



ISSN Print 2231 – 3648
Online 2231 – 3656

Available Online at: www.ijpir.com

EVALUATION OF TETANUS ANTITOXIN TITERS OF SERA FROM IMMUNIZED GUINEA PIGS DETERMINED BY TOXIN NEUTRALIZATION TEST AND STANDARDISATION OF ENZYME-LINKED IMMUNOSORBENT ASSAY

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Abstract

Enzyme-linked immunosorbent assay (ELISA) and toxin neutralization (TN) test were comparatively evaluated for determination of antibodies to tetanus toxin in sera of immunized guinea pigs and mice at different intervals. Tetanus specific IgG antibodies by ELISA were found in sera of guinea pigs at ninth day after inoculation and in mice sera at seventh day after inoculation when there were no TN antibodies. Two weeks after injection of mice with aluminium phosphate adsorbed tetanus toxoid, anti-tetanus toxin IgG levels determined by ELISA were 35 to 40 times higher than the TN titers. At 4 weeks, ELISA IgG antibody levels of mice and guinea pig sera were still statistically significantly higher than TN titers but with a good correlation between ELISA and TN titers (correlation coefficient of mice sera 0.89). Therefore ELISA results on sera before 6 weeks of immunization determined against a hyper-immune reference serum do not give a true picture of TN levels. The quality control testing of biological especially potency test is a subject on its own. The biological standardizations of each procedure requires meticulous planning and execution. The potency test for tetanus toxoid as specified in Indian pharmacopoeia is biological assay of tetanus antitoxin in immunized guinea pigs. An in vitro serological assay system – ELISA to assess the potency of tetanus toxoid was standardized and results compared with the conventional biological assay.

Keywords: ELISA, toxin neutralization test, IgG antibody.

Introduction

The toxin neutralization (TN) test in mice is a highly sensitive, reproducible and accurate technique for measuring tetanus antitoxin in sera. However, the test is cumbersome, requiring large number of laboratory animals active and stable tetanus toxin. Well trained staff and relatively large

volumes of sera. Therefore, several in vitro serological tests for titration of tetanus antitoxin have been developed. Although in vitro tests such as indirect haemagglutination,^{1,2} several version of Enzyme linked immunosorbent assay (ELISA)^{3,4,5,6,7,8} and Toxin binding inhibition test⁹

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have been widely used in many laboratories. None of these has been universally accepted as a substitute for the TN test. ELISA has been widely used in recent years for titration of tetanus diphtheria antitoxins in human and animal's sera due to simplicity ease of automation, availability of stable reagents and objective interpretation. Different groups assign antibody concentrations by different methods including titers, international or antitoxin units (IU or AU)^{3,4,5,6,7,10} and weight based units $\mu\text{g} / \text{ml}$.¹¹ In most reports, ELISA results have not been directly compared with TN in mice results making a comparison of ELISA results from various laboratories extremely difficult. In the present study tetanus antibody levels in sera of immunized mice and guinea pigs were measured by ELISA at different intervals after immunization and correlated with TN titres.

Materials and Methods

1. Lf determination of tetanus toxoid by using flocculation method.¹²

Preparation of 10% tri Sodium Citrate

Solution:

10.0g of tri sodium citrate was weighed and transferred in to the 100 ml bottle and the volume was made to 100 ml using WFI /Miii Q water. Stored at room temperature for not more than 6 months.

Test Procedure:

10ml of the sample was taken in a 15 ml centrifuge tube. Centrifuged at 2000 rpm for 10 minutes. Supernatant was collected in another tube and label it as supernatant. The supernatant contains Lf from the vaccine sample that was left unadsorbed. Store at 2-8°C for testing at a later stage. To the sediment of the test tube, labeled as Elute add 10% tri sodium citrate solution to make up the volume to 10 ml. Incubated at 35-37°C for about 16 hours. Tetanus Toxoid shall elute in this mixture and is tested for Lf that is contributed by adsorbed tetanus toxoid in the sample. 10 ml of the sample was taken in another test tube and label it as Total, add 10.0 g of tri sodium citrate. Mixed well and incubated at 35-37°C for about 16 hours. It contains total Lf contributed by both adsorbed as well as unadsorbed tetanus toxoid. The Lf in supernatant was estimated by performing the Lf test taking antitoxin concentration in the range of 2 Lf to 10 Lf with the difference of 1Lf. Add 0.85% sterile physiological saline was added in the tubes

to make up the volume to 1.0 ml. Add 1.0 ml of the supernatant sample to all flocculation tubes. The Lf in Elute was estimated by performing the Lf with the difference of 1 Lf. Add 0.85% sterile physiological saline in the tubes to make up the volume to 1.0 ml. 1.0 ml of the Total sample was added to all flocculation tubes. Keep the reaction mixture at 45-50°C in water bath in such a way that at least half of the reaction mixture is in the water. The tubes were observed for the flocculation reaction to occur. Mixture flocculating first is that which contains the most nearly equivalent quantities of Tetanus Toxoid.

