Replacement of the Toxin Neutralization Test in Mice by *in vitro* Serological Assay Systems for Potency Testing of Tetanus Toxoid Vaccines for Veterinary Use: Results of a European Collaborative Study

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Summary

This paper describes the results af a collaborative study to the use of in vitro serological assay systems in the assessment of the potency of tetanus toxoid in single and multicomponent vaccines for veterinary use. According to the procedure described in monograph 697 of the European Pharmacopoeia (method A) these products are currently tested by toxin neutralisation tests in mice.

The collaborative study was performed in seven laboratories throughout Europe. Nine commercial vaccines, representing the range of products available, and one experimental tetanus toxoid preparation were tested by immunization of groups of rabbits and guinea pigs. Individual and pooled serum samples were titrated for levels of tetanus antitoxin by indirect enzyme-linked immunosorbent assay (ELISA), toxin binding inhibition (ToBI) test, passive haemagglutination (HA) test and by the toxin neutralization (TN) test in mice. It was found that estimates of potency by in vitro tests and by the TN test were in good agreement for the various vaccines tested and for antitoxin titres of individual serum samples. Significant intralaboratory variation occured less frequently for ELISA and ToBI than for HA. The frequency of significant interlaboratory variation was acceptable for ELISA and for ToBI but larger variation was observed for HA.

It is concluded that ELISA and ToBI are suitable in vitro assay systems for assessing the potency of tetanus toxoid in batches of single and multicomponent vaccines for veterinary use. Rigid standardization of HA test is essential before this test can be used for the same quality control purpose.

Zusammenfassung: Ersatzmethoden für den Toxinneutralisationstest in der Maus. Ergebnisse eines europäischen Ringversuchs.

Zur Wirksamkeitsprüfung von Tierimpfstoffen gegen Tetanus wird ein Toxinbelastungsversuch an Labortieren im Europäischen Arzneibuch verlangt. Hierzu werden zwei Methoden A und B beschrieben. Beide Tierversuche erfordern hohe Tierzahlen und sind so angelegt, daß ungefähr die Hälfte der Tiere unter erheblichem Leiden an Tetanus verendet. In den letzten Jahren wurden einige immunologische Methoden zur Bestimmung von Tetanus-Antikörpern entwickelt. In einem europäischen Ringversuch wurden drei Testsysteme (Haemagglutinationstest, Toxoid-ELISA und Toxinbindungsinhibitions-(ToBI)-Test) auf ihre Eignung als Alternativmethoden geprüft. Die Ergebnisse zeigen, dass der Toxoid-ELISA und der ToBI-Test geeignete Alternativen zum in vivo Toxin-Neutralisationstest der Methode A darstellen.

Keywords: Tetanus toxoid, potency testing, serological test systems, collaborative study

Introduction

The year 1889 was a breakpoint in the history of tetanus. This infectious disease, already described by Hippocrates, was one of the world's greatest killers, both in human and animal populations. No therapy applied in those days, such as bleeding and aloe extract, was very effective to the state of spastic paralysis of tetanus victims. In 1889 the causative agent, *Clostridium tetani*, was identified by the bacteriologist Kitasato, a colleague of von Behring. It marked a milestone as from then on the development of an effective therapy and prophylaxis became possible. In 1890 von Behring and Kitasato discovered the therapeutic value of tetanus antitoxin and in 1920 Ramon succeeded in rendering tetanus toxin harmless by formaldehyde, while still remaining its antigenic power. The product was called ‚toxoid'. With the availability of immunological products as tetanus antiserum and tetanus toxoid the need for a quality control of these products emerged. Already in 1897 Ehrlich described an assay method for the estimation of the potency of tetanus antitoxin, based on the principle of Toxin neutralization (TN) in laboratory animals. In 1937 Prigge developed the concept of the so-called „Kollektivversuch" for the potency testing of toxoid vaccines in which information on a dose-response and ED50 (Effective Dose

50%; the dose of vaccine which protects 50% of the immunized animals against the lethal or clinical effects of a challenge with the virulent agent) was obtained. Both methods; the TN test and the „Kollektivversuch" are still used. To cxpress it even more forcibly, both methods form the back-bone for potency testing in the European Pharmacopoeia monograph on tetanus vaccines for veterinary use (Council of Europe, 1990).

