different clostridial strains.

Toxins	Concentration	Antitoxin	Concentration
Beta toxin of C. perfringens type B	1L+/10	Beta antitoxin of C. perfringens type B	One I.U./ml
Epsilon toxin of C. perfringens type D	1L+/100	Epsilon antitoxin of C. perfringens type D	
Alpha toxin of C. novyi type B	1L+/10	Alpha antitoxin of C. novyi type B	
Tetanus toxin	0.1 Lf/ml	Tetanus antitoxin	

Reproducibility of ToBi:

The reproducibility of ToBi was determined by repeated testing of 10 serum samples for several weeks. The inter and intra assay coefficient of variance was determined as described by (Dawson-Sounders and Trapp, 1990).

RESULTS and DISCUSSION

Despite the serum neutralization assay in mice for measuring clostridial antitoxin is laborious and expensive and uses large number of laboratory animals, it is still the in vivo method of choice for demonstrating the protective (neutralizing) antitoxin. The need for simple in vitro test, which makes possible rapid titration of sera for potency testing of clostridial vaccines has been previously recognized (Peel, 1980). To discriminate neutralizing from non-neutralizing antitoxins is an essential prerequisite for many techniques that replacing the TN. In the present study, the ToBi test is based on the detection of free toxin in toxin-antitoxin mixture by an ELISA with preoxidase-labeled antitoxin and the only difference between the ToBi and in vivo TN test being the way in which free toxin is detected (Hendriksen *et al.*, 1991).

During this work, pooled sera collected from rabbits vaccinated with the prepared polyvalent clostridial vaccine was titrated using both TN and ToBi tests. The results illustrated in Table (2) revealed that the antitoxin values measured by TN test were 10,5,5 and 20 I.U. for Beta, Epsilon, Alpha, and Tetanus antitoxin respectively. On the other hand, the corresponding antitoxin values determined by ToBi were 11,5,5 and 21 respectively. The obtained antitoxin titers satisfy the requirements of British Veterinary Standard for all vaccine components.

By inspecting the data shown in Table (2), it is clear that there is a significant correlation coefficient r= 0.89 between both TN and ToBi test in the estimation of antitoxin level, as also documented by (Hendriksen *et al.*, 1991).

In vaccinated sheep Table (3), statistical analysis revealed that there is a good correlation between TN and ToBi for their sera (for Beta antitoxin r= 0.999, Epsilon antitoxin r= 0.994, *C. novyi* type B antitoxin r= 0.986, tetanus antitoxin r= 0.999) $p<0.05$. Such results almost agree with these of (Hendriksen *et al.*, 1989; Marcjanna *et al.*, 1989; Hendriksen *et al.*, 1991) as they used ToBi instead of TN and in vitro neutralization test in vero cells for titration of tetanus and diphtheria antitoxin.

The reproducibility of the test for the 10 sera samples that tested for 5 times proved that the ToBi test was reliable. Statistical analysis of the obtained results showed that the intra assay coefficient of variance was (1-4%) Where the inter assay coefficient of variance was (0-36%)

which are very satisfactory and within the normal range (Dawson-Sounders and Trapp, 1990).

In conclusion, the ToBi was found to be a practically simple; reproducible, and quick to perform and it is correlated well with TN test, it could be also act as non expensive, alternative to TN test by saving for the laboratory animals needed for testing the potency of multicomponent clostridial vaccines.

Table 2: Antitoxic values of pooled rabbit sera vaccinated with polyvalent clostridial vaccine as estimated by serum neutralization test and toxin binding inhibition test.

Test	Antitoxin titer (IU/ml)			
	C. perfringens type B	*C. perfringens* type D	*C. novyi* type B	*C. tetani*
Serum neutralization test	10	5	5	20
Toxin binding inhibition test	11	5	5	21

Table 3: Immune response of sheep to polyvalent clostridial vaccine as measured by TN and ToBi test.

Time after vaccination	Antitoxin titer (I.U./ml)							
	C. perfringens Type B Beta antitoxin		*C. perfringens* Type D Epsilon antitoxin		*C. novyi* Type B Alpha antitoxin		Tetanus antitoxin	
	TN	ToBi	TN	ToBi	TN	ToBi	TN	ToBi
Prevaccination	0	0	0	0	0	0	0	0
4 weeks	5	5	3	3	2	2	5	5
7 weeks	20	21	10	10	5	6	20	21
10 weeks	15	15	8	7	5	5	20	20
13 weeks	10	10	5	5	4	4	15	15

I.U.= International Unit.

REFERENCES

British veterinary pharmacopoeia, (1993): HMSO, London, pp. 118.

Dawson-Sounders, B., and Trapp, R.G. (1990): Basic and clinical biostatistics Appleton and lange, Norwalk CT.

Ebert, E.; Oppling, V.; Werner, E., and Cussler, K. (1999): Development and prevalidation of two different ELISA systems for potency testing of *C. perfringens* type B and epsilon toxoid containing veterinary vaccines. FEMS Immunol. Med. Microbiol. 24: 299-311.

EL-Idrissi, A.H., and Ward, G.E. (1992): Development of double sandwiches ELISA for Clostridium perfringens beta and epsilon toxins. Vet. Microbiol. 31: 89-99.

Frerichs, G.N., and Gray, A.k. (1973): Immune response of rabbits to multicomponent clostridial vaccine. J. Biol. Stand. 1:33-36.

Gadalla, M.S.; Farrag, I., and Sharaf, D. (1974): Effect of growth requirement on the improvement of clostridial vaccines. J. Egypt. Vet. Med. Ass. 34 (112):19-28.

Hagenaars, A.M.; Van Delft, R.W., and Nagel, J. (1984): Comparison of ELISA and toxin neutralization for detection of tetanus antibodies. J. Immunoassay, 5: 1-11.

Hendriksen, C.F.M.; Gun, V.D.J.; Nagel, J., and Kreeftenberg, J.G. (1988): The toxin binding inhibition test as a reliable in vitro alternative to toxin neutralization test in mice for the estimation of tetanus antitoxin in human sera. J. Biol. Stand. 16: 287-297.

Hendriksen, C.F.M.; Gun, V.D.J., and Kreeftenberg, J.G. (1989): Combined estimation of tetanus and diphtheria antitoxin in human sera by in vitro toxin binding inhibation (ToBi) test. J. Biol. Stand. 17:181-190.

Hendriksen, C.F.M.; Gun, V.D.J.; Marsman, F.R., and Kreeftenberg, J.G. (1991): The use of the in vitro toxin binding inhibition (ToBi) test for the estimation of the potency of tetanus toxoid. J. Biol. Stand. 19: 23-29.

Marcjanna, G.S.; Victor, J.W.; Cloristopher, J.T., and Peter, L.P. (1989): The detection of *Clostridium perfringens* epsilon antitoxin in rabbit serum by monoclonal antibody based competition ELISA. J. Biol. Stand. 17: 117-124.

Peel, M.M. (1980): Measurement of tetanus antitoxin 1-Indirect haemagglutination. J. Biol. Stand. 8:177-189.

Rijks, I. (1980): Instructions for the preparation of vaccines. Rijiks Institute Voor Volksgezondheid Bilthoven, The Netherland.

Simonson, O.; Kjeldsen, K.; Bentzon, M.W. and Heron, J. (1987): Susceptibility to diphtheria in populations vaccinated before and after elimination of indigenous diphtheria in Denmark. Acta. Pathol. Microbiol. Immunol. Scand. 13: 229-234.