Quality Assurance of *C. perfringens* Epsilon Toxoid Vaccines – ELISA *Versus* Mouse Neutralisation Test

Ute Rosskopf-Streicher, Peter Volkers, Kerstin Noeske and Esther Werner
Paul-Ehrlich-Institut, D-Langen

Summary
Clostridium (C.) perfringens is a Gram-positive anaerobic spore-forming bacterium. Disease caused by C. perfringens infection is called enterotoxaemia. C. perfringens strains are classified on the basis of the lethal exotoxins formed by the bacteria. Epsilon toxin is one of the major lethal toxins and is formed by C. perfringens types B and D.
C. perfringens is an ubiquitous bacterium. Infection occurs via food, water, animal litter or soil. Affected animals include mainly sheep, pigs and cattle. C. perfringens infection manifests as pulpy kidney disease and diarrhoea in suckling lambs. Enterotoxaemia development is peracute in most cases. Animals die suddenly while grazing on the pasture, without any prior signs of disease. Therefore, treatment is possible only in very rare cases.
Suitable immunoprophylactic measures are the treatment of choice to combat the disease: Vaccines and immunosera have therefore been used extensively for a long time.
The requirements for quality, efficacy and safety testing of the inactivated vaccines are laid down in the Ph. Eur. in the monograph: Clostridium perfringens vaccines for veterinary use. After a marketing authorisation is attained, the product batches must be tested in laboratory animal models for their potency against all vaccine components (Pharmeuropa, 1997).
For potency testing (batch control) of C. perfringens types B and D, the induction of specific antibodies against epsilon toxin in rabbits must be verified. For this purpose, 10 rabbits are immunised twice with the product to be tested. Their blood is taken 14 days after the last immunisation and the serum is pooled. The pooled serum is then tested for its protective effect. This is done by means of the toxin neutralisation test in mice (optionally also in guinea pigs) in comparison with an international reference serum. The evaluation criterion is the death rate of the mice in the test and reference groups after administration of lethal doses of epsilon toxin. The exact potency of the test serum is given in International Units (IU). The tested serum must show a minimum content of 5 IU.
This in vivo method requires a very high number of experimental animals. Approximately 400 mice (or 50 guinea pigs) are used per vaccine batch.
The monograph for C. perfringens vaccines, which has recently been revised, expressly indicates that a validated serological method may be used for batch testing. In addition, a reference

Zusammenfassung: Wirksamkeitsprüfung von *C. perfringens* Epsilontoxoid-Impfstoffen – ELISA *versus* Mausneutralisationstest
Clostridium (C.) perfringens ist ein grampositives Bakterium und anaerober Sporenbildner. Krankheiten, die durch C. perfringens Vergiftungen entstehen, werden als Enterotoxämien bezeichnet. Anhand der letalen Exotoxine, welche die Bakterien bilden, erfolgt die Typendifferenzierung von C. perfringens. Das Epsilontoxin ist eines dieser letalen Haupttoxine, die durch C. perfringens des Typs B und D gebildet werden.
Der Erreger ist ubiquitär zu finden, eine Aufnahme erfolgt über das Futter, Wasser, Einstreu oder Erdboden. Betroffen sind vor allem Schafe, Schweine und Rinder. Die Erkrankung äußert sich beim Schaf durch die sogenannte Breinierenkrankheit und Durchfälle bei Sauglämmern.
Der Verlauf der Enterotoxikämie ist in den meisten Fällen perakut, die Tiere verenden ohne vorherige Krankheitserscheinungen plötzlich auf der Weide und sind daher therapeutisch nur in sehr seltenen Fällen behandelbar.
Geeignete immunprophylaktische Maßnahmen sind das Mittel der Wahl bei der Bekämpfung der Krankheiten: Impfstoffe und Immunsera werden daher schon seit langem in großem Umfang eingesetzt.
Die Anforderungen zur Qualität, Sicherheit und Wirksamkeit an die inaktivierten Impfstoffe sind im Europäischen Arzneibuch in der Monografie: Clostridium perfringens Impfstoffe für Tiere festgeschrieben. Die Produkte müssen nach Erteilung einer Zulassung chargenweise in Labortiermodellen auf ihre Wirksamkeit gegenüber allen Impfstoffkomponenten überprüft werden.
Für die Wirksamkeitsprüfung (Chargenprüfung) von C. perfringens Typ B und D ist die Induktion spezifischer Antikörper in Kaninchen gegen das Epsilontoxin zu belegen. Hierfür werden zunächst 10 Kaninchen mit dem zu prüfenden Präparat zweimalig immunisiert, das Blut der Tiere 14 Tage nach der letzten Impfung entnommen und das Serum gemischt. Dieses Mischserum wird dann auf seine schützende Wirkung überprüft. Dies erfolgt mittels Toxinneutralisationstest in Mäusen (wahlweise auch Meerschweinchen) im Vergleich zu einem internationalen Referenzserum. Auswertungskriterium ist die Todesrate der Mäuse in den Prüf- und Referenzgruppen nach Verabreichung letaler Dosen von Epsilontoxin. Die genaue Wirksamkeit des Prüfimpfstoffes wird in Internationalen Einheiten (IE) angegeben. Das geprüfte Serum muß einen Mindestgehalt von 5 IE aufweisen.