Potency testing of tetanus vaccines for veterinary application according to the European Pharmacopoeia

For potency of tetanus vaccines for veterinary use two methods, A and B, are described in the European Pharmacopoeia (Council of Europe, 1990). Method A is an indirect protection test and is based on primary (day 0) and booster (day 28) immunization of 10 guinea pigs, followed by bleeding of the animals at day 42 and pooling of the sera. Thereafter, the potency of the pooled sera is determined in TN test, by comparing the dose necessary to protect mice or other suitable animals against the toxic effects of a fixed dose of tetanus toxin with the quantity of a reference preparation of tetanus antitoxin, calibrated in International units (IU), necessary to give the same protection. The TN test should be repeated at least once. When tetanus vaccine for veterinary use is presented as a mixed vaccine for use in animals other than *Equidae* and the potency of the other component(s) of that vaccine is normally performed in rabbits, potency testing may also be carried out in rabbits. Potencies should exceed 7.5.IU/ml in the case of tests in guinea pigs, except when the vaccine is intended for use in *Equidae*. Then the potency of the pooled sera should not be less than 30 IU/ml. In the case of potency tests in rabbits the potency of the pooled sera should exceed the level of 2.5 IU/ml. It should be noted that no information is given in the Monograph as to the rational behind the minimal levels stated.

Method B in the EP Monograph is a direct protection test which strongly resembles the potency test on tetanus vaccines for human use. In Method B groups of mice are immunized only once with the vaccine under study and a reference toxoid, respectively, and challenged after 28 days with a fixed dose of tetanus toxin. Potency is calculated by parallel-line probit analysis. Method B may be used only for those preparations for which it has been shown to be suitable. In particular, the method may not be suitable for vaccines with an oily adjuvant or for multicomponent vaccines.

With regard to animal welfare, both methods include serious disadvantages, being the degree of distress and suffering inflicted on the animals, and the large numbers of animals required, being about 10 guinea pigs or rabbits and at least 50 mice for Method A, half of them suffering or dying from tetanus. Therefore it is felt that replacement or refinement of the direct or indirect protection test is urgently needed, however, without jeopardizing the testing of quality of the vaccine.

In vitro serological assay systems

As the immunogenicity of tetanus toxoid is affected not only by its quantity, but also by its quality and by other components (e.g. adjuvant), it generally is felt that some kind of biological test system will be needed to estimate potency. Procedures which have been developed last decade are based on immunization of laboratory animals with toxoid, followed by serum titration in *in vitro* test systems, preferably based on a functional parameter. Unfortunately, the only biological activity of Tetanus toxin known is the inhibition of neurotransmitter release in nerve cells (Habermann und Dreyer, 1986). This rather specific activity limits the possibility of developing a functional *in vitro* model, such as the titration of antiserum by Toxin neutralization in cultures of nerve cells or neuroblastoma cells. So far, studies in this direction have not been very successful (Hendriksen et al., 1988). Instead, several non-functional *in vitro* models have been established, based on immunological techniques, such as the haemagglutination (HA) test (Pitzurra et al., 1983), Toxoid-Enzyme Linked Immunosorbent assay (ELISA) (Melville-Smith et al., 1983) and Toxin binding inhibition (ToBI) test (Hendriksen et al., 1988). These methods have shown to be very useful in the estimation of tetanus antitoxin concentration, although for Toxoid-ELISA overestimation of tetanus antitoxin has been reported (Simonsen et al., 1986) with low titre antisera.

In 1991, a collaborative study in seven laboratories throughout the European Union (EU) was initiated to evaluate the suitability of the *in vitro* serological assay systems to provide valid and reproducible measurements of the potency of tetanus toxoid preparations for veterinary use, according to Method A of the EP. The study was financially supported by Directorate General (DG) XI of the EU.

The collaborative study: design

Seven laboratories (of which 6 national control laboratories) from different EU countries participated in the study (Annex I). First an informal pilot study was carried out in all participating laboratories to gain experience with the *in vitro* assay systems, to evaluate the study protocol and technical instructions and to pre-screen materials.

