

Replacement of the *in vivo* Neutralisation Test for Efficacy Demonstration of Tetanus Vaccines *ad us. vet.*

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Summary

The bacterium *Clostridium (C.) tetani* is an ubiquitous pathogen. This anaerobic, gram-positive bacterium can form spores and can be found in the whole environment. It enters the body via injuries of the skin and wounds where it releases the neurotoxin "tetanospasmin" (= tetanus toxin). The animals most susceptible to tetanus infection are horses and sheep. Only active immunisation by tetanus vaccine provides effective protection against tetanus intoxication.

The marketing authorisation requirements stipulate that efficacy of tetanus vaccines *ad us. vet.* must be demonstrated in all target animal species via determination of neutralising tetanus serum antitoxin concentrations. The standard method used for this purpose is still the toxin neutralisation test (TNT), as it quantifies the tetanus toxin-neutralising effect of tetanus serum antibodies *in vivo*. In this test, tetanus toxin is added to dilutions of serum from vaccinated horses and sheep. The serum dilutions are then administered to mice or guinea pigs, which are observed for toxic symptoms.

Against the background of animal protection, the goal of one project of the Paul-Ehrlich-Institut (Bundesministerium für Bildung und Forschung (Federal Ministry for Education and Research), 0312636) was to establish an alternative to the toxin neutralisation test, enabling the testing of efficacy of tetanus vaccines with serological *in vitro* methods. For this purpose, a so-called double antigen ELISA (DAE) was established which enables the testing of sera of different species in one assay. In addition, the sera were tested in an indirect ELISA for horses and sheep separately.

Altogether, ten groups of horses and eight groups of sheep were immunised with ten animals per group each. The tetanus vaccines comprised almost all products authorised for the German market at the start of the project.

564 horse sera and 257 sheep sera were tested using the two ELISA methods. Some sera were also tested *in vivo*. The kinetics of antibody responses were recorded. The *in vitro* DAE method seems to be suitable to replace the mouse neutralisation test used for the detection of tetanus antitoxin in sera of target animal species. The comparison of some sera in the ELISA and the TNT showed good equivalence of results. Nevertheless, before an ELISA titre in horse and sheep sera indicating unambiguous protection against tetanus can be fixed, further comparative assays of low titre sera in the TNT and the DAE will have to be performed.

Zusammenfassung: Ersatz des *in vivo* Neutralisationstests zum Wirksamkeitsnachweis von Tetanusimpfstoffen *ad us. vet.*

Das Bakterium *Clostridium (C.) tetani* ist ein ubiquitärer Krankheitserreger. Das anaerobe, gram-positive Bakterium ist ein Sporenbildner und gelangt über Hautverletzungen und Wunden in den Organismus. Dort setzt es das Neurotoxin „Tetanospasmin“, auch bekannt als Tetanustoxin, frei. Die empfänglichsten Tierarten für Tetanus sind das Pferd und das Schaf. Nur die aktive Immunisierung mit Tetanus-Impfstoffen *ad us. vet.* bietet einen sicheren Schutz gegenüber einer Tetanusintoxikation.

Im Rahmen der Zulassung von Tetanus-Impfstoffen *ad us. vet.* müssen Versuche zum Nachweis der Belastbarkeit des Impfschutzes bei allen Tierarten, bei denen die Impfstoffe eingesetzt werden sollen, durchgeführt werden. Dies wird oft noch anhand eines Toxinneutralisationstests (TNT) praktiziert. Dazu werden Serumverdünnungen geimpfter Pferde bzw. Schafe mit Tetanustoxin versetzt und Mäusen oder Meerschweinchen appliziert, die dann auf Tetanus spezifische Symptome hin beobachtet werden.