2. Selection of test animals on guinea –pigs¹²

Healthy guinea – pigs was used from the same stock and weighing between 250 and 350 g. Distribute them into six groups of sixteen each. The guinea – pigs should all be of the same sex or the males and females should be distributed equally between the groups. If the challenge toxin to be used has not been shown to be stable or has not been adequately to serve as unvaccinated controls.

3. Immunization of guinea pigs¹²

Inject subcutaneously on each of two occasions separated by an interval of not more than weeks, one tenth of the stated human dose diluted to 1 ml with saline solution into each of 9 normal, healthy guineas – pigs weighing between 250 and 350 g. Not more than 2 weeks after the second injection. Collect the serum from each animal and carry out the biological test for tetanus antitoxin, described under Tetanus antitoxin or any other method approved by National Regulatory Authority.

4. Collection of blood and separation of serum

Preparation of serum samples: For preparation of serum samples, the following technique has been found suitable. Invert the tubes containing blood samples 6 times and allow standing 37°C for 2 h, then 4°C for 2 hours; centrifuge at room temperature at 800 g for 20 min. transfer the serum to sterile tubes and store at a temperature below - 20°C. At least 40 % yield of serum is obtained by this procedure.

5. Toxin neutralization test¹²

Tetanus anti toxin preparation

The 915 International Units Per Vial Tetanus Anti toxin (*in vivo*) was mixed with 91.5 ml of normal saline (1 ml contain 10 units of AT) and from this

solution 0.1 ml was taken and mixed with 19.9 ml of normal saline, This standard preparation contain 0.05 units/ml.

Reference standard preparation

Low concentration of toxin preparation,
Substantiated concentration of toxin preparation,
High concentration of toxin preparation.

Diluted sera preparation

Sub dilution sera preparation Each test tube 0.9 ml of normal saline was taken and 0.1 ml of guinea pigs serum was added

Diluted serum test preparation

The 0.6 ml of normal saline, 0.6 ml of SCTP was taked and 0.3 ml of SDSP solution was added.

Animal inoculation

Neat serum preparation and Diluted serum test preparation was stored in room temperature at one hour after one hour each test taken 0.5.ml was injected for two mice and observed the mice at four days.

ELISA

Standardization of in house elisa for determination of ELISA IgG antibodies against TT raised in guinea pigs.

Coating of Antigen:

Tetanus toxoid coated 0.5 LF of commercial TT was coated and stored at 4⁰c for over night.

Preparation of reagents for conducting ELISA

Peroxidase conjugate preparation Peroxidase conjugated rabbit or goat antibody directed against guinea – pig IgG,Carbonate coating buffer pH 9.6 Dissolve 1.59 g of anhydrous sodium carbonate and 2.93g of sodium hydrogen carbonate in 1000 ml of water. Distribute into 150 ml bottles and sterilize by autoclaving at 121⁰c for 15 min

Determination of potency

Toxin neutralization test

,Phosphate Buffered Saline Preparation PH7.4 Dissolve with stirring 80 g of sodium chloride, 2.0 g of potassium dihydrogen phosphate, 14.3 g of disodium hydrogen phosphate dihydrate and 2.0 g of potassium chloride in 1000 ml of water. Store at room temperature to event crystallization. Dillute to 10 times its volume with water before use, Citric Acid Solution Preparation Dissolve 10.51g of citric in 1000 ml of water and adjust the solution to pH 4.0 with a 400g/l solution of sodium hydroxide, Washing Buffer Preparation PBS containing 0.5g/l of polysorbate 20, Diluent block buffer preparation PBS containing 0.5 g/l of polysorbate 20 and 25g/l of dried skimmed milk, Peroxidase Substrate Preparation Shortly before us. Dissolve 10 mg of diammonium 2.2- azti bobis 93 ethylebenzthizoline 6 sulphonate) (ABTS) in 20 ml citric and solution. Immediately before use add 5 µl if strong hydrogen peroxide solution.