For the collaborative study groups of 10 rabbits and 10 guinea pigs were immunized with each of nine commercial single and multicomponent tetanus toxoid vaccines and one experimental preparation; a monovalent tetanus toxoid heated for 36 hours at 56°C. Vaccines selected, included a wide range of products, both with regard to formulation as with regard to adjuvant products used. A specification is given in

Table 1. Animals were immunized according to method A of the EP at one of the coordinating laboratories. For the study, both individually rabbit serum samples (n=100), mixed serum samples from pairs of two guinea pigs (n=50, 5 per vaccine) and pooled serum samples (n=20; one pool per vaccine and animal species) were used.

Participants were asked to perform the HA test, the Toxoid ELISA and the ToBI test in triplicate on different days, starting each with independently prepared dilutions of freshly made solutions. Descriptions of the methods used can be found elsewhere (Hendriksen et al., 1993). Each laboratory used the same standard operating protocol for performance of test. In the organizing laboratories the potency of the 20 pooled serum samples and of 10 rabbit and 10 guinea pig serum samples were also determined in duplicate in the *in vivo* TN test.

Each laboratory was provided with an equal part of the code-labelled serum samples and principle materials for the *in vitro* assays (i.e. microtiter plates, conjugates, tetanus toxin/toxoid, sensitized red blood cells). In addition, laboratories were supplied with rabbit and guinea pig reference serum, which were produced and calibrated at the organizing laboratories.

Raw data of the participants were elaborated at the organizing laboratories and intra- and interlaboratory variation in HA test, Toxoid ELISA and ToBI test was determined by one-way analysis of variance (anova). In addition *in vivo* data and *in vitro* data were used to estimate correlation.

The collaborative study: results

Only a selection of data will be presented here.

Large intralaboratory variation was seen with the HA test, statistical significant at $p < 0.05$. When triplicate HA tests in all participating laboratories are taken together it showed statistical significant variation in 30 out of 70 triplicate tests for guinea pig sera and for 19 out of 68 triplicate tests for rabbit sera. Intralaboratory variation was smaller for Toxoid-ELISA (14/70 and 15/70, respectively) and ToBI (15/70 and 10/70, respectively). Large intralaboratory variation in HA test most probably is related to optical reading of the test, which, to a certain extent, is subjective.

Table 1: Specifications of the tetanus toxoid preparationsused in the interlaboratory collaborative study

Tetanus toxoid	Adjuvant	Animal(s)
Combi/Influenza	Saponin	Horse
Combi/Influenza	Alum.Phosphate	Horse
Combi/Influenza	Alum.Hydroxide	Horse
Combi/Influenza	Alum.Hydroxide	Horse
Mono	Alum.Hydroxide	All species
Multi Clostridial	Alum.Hydroxide	Sheep
Multi Clostridial	Aluin	Sheep, pig, cattle
Multi Clostridial	Alum.Hydroxide	Sheep, goat, cattle
Multi Clostridial	Water/Oil	Sheep, goat, cattle
Mono (experimental)	Alum.Phosphate	–

For interlaboratory comparison estimates of potency of each vaccine obtained in one assay performance were combined using the arithmetic mean of individual serum samples. Thus for each vaccine and for each laboratory three values were obtained per *in vitro* test system. Thereafter interlaboratory variation was measured by one-way anova at $p < 0.05$ for each vaccine, resulting in 20 estimates (10 data of tests in rabbits and 10 of tests on guinea pigs) per *in vitro* test system. Interlaboratory variation was seen especially with the HA test (in 18 out of 20), but variation also frequently occurred for the ELISA (10/20) and ToBI test (8/20). However, in most cases variation was due to deviating results of one or two participating laboratories.

The antitoxin levels of the 40 serum samples obtained by TN test, were in the range of 2.6 IU/ml to 266 IU/ml. The relationship between these data and those measured by the *in vitro* serological assay systems is presented for one of the participating laboratories in Figure 1 (guinea pigs) and Figure 2 (rabbits). Good agreement was demonstrated in our study between the Toxoid-ELISA and TN test and between ToBI test and TN test (Table 2). Agreement between HA test and TN test was less pronounced. However, this might be due to the large variance in test results.

Conclusions and recommendations

From the results of the collaborative study it was concluded that Toxoid-ELISA and ToBI test can be used as a valid alternative to the *in vivo* TN test for the estimation of potency of tetanus toxoid for veterinary use according to Method A of the EP. Further standardization of the HA test will be needed before this test can be introduced in potency testing.