Ziel eines Projektes des Paul-Ehrlich-Instituts (Bundesministerium für Bildung und Forschung, 0312636) war es, unter dem Aspekt des Tierschutzes eine Alternativmethode zum TNT zu finden, die es ermöglicht, die Wirksamkeit von Tetanus-Impfstoffen im Zieltier mittels serologischer *in vitro* Methoden zu prüfen. Dazu wurde ein so genannter Doppelt-Antigen ELISA (DAE) etabliert, der es ermöglicht, Seren verschiedener Spezies in einem Ansatz zu prüfen. Zusätzlich wurden die Seren in einem indirekten ELISA jeweils für Pferd und Schaf separat untersucht.

Zehn Gruppen für das Pferd und acht Gruppen für das Schaf mit jeweils zehn Tieren pro Gruppe wurden immunisiert. Die Tetanus-Impfstoffe umfassten fast alle Produkte, die zu Beginn des Projektes auf dem deutschen Markt zugelassen waren.

Insgesamt konnten 564 Seren vom Pferd und 257 Seren vom Schaf mit den beiden ELISA Methoden untersucht werden. Einige der Seren wurden parallel auch *in vivo* überprüft. Die Kinetik der Antikörperentwicklung konnte erfasst werden. Der DAE scheint als *in vitro* Methode zum Ersatz des TNT geeignet zu sein. Der Vergleich einiger Seruntiter im ELISA und im TNT zeigte gute Übereinstimmungen. Ein eindeutig gegenüber Tetanus schützender ELISA Titer im Pferd bzw. Schaf kann erst nach weiteren Vergleichsuntersuchungen niedrig-titriger Seren im TNT und DAE fixiert werden.

Keywords: *C. tetani*, Tetanus toxoid vaccines, replacement animal test, mouse neutralisation test, European pharmacopoeia, license, alternative methods, double antigen ELISA, tetanus antitoxin titres

1 Introduction

Active vaccination of horses against tetanus has been available since 1931 (Jansen und Knoetzke, 1979). Tetanus vaccines rank among the so-called toxoid vaccines, since inactivation of the immunogenic components, i.e. tetanus toxin (tetanospasmin), is performed by treatment with formaldehyde which induces toxoid formation. Immunisation using the toxoid provides effective protection against disease. In veterinary medicine, such vaccines are used commonly for horses, and also sheep, mainly because of their sensitivity against tetanus.

Within the scope of marketing authorisation procedures for veterinary tetanus vaccines, efficacy studies in the target animal species must be performed. The legal basis for these studies can be found in the *European Pharmacopoeia* (*Ph.Eur.*, Monograph 0697) and the Directive 2001/82/EC (Directive 2001/82/EC). This includes the following tests:

- Efficacy of the vaccine in the target animal at a minimum vaccination age
- Onset of immunity after primary immunisation
- Duration of immunity
- Status of immunity in the target animal after repeat vaccination
- Efficacy for each target animal species and target animal category (e.g. pregnant animal, young animal) separately and
- Efficacy compared with all antigens contained in the vaccine separately.

Efficacy testing of tetanus vaccines *ad us. vet.* occurs indirectly, since direct challenge tests in horses or other target species have so far not been performed for reasons of animal welfare.

For the demonstration of efficacy of tetanus vaccines *ad us. vet.*, a serological standard method has been used so far, i.e. the toxin neutralisation assay (TNT), equivalent to the previous Method A of Monograph 0697 used for batch potency testing of tetanus vaccines *ad us. vet.* (last described in the 4th edition *Ph.Eur.* 2002:0697). For this *in vivo* assay, serum dilutions of vaccinated horses or sheep

are mixed with a standardised tetanus toxin concentration, and administered to laboratory animals (mice or guinea pigs). The neutralising tetanus antitoxin titres thus obtained are compared with the protective tetanus antitoxin titres described in the literature. These are to 0.01 International Units (IU) for horses and 0.1 IU for sheep (Löhner and Radvila, 1970; Radvila and Löhner, 1965; Liefmann, 1980; Gräßer, 1981). The TNT involves a great deal of suffering for the laboratory animals, since they undergo tetanus intoxication. Half the animals die during the assay which extends over a period of several days.