Results and discussion

ELISA specific antitetanus IgG antibodies appear in the sera of guinea pigs at 42 days of immunization of 2 doses of tetanus toxoid. The antibodies measured by conventional toxin neutralization test correlated well with Elisa.

Lf determination of tetanus toxoid by using flocculation method.

The tetanus toxoid LF was identified. 0.5 ml contain 5 LF commercial tetanus toxin was identified.

Selection of test animals on guinea –pigs

The guinea-pig was selected and used for the test weight 250-350g.

Immunization of guinea pigs

The 12 guinea-pigs after TT injected and one control guinea-pigs the conditions was observed daily.

Table No. 01: Symptoms in mice

Stages	Normal	✓
I	slight stiffness to hind limb	+
II	cramping of hind limb with scoliosis	++
III	serious cramping pronounced scoliosis	+++
IV	Death	D

Table No. 02: Observation reference standard

S.No.	Unit age tested for (IU/ml)	Mice	Observation Days / Date			
			1	2	3	4
1	HC	1	✓	++	D	-
	0.5ml	2	✓	++	D	-
2	SC	1	✓	++	+++	D
	0.5ml	2	✓	++	+++	D
3	LC	1	✓	✓	✓	✓
	0.5ml	2	✓	✓	✓	✓

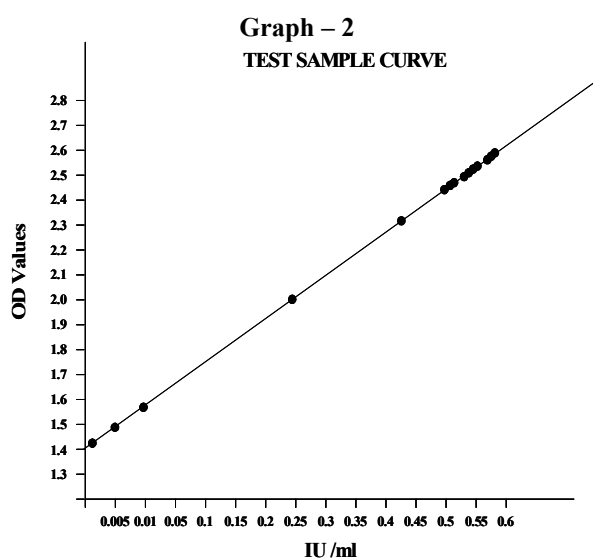
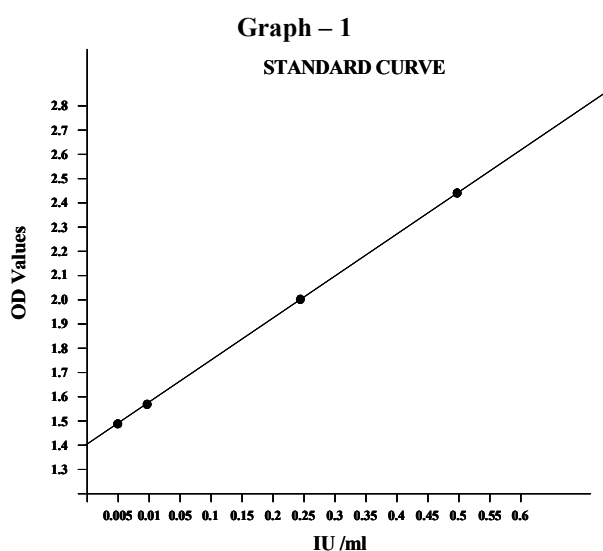
The dose neutralized mixture (normal saline+ toxin+ sera) injected mice were observed daily.

1. HC dose should stage IV symptoms on 3th day due to high level of toxin.

2. SC dose should stage IV on 4th days as the unitage of antitoxin is 0.5IU/ml.

3. LC dose mice should stage I.

Standardization of in-house Elisa for determination of Elisa IgG antibodies against TT raised in guinea pigs.



Discussion

The immunized guinea-pig was observed daily. All guinea-pig were normal and healthy. Food and water was provided daily & housed in a quiet environment. 9th day Bleeding by cardiac puncture under anaesthesia (ether), 20th day Bleeding by cardiac puncture under anaesthesia, 30th day Bleeding by cardiac puncture under anaesthesia (ether), 43rd day Bleeding by cardiac puncture under anaesthesia, Collection of blood and separation of serum The blood was collected and sera was separated stored at -20°C.