Received 18 December 2003; received in final form and accepted for publication 17 February 2004

serum known as clostridium multicomponent serum has been available since 2000. The objective is to test vaccine batches against this reference and by means of a competitive ELISA developed in the precursor project, using a monoclonal antibody for direct determination of specific antitoxins in rabbit sera.
This ELISA method was subjected to an international validation to verify whether the protocol and the precision can be transferred within and between the participating laboratories.

Diese in vivo Methode ist mit einem sehr hohen Verbrauch an Versuchstieren verbunden. Pro Impfstoffcharge werden ca. 400 Mäuse (oder 50 Meerschweinchen) benötigt.
Die zwischenzeitlich überarbeitete Monografie für C. perfringens Impfstoffe weist ausdrücklich darauf hin, daß eine validierte serologische Methode für die Chargenprüfung verwendet werden kann. Seit dem Jahr 2000 steht auch ein Referenzserum, ein sogenanntes Clostridium-Multikomponentenserum, zur Verfügung. Mit dieser Referenz und mit der im Vorläuferprojekt entwickelten Testmethode eines ELISAs unter Verwendung eines monoklonalen Antikörpers zur direkten Bestimmung spezifischer Antitoxine in den Kaninchenseren sollen zukünftig Impfstoffchargen geprüft werden.
Dieser ELISA wurde einer internationalen Validierung unterzogen, um die Übertragbarkeit des Protokolls und die Präzision innerhalb und zwischen den teilnehmenden Labors zu überprüfen.

Keywords: C. perfringens vaccines, replacement animal test, competitive ELISA, biological reference preparation, validation study

1 Introduction

Diseases of *C. perfringens* intoxication are called enterotoxaemias. Five major toxins are produced by bacteria of the *C. perfringens* types. Epsilon toxin is one major lethal toxin formed by *C. perfringens* of the B and D types.

In sheep, the sickness manifests as diarrhoea in suckling lambs and so-called pulpy kidney disease. On the basis of the acute progression immunisation is an appropriate means to protect the animals in addition to avoiding a change of feeding.

Toxoid vaccines are commonly used for active immunisation. In Germany, four multivalent vaccines containing epsilon toxoid are licensed. For each batch of vaccine, quality, safety and efficacy must be demonstrated according to the European Pharmacopoeia (Ph. Eur.) monograph 0363: "*Clostridium perfringens* vaccine for veterinary use" (Pharmeuropa, 2002).

Therefore, 10 rabbits are immunised twice with the test product, bled two weeks later, and the sera are pooled. The potency (induction of specific antibodies) is measured by comparing the quantity of the serum necessary to protect mice (or other suitable animals) against a fixed dose of toxin with the quantity of reference serum required. A content of 5 IU epsilon antitoxin per millilitre of *C. perfringens* vaccine of types B and D is prescribed. The revision of the monograph explicitly favours validated serological methods for controlling vaccine batches above the *in vivo* test (see Tab. 1).

With the production of a specific monoclonal antibody against epsilon toxin and the development of an ELISA for the quantification of antibody induction in vaccinated rabbits by Elvira Ebert (Ebert et al., 1998), a first important step was taken towards replacing the mouse neutralisation test. The next step was the supply of the "Clostridia rabbit (multicomponent) antiserum BRP batch 1" by the European Directorate for the Quality of Medicines (EDQM), allowing the evaluation of test sera by means of this reference, which has a defined content on epsilon antitoxin of 11 IU (Lucken et al., 2001).