The revised *Ph.Eur.* monograph (*Ph.Eur.*, 1/2003:0697) stipulates in the Section "Immunogenicity": "It shall also be demonstrated for each target species that the vaccine, administered by the recommended route, stimulates an immune response consistent with the claims for the product (for example, induction of antitoxic antibodies or induction of protective levels of antitoxic antibodies)." For potency testing of vaccine batches, serological *in vitro* methods were included in the monograph.

The goal of the present project (*Bundesministerium für Bildung und Forschung* (Federal Ministry of Education and Research), 0312636) from the point of view of animal protection was to establish a

suitable *in vitro* method for serological testing of the efficacy of tetanus vaccines in the target animal.

At the start of the project, the aim was to test all tetanus vaccines authorised for veterinary use in Germany. External co-operation partners could be found for the project, and, except for one horse vaccine, all products marketed in Germany were tested. The studies were carried out over a period of up to two years.

The so-called double antigen ELISA (DAE) was chosen and established as the standard ELISA method used in this project. This method, described by Aggerbeck et al. (1996) and Kristiansen et al. (1997), showed good correlations with established *in vivo* toxin neutralising assays with over 200 human sera tested. Thanks to the test design, sera could be examined independent of the animal species. In addition, one indirect ELISA for the horse and one for the sheep were established to test the sera in a second system. The test design is shown in Table 1. Some of the sera were also tested in parallel by TNT by two external laboratories for the verification of their neutralising tetanus antitoxin concentration *in vivo*.

For both *in vitro* test systems, the DAE and the indirect ELISA, validation was performed including important parameters like test specificity, accuracy, preci-

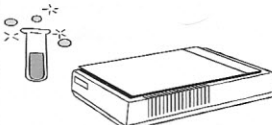
Serum of horses or sheep	
with animal test	without animal test
	
Injection of a mixture of horse or sheep serum dilutions with tetanus toxin into mice or guinea pigs. Reference group with standard preparation of tetanus antitoxin (analogous with the previous method A of the monograph for batch potency testing)	Direct examination of horse or sheep sera regarding the tetanus antitoxin content by means of ELISA.
Determination of the efficacy by means of the survival rate in comparison with the reference group	Determination of the efficacy by comparison with an International Standard or reference serum with a standardised tetanus antitoxin content in IU.

Fig. 1: Efficacy testing of tetanus vaccines *ad us. vet.* for marketing authorisation procedures

sion, linearity and analytical sensitivity (quantification limit).

2 Animals, material and methods

2.1 Animals

2.1.1 Horses

Ten vaccine groups of ten animals each were formed. The animals' ages ranged between four and five months, and they had not previously received tetanus immunisations. The vaccines were tested at three studs. The animals at the stud Radegast exclusively belonged to the breed 'Saxony-Anhalt'. The animals immunised at the stud Marbach were Arabs, Württemberg, Trakehner and black-forest cold blood. The stud at Meura consisted entirely of Haflinger.

2.1.2 Sheep

The group size also consisted of ten animals per vaccination group. Eight groups were formed. The vaccines were tested at two facilities. The Institute for Tropical Animal Hygiene at Göttingen tested five vaccines. The animals tested belonged to the following breeds: Schwarzkopf, Rhön sheep, Chamoise and Wilshire horn. The three remaining vaccines were tested on Schwarzkopf sheep at the experimental farm Marienfelde in Berlin.

2.2 Material

2.2.1 Vaccines

The vaccines used for both animal species were coded using a number. Horse vaccines were preceded by a P, so that products were given the codes P1 to P10. In the same way, sheep vaccines were given the codes S1 to S8.

2.2.1.1 Horse vaccines

Four pure tetanus toxoid vaccines were examined; one product was tested twice, but at different studs. The vaccines differed regarding their tetanus toxoid content, the adjuvant and their content of inactivation agents and preservatives. The six combination products tested included influenza antigen as additional component. These products also differed in their tetanus toxoid content, the influenza strains, adjuvants, and preservatives.