Correlation between TN and ELISA

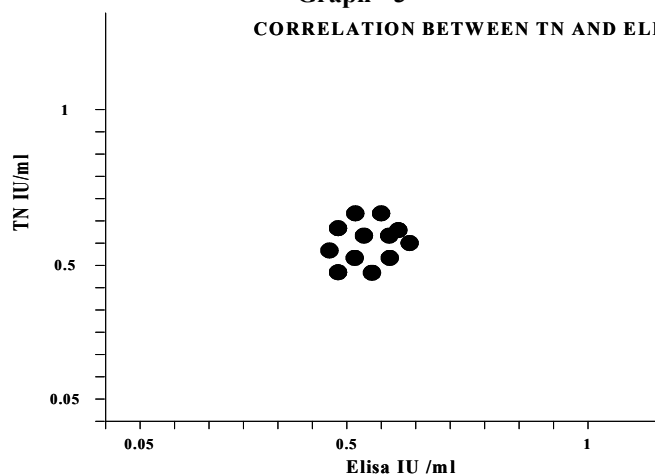
The toxin neutralization test and Elisa was correlated by statistical methods 12 guinea pigs sera by TN should be more than 0.5 IU/ml geometric mean of titers detected by ELISA is

0.531 IU after 6 weeks of immunization this shows that titers were similar then by TN or ELISA. On receiving one dose of sera from guinea pigs on 9th day showed a titer of TT (1 in 10). 0.0025IU, 0.005IU ON 20th day and 0.01 IU on 30th day. The titers of antibodies that could not be detected by TN were detectable by ELISA. After 30 days i.e. after receipt of 2 doses of TT the guinea pig sera showed a level of 0.531 IU by Elisa showing good correlation with antibodies titers by TN. The antibody levels by ELISA valued from 0.45IU to 0.572IU/ml indicate that if values of 9 out of 12 guinea pigs are taken they are all well about 0.5IU/ml which is also the level detectable by antibody titration method also the increase in antibody titer 1.2 times the minimum level detected amongst the 12 guinea pig sera. Further the increasing in titer of antibodies from 9 day to 30

day from 0.0025 to 0.01IU is well correlating. This detection not possible by TN. This show the levels acceptable by the **ELISA methods have been proven.** There were no statically significant

difference between TN test and ELISA 12 guinea pig serum pools ($p > 0.11$). The sensitivity & specificity of the ELISA test was 100% as proved in this study.

Graph - 3
CORRELATION BETWEEN TN AND ELISA



Conclusion

The results of this project affirm the previous observations that in vitro system of assay like ELISA are more quick, sensitive, cost effective, appropriate. Further quantifications of antibodies is an added advantage. The findings that the reduction of animals used for potency of TT when compared to biological assay contributes to the advantages of using ELISA as a routine to assess potency of TT. A good correlation can be seen between titers of antibodies in neutralization toxin test and ELISA. Tetanus toxoid is one of the most used immunogens available to protect against tetanus. A considerable reduction and retirement of the use of animals can be achieved by introduction of ELISA as a routine for potency of tetanus toxoid. Substantial reduction of number of mice and quantitative data generated boosts up the image of in vitro assay. ELISA is easy to perform, sensitive, highly reproducible and require only small quantities of sera and time saving and objective. From the study it was concluded that ELISA standardization done by me in the department of biological control (King Institute) is a Promoting alternative to the biological potency assay of tetanus toxoid. Rapid technique in the based on application of ELISA. eg : Immuno comb can be standardized in future. Therefore the vaccine formulation did not influence the correlation between TN and ELISA titers of immunized guinea pig sera. Other workers have also reported good correlations between TN and ELISA titers of human sera.³ Simonsen et al. found good correlation between ELISA and TN titers of

sera from vaccinated human populations but no correlation with sera from persons having an incomplete vaccination history. Our findings of a discrepancy between ELISA and TN titers of immunized animals early during the course of immunization have implications on the studies where antibody levels are measured by ELISA only. Therefore, we suggest that the ELISA titers of sera from immunized guinea pig or mice should be interpreted cautiously up to 6 weeks after a single immunization when the results are expressed as titers in weight units ($\mu\text{g/ml}$) or EAU/ml determined against a hyper-immune serum. To overcome the problem of discrepancies between ELISA and TN titers, one possibility may be the use of an ELISA reference serum (calibrated by TN test) with similar affinity antibodies or similar immunization status as the test serum.

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