A major disadvantage of the Toxoid-ELISA is that marked overestimation of potency has been demonstrated at TN test antitoxin levels < 0.16 IU/ml, indicating that the Toxoid-ELISA only poorly discriminates between neutralizing and non-neutralizing tetanus antibodies. Although this is considered to be a major drawback for clinical studies, it is not for potency testing as minimum requirements for tetanus vaccines for veterinary use are well above the critical level in which significant overestimation of potency in Toxoid-ELISA might be expected. As potency calculations of serum samples in Toxoid-ELISA and ToBI test are based on parallel-line analysis, using dose-response (optical density) relation in immunoassay, differences in avidity between reference sample and test sample may interfere with estimation of serum potency. Avidity

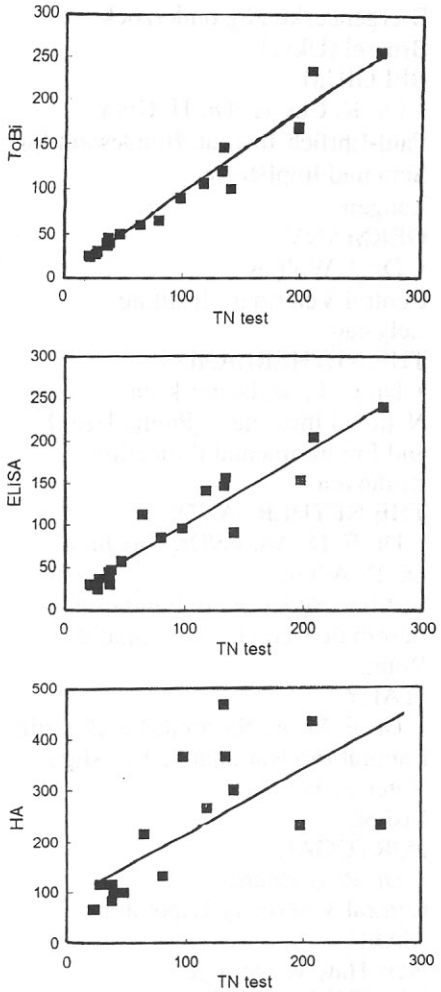

Figure 1: Correlation of *in vitro* test systems with *in vivo* TN test in the estimation of guinea pig samples.

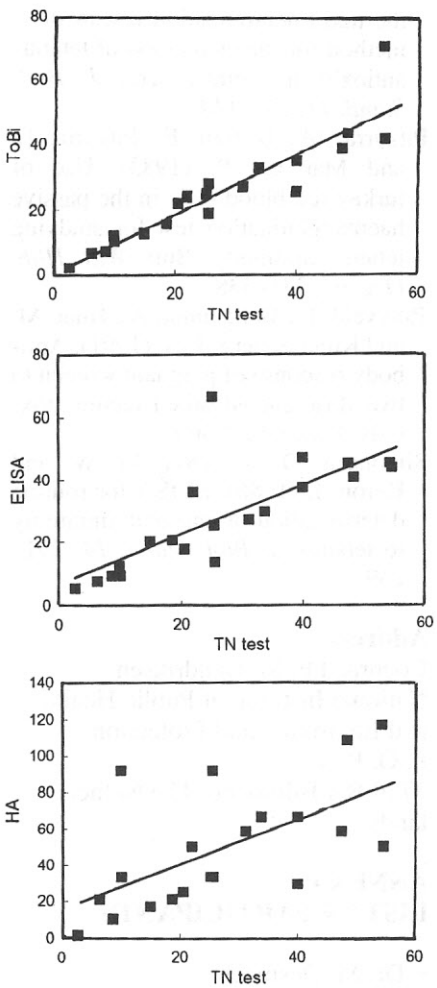

Figure 2: Correlation of *in vitro* test systems with *in vivo* TN test in the estimation of rabbit serum samples.