Tab. 1: ELISA principles

double antigen ELISA	indirect ELISA
5) substrate: TMB/H ₂ O ₂	4) substrate: TMB/H ₂ O ₂
4) streptavidin, peroxidase labelled	
3) tetanus toxoid, biotinylated anti-horse or anti-sheep	3) conjugate (peroxidase labelled)
2) addition of serum: species independent horse or sheep	2) addition of serum: specific
1) solid phase: tetanus toxoid	1) solid phase: tetanus toxoid

2.2.1.2 Sheep vaccines

Two monovalent vaccines and six vaccines, which were combinations with one or more additional antigens, were tested in the sheep. These included antigens of *C. chauvoei*, *C. haemolyticum*, *C. novyi*, *C. perfringens*, *C. septicum*, *Mannheimia haemolytica* and *Pasteurella trehalosi*. These vaccines differed in the type of adjuvant, the inactivation agent and/or preservative as well.

2.2.2 Sera

2.2.2.1 International WHO Standard "Tetanus-Antitoxin"

One ampoule is indicated to contain 1400 IU. The freeze-dried antitoxin was initially diluted to 2 IU per ml in phosphate buffer/PBS, portioned (1 ml) and stored at -20 °C.

2.2.2.2 NIBSC Standard "Tetanus-Antitoxin" (3rd British Standard)

1 ml horse serum containing 230 IU tetanus antitoxin. The standard was initially diluted to 5 IU, portioned at 0.5 ml each, and stored at -20 °C.

This standard was used to verify the tetanus antitoxin concentration of 2 IU of the WHO standard dilution in the DAE.

2.2.2.3 Internal reference serum horse

The reference serum was obtained from two vaccine groups. For this purpose, blood collections from animals fourteen days after the 2nd immunisation (third blood collection) were used. The reference serum was calibrated in the DAE against the WHO standard dilution of 2 IU. After freeze-drying, it was stored at 1.5 ml each at 2°-8 °C.

2.2.2.4 Negative serum horse

Foetal horse serum #06618-203 from PAA, Germany, was used. Aliquots of 0.5 ml each were filled, freeze-dried and stored at 2°-8 °C. The serum was tested *in vivo* for its tetanus antitoxin content.

2.2.2.5 Internal reference serum sheep

Serum samples of the third blood collection were used to prepare the reference serum (after two immunisation procedures). Sera from six groups of vaccines were included in the serum pool. Calibration was performed in the DAE with the WHO standard. After freeze-drying, 1.5 ml aliquots of the serum were stored at 2°-8 °C.

2.2.2.6 Negative serum sheep

The serum pool was prepared from individual sera of unvaccinated lambs, portioned, freeze-dried and stored at 2°-8 °C. The serum was tested for its tetanus antitoxin content *in vivo* as well.

2.2.3 Tetanus toxoid

Tetanus toxoid, batch no. #8501, RIVM, The Netherlands, was used. It contains 150 Lf/ml tetanus toxoid and is a purified tetanus toxoid for the production of vaccines for both human and veterinary use. It complies with the requirements of *Ph.Eur.*

2.2.4 Biotin-marked tetanus toxoid

For the production of biotin-marked tetanus toxoid, the toxoid mentioned in Section 2.2.3 and Biotin-X-NHS Kits (#203187) by Calbiochem, purchased via Merck were used. Glycerol was added to the biotinylated toxoid 1+1 and stored at -20 °C.



2.3 Methods

2.3.1 Immunisation and bleeding procedures

2.3.1.1 Immunisation and bleeding procedure – horse

The animals received the vaccinations according to the manufacturers' specifications. Primary immunisation with two injections was carried out at an interval of 4 weeks (in one case 6 weeks). The animals received additional primary immunisations and booster vaccinations at the intervals specified by the manufacturer, where possible. Blood was collected on the day of the first injection (day zero) to record the initial serological status of the animals. The second blood collection was performed on the day of the second injection (as a rule four weeks after the first injection) and a third 14 days later. In the following period, blood was collected half-yearly. For additional primary and/or booster vaccinations, blood was collected from the animals on injection day, and where possible, 14 days later. In three groups, blood was collected eight times, corresponding to an observation period of two years. In the remaining groups, blood was collected between five and seven times.