2 Animals, material and methods

2.1 Laboratory animals

Rabbits of the breed "White New Zealand" were purchased from Charles River, Kisslegg, Germany. Body weight was in the range of 1,800 g, and age was between 3 and 6 months at the time of immunisation. Animals were kept in floor husbandry and were fed with raw vegetables and commercial pellets; water was available *ad libitum.*

2.2 Vaccines

Four *C. perfringens* epsilon toxoid vaccines of different compositions were in-

Tab. 1: Potency test of *C. perfringens* toxoid vaccines

up to now	in the future
2-fold immunisation of 10 rabbits test category: low exposure	2-fold immunisation of 10 rabbits test category persisting
mouse neutralisation test (*in vivo*) test category: severe suffering	serology (*in vitro*) test category replaced
Amount of animals per batch: • 10 rabbits	Amount of animals per batch: • 10 rabbits
• approx. 400 mice or 50 guinea pigs	• no more mice or guineapigs

cluded (Tab. 2). All vaccines are combination products and are licensed for the German and European markets.

2.3 Immunisation procedure

Each vaccine was administered to 10 rabbits as required in the monograph "*Clostridium perfringens* vaccines for veterinary use". The animals received one dose as stated on the label and a second dose after four weeks. Blood was taken two weeks after the second immunisation by cardiac puncture under anaesthesia. The samples were centrifuged (6,000 g for 15 min), and equal volumes of individual sera of one vaccine group were combined to produce a serum pool. Vials with 1 ml serum each were freeze-dried and stored at 2°-8°C.

2.4 Reference serum

The Clostridia rabbit (multi-component) antiserum BRP Batch 1 was used as reference with an assigned activity of 11 IU *C. perfringens* epsilon antitoxin per vial.

2.5 Negative serum

The negative control serum was prepared by taking blood from unvaccinated rabbits. The status of the animals was specified pathogen free (spf). The serum preparation was performed as described in 2.3.

2.6 Monoclonal antibody

The monoclonal antibody (mAb) 5B7 against epsilon toxin was generated in hybridoma cells at the Paul-Ehrlich-Institut cultured *in vitro* (miniPERM). In a serum neutralisation test (SNT) using MDCK cells, no cytopathogenic effect (CPE) was observed after addition of trypsin-activated epsilon toxin at a dilution of 1:6,400 of the hybridoma supernatant. Protection was also shown in a mouse challenge test: The supernatant, diluted 1:10, was mixed separately with 10 different toxin concentrations each. The mice were protected even against the highest amount of toxin (270-fold LD50) (Ebert et al., 1999).

2.7 ELISA

The applied method is an indirect competitive ELISA. First, the epsilon toxin is used as antigen to coat the multi-well plates. In a second step, the serum samples are titrated and pre-incubated. Without washing the plate, the purified mAb 5B7 against epsilon toxin is added as competition against the polyclonal sera being tested. In a further step, peroxidase labelled goat anti-mouse IgG binds to the mAbs. The reaction is visualised by addition of tetramethyl-benzidine/hydrogen peroxide as substrate. Controls included on each plate are a negative serum and

Tab. 2: Vaccine constituents

Antigen (No. of vaccines)	Adjuvant (No. of vaccines)	Preservative (No. of vaccines)
C. perfringens type B (4)	Aluminium hydroxide (2)	Thiomersal (3)
C. perfringens type C (4)	Alaun (1)	Formaldehyde (2)
C. perfringens type D (4)	Emulsified Oil (1)	
C. chauvoei (3)		
C. septicum (3)		
C. novyi type B (3)		
C. novyi type D (1)		
C. tetani (3)		
E. coli (1)		
Mannheimia haemoloytica (1)		
Pasteurella trehalosi (1)		

The numbers in brackets are representing the amount of vaccines in which the constituent is to find e.g.: *C. perfringens* type B (4) indicates, it is included in all used vaccines.