Table 2: Correlation coefficient of *in vitro* test systems with *in vivo* TN test in the estimation of serum samples (N=40) in seven laboratories.

in vitro	Laboratory						
test system	A	B	C	D	E	F	G
HA test	0.877	0.949	0.795	0.728	0.680	0.917	0.792
ELISA	0.965	0.957	0.955	0.968	0.804	0.924	0.957
ToBI test	0.909	0.971	0.933	0.981	0.916	0.876	0.983

problems are to be expected with the horse antitetanus reference serum currenly used. Therefore, two animal model homologous reference preparations; the guinea pig tetanus reference serum and the rabbit tetanus reference serum, were produced for the collaborative study. Implementation of *in vitro* serological test systems in EP requirements for veterinary tetanus toxoid potency testing should therefore go together with the production and calibration of two new reference tetanus antitoxin preparation; a guinea pig and a rabbit standard preparation. To anticipate on avidity problems, the quality of the reference preparations should be comparable to that of test sera. Initiatives for the production of these preparations should be taken by the EP.

Discussion

A rather surprising finding in the collaborative study was the estimated high potency, both in the *in vitro* tests and in the TN test, of the experimental toxoid preparation, heated for about 36h at 56°C, and therefore, expected to have an inferior quality. In an additional study, based on Method B of the EP, however, the potency was found to be low (135 IU/ml) and did not meet the minimum requirement for method B (> 150 IU/ml). This means that Method A and Method B gave conflicting results with regard to this experimental preparation. Apart from the effect of boostering and difference of animal species, no closely reasoned explanation can be given for this conflicting finding. Similar results have been described by Lyng (1992) and Relyveld et al. (1991) and prompts us to challenge the rational of two (qualitative) different methods (A and B) for the estimation of potency of tetanus toxoid for veterinary use. Considering the well known effect of the strain of mice (Hardegree et al., 1972; Lyng and Nyerges, 1984) on the estimation of potency according to Method B, preference is given to Method A and to omit Method B in Monograph 697. Information, relating estimates of potency obtained by Method A or Method B to protecting antibody levels in the target species would scientifically underpin such a decision.

References

Council of Europe. (1990) Vaccinum tetani ad usum veterinarium. *European Pharmacopoeia*, 697.

Habermann, E. and Dreyer, F. (1986). Clostridial neurotoxins: handling and action at the cellular and molecular level. *Curr. Top. Microbiol.*

Immunol. 129, 93–179.
Hardegree, M. C., Pittman, M. and Maloney, C. L. (1972). Influence of mouse strain on the assayed potency (unitage) of tetanus toxoid. *Applied Microbiology 24, 1*, 120–126.
Hendriksen, C. F. M., Van der Gun, J. W., Nagel, J. and Kreeftenberg, J. G. (1988). The toxin binding inhibition test as a reliable *in vitro* alternative to the toxin neutralization test in mice for the estimation of tetanus antitoxin in human sera. *J. Biol. Stand. 16*, 287–297.
Hendriksen, C. F. M., Woltjes, J., Van de Gun, J. W., Akkermans, A. M., Marsman, F., Verschure, M. H. and Veldman, K. (1993). Interlaboratory validation of *in vitro* serological assay systems for potency testing of tetanus toxoid vaccines for veterinary use. *Biologicals,* in press.
Lyng, J. (1992). EP/DT study on *in vitro* methods for estimating antibodies to diphtheria and tetanus toxoid. An international collaborative study. *WHO document, BS/92.1689.*
Lyng, J. and Nyerges, G. Y. (1984). The second international standard for tetanus toxoid (adsorbed). *J. Biol. Stand. 12*, 121–131.
Melville-Smith, M. E, Seagroatt, V. A. and Watkins, J. T. (1983). A comparison of enzyme-linked immunosorbent assay (ELISA) with the toxin neutralization test in mice method for the estimation of tetanus antioxin in human sera. *J. Biol. Stand. 11*, 137–144.
Pitzurra, M., Bistoni, F., Pitzurra, L. and Marconi, P. (1983). Use of turkey red blood cells in the passive haemagglutination test for studying tetanus immunity. Bull. *Wld. Hlth. Org. 61*, 331–338.
Relyveld, E., Bengounia, A., Huet, M. and Kreeftenberg, J. G. (1991). Antibody response of pregnant women to two different adsorbed tetanus toxoids. *Vaccine 19*, 369.
Simonsen, O., Bentzon, M. W. and Heron, I. (1986). ELISA for routine determination of antitoxic immunity to tetanus. *J. Biol. Stand. 14*, 231–239.

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