2.3.1.2 Immunisation and bleeding procedure – sheep

The animals received primary immunisation consisting of two injections at a six-weekly (for one product four-weekly) interval. Route of administration, dosage, and injection interval complied with the manufacturers' specifications. The first blood collection was performed simultaneously with the first injection, the next on the day of the second injection, and a third 14 days later. Of eight vaccine groups in total, six were bled three times each. In one group, it was possible to perform the first booster immunisation one year after the second primary immunisation; however the remaining stock consisted of six animals by then. At that time, a fourth blood collection was carried out, and a fifth blood collection 14 days after the repeat immunisation. In another group, blood could still be collected from four animals one year after primary immunisation.

2.3.1.3 Serum samples of horses and sheep

The blood samples were centrifuged (2000 g; 10 min) to collect the animals' serum. All samples were portioned and frozen at -20 °C. The samples were labelled with the name of the vaccine, name of stud or institute, name or number of the animal, and the date and number of the blood collection.

2.3.2 ELISA

2.3.2.1 Double antigen ELISA (DAE) horse and sheep – principle

The principle of the DAE is that of an indirect sandwich ELISA. The tetanus toxoid (antigen) is bound to the surface of a microtitre plate. In the second step, the antibodies contained in the serum bind to the antigen. In this step, it is possible to examine sera of different species. Then, an incubation step is performed with biotinylated tetanus toxoid. In another step, streptavidin (peroxidase conjugated) is added. The substrate hydrogen peroxide and the chromogen tetramethylbenzidine (TMB) make the reaction visible and the extinction is measured at 450 nm.

The International WHO standard (equine) with a defined tetanus antitoxin concentration in IU, one negative horse or sheep serum for system control and a conjugate control are applied to each plate. The WHO standard and the test sera are titrated using seven dilution steps in double determinations. The result is calculated by comparing the test sera with the WHO standard (detailed information on the test instructions can be obtained from the author).

2.3.2.2 Indirect ELISA horse and sheep – principle

In this assay, tetanus toxoid is applied to the microtitre plate as well. In the second step, serum antibodies are bound to the antigen. These, again, are detected by a peroxidase-marked secondary goat anti-horse antibody for the detection in horse sera or donkey anti-sheep for the detection in sheep sera. The substrate and stop reactions are equivalent to those described for the above ELISA.

The standard used is an internal reference serum from horse or sheep on each

microtitre plate. A negative horse or sheep serum serves as control. A conjugate control is also performed. Titration of the internal reference serum and test sera is analogous with the DAE. The result is calculated by comparing the test sera with the homologous internal reference serum calibrated in the DAE against the WHO standard.

2.4 Statistical methods

The data were evaluated using CombiStats, Version 1.0 (EDQM, 2000). The program, among other things, also tests the requirements for the parallel model (linearity and parallelity), and calculates the relative potency as compared with predefined standards. It fulfils the specifications for statistics contained in the *European Pharmacopoeia* (Ph.Eur. 5.3.).

3 Results

Altogether, nine vaccines for horses (but ten vaccine groups, since one product was tested at two different studs), and eight vaccines for sheep were tested in groups of ten animals each (original group size). Blood was collected five to eight times in the horse groups. This corresponds to a period of up to two years. Within the sheep groups, blood was collected three times each for six groups. Additional collections were performed in two of the groups. As a result, 564 horse sera and 257 sheep sera were tested for their tetanus antibody content, both in the DAE and in the indirect ELISA.

All four ELISA systems distinguished well between positive sera and the corresponding negative sera and therefore their specificity could be demonstrated. The quantification limit for the indirect ELISA horse and sheep and the DAE sheep was 0.0002 EU. For the DAE horse a limit of 0.00014 EU was given. Precision (repeatability, intermediate precision, reproducibility) and linearity could be demonstrated as well.

The tetanus antitoxin status of all animals was recorded at the beginning of the study with the first blood collection, which was carried out simultaneously with the first immunisation. The animals' responses to the vaccinations could be de-

tested in the respective serum samples using both test systems. Exemplary results of one horse group (P1) and one sheep group (S2) are shown in the Figures 2 and 3, respectively. For the group P1 a mild increase was shown after the first immunisation (2nd blood collection = bc), and after the second injection an increased immune reaction (booster effect) was shown at the third bc. The decrease in the titres over time could be observed in the blood collected between the vaccinations (4th bc, 6 months after 2nd vaccination), and a new sharp increase after a further vaccination (6th bc, two weeks after 3rd vaccination). High antibody titres of more than 7 ELISA units/ml on average were detectable even six months later. For vaccine group S2 again a mild increase of antitoxin titres was visible after the first vaccination. The booster effect of the second vaccination was apparent in the values of the samples of the third blood collection.

Verification of some of the sera *in vivo* via TNT tended to result in good equivalence. However, only a small portion of serum samples could be examined so far compared with the total (the results of these investigations will be published elsewhere). The tests were carried externally by two marketing authorisation holders where the different sera were tested by one laboratory each.

4 Discussion

For the batch potency testing of tetanus vaccines *ad us. vet.*, which was originally performed as immunisation-challenge experiments or tests including immunisation and serology with TNT in small laboratory animals such as guinea pigs or mice, alternative *in vitro* serological methods were included in the *Ph.Eur.* monograph 01/2003:0697. These methods include a so-called toxin binding inhibition test (ToBI) (Hendriksen et al., 1988), which detects tetanus toxin binding antibodies in the test sera on the basis of its test structure. The test thus produces results very close to those obtained by the *in vivo* TNT test. Trials of the ToBI test showed that this test was not suitable for the purposes of the present project. Pre-incubation of the sera and transfer to a second plate is

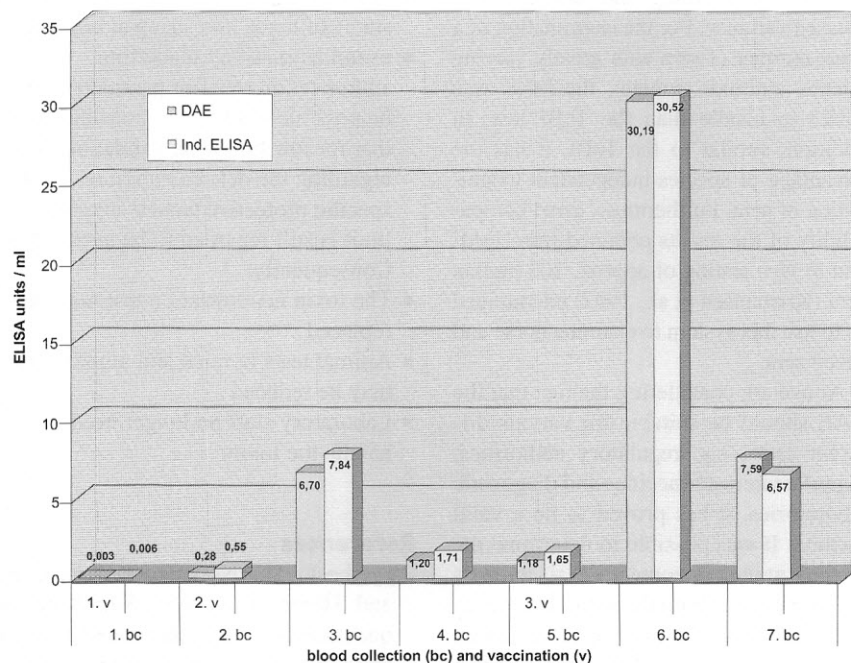


Fig. 2: Antitoxin titre curve (median values of ten sera per blood collection) of vaccine group P1. Results, DAE and indirect ELISA

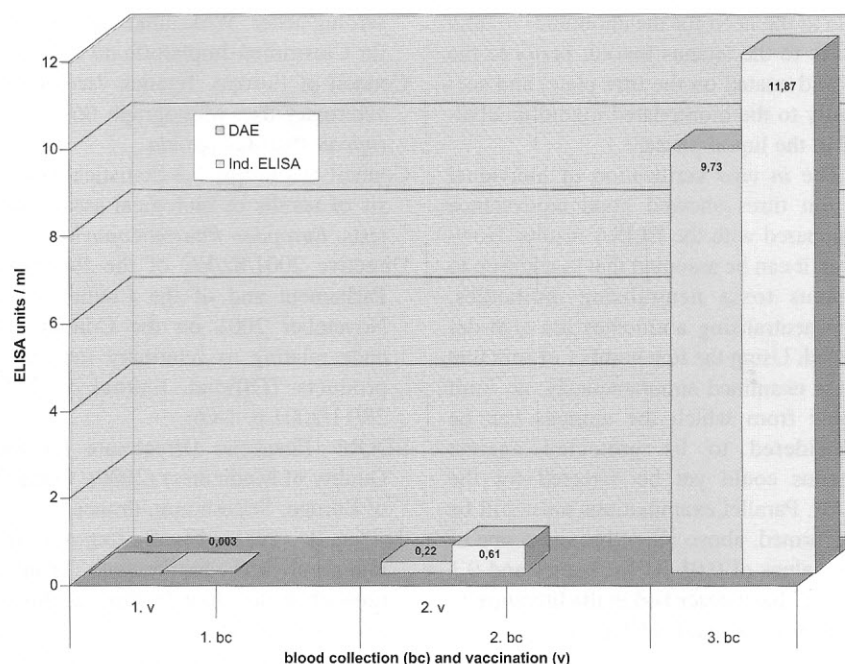


Fig. 3: Antitoxin titre curve (median values of ten sera per blood collection) of vaccine group S2. Results, DAE and indirect ELISA



very time-consuming and results in increased variance. For the examination of a great number of sera with greatly varying tetanus antitoxin content, the DAE was easier to handle than the ToBI test. In addition, similar to the ToBI, it has the advantage of species-independent examination of sera. Furthermore, good comparability of the results obtained from DAE and *in vivo* testing of approx. 200 human sera (Kristiansen et al., 1997) encouraged us to use this system to examine horse and sheep sera.

Above all, considering the fact that the DAE should be suitable for various different users, e.g. regulatory authorities, manufacturers of vaccines and diagnostic laboratories, it has proven to be a valid method. It was possible to determine the tetanus antitoxin content of all animals over time using both the DAE and the indirect ELISA. Concerning low serum titres, slightly larger deviations were found in both test systems. Over all, the indirect ELISA seems to overrate sera. This observation is also described by Kristiansen et al. (1997). Based on its assay structure, the DAE is more specific, due to the need for the antibodies to bind twice to the tetanus toxoid: firstly to the toxoid coated on the titre plate, and secondly to the biotinylated toxoid incubated in the liquid phase.

The *in vivo* verification of individual serum titres showed good equivalence compared with the ELISA results. However, it can be assumed that in addition to tetanus toxin neutralising antibodies, non-neutralising antibodies are also detected. Using the low number of sera that were examined simultaneously, no limit value from which the animals can be considered to be protected against tetanus could yet be detected for the DAE. Parallel examinations must still be performed, above all within the scope of the values of 0.01 IU for horses and 0.1 IU for sheep described in the literature to be protective against tetanus.

5 Conclusions

In conclusion, we can state that the double antigen ELISA is characterised by:

- An easy-to-use test set-up
- Species-independent usability

- detectability of the tetanus antitoxin status of horse and sheep at any time
- examination of all marketing authorisation relevant parameters may be possible (taking into consideration that for low titre sera, validation data regarding the determination of a test-specific protective tetanus antitoxin limit is still regarded to be necessary) Consequently:
- The toxin neutralisation test may be replaced
- Animal tests in mice and guinea pigs may be reduced
- Laboratory staff no longer need to handle the toxin.

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