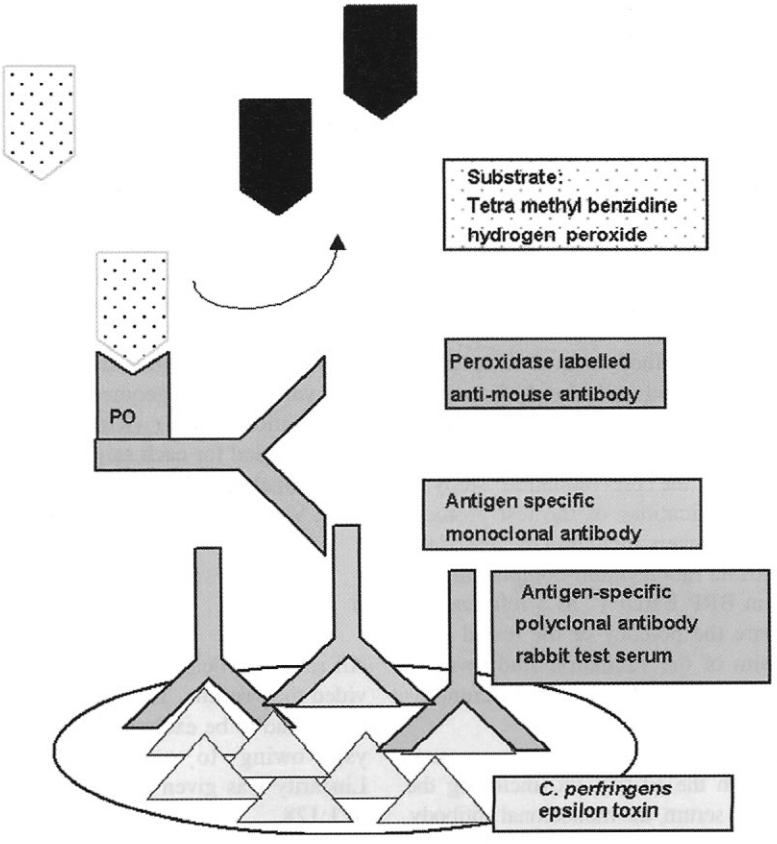

Fig. 1: Principle of the indirect competitive ELISA.

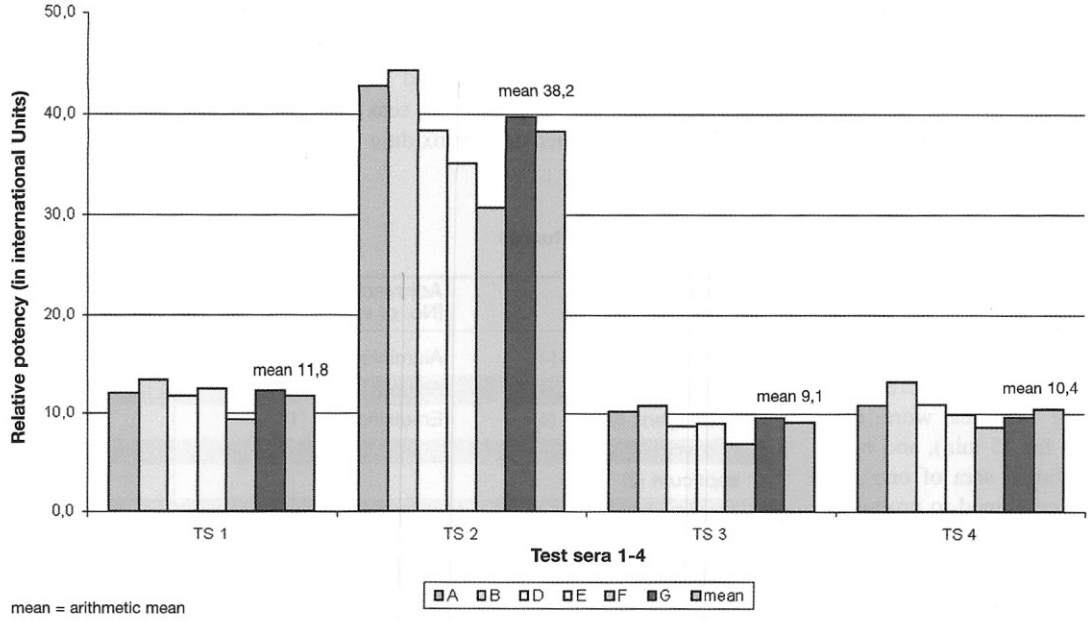

Fig. 2: Relative potency values calculated for each test serum, representing the mean value of four measurements in each laboratory. Required content per millilitre vaccine: 5 IU.

the conjugate control. The tested sera are compared with the reference serum (clostridia rabbit antiserum, BRP Batch 1) also titrated on each plate. Figure 1 shows the principle of the test.

2.8 Study design

The study was performed in seven laboratories of different international authorities. In order to assure anonymity, each laboratory was coded with a capital letter: Laboratory A to Laboratory G.

The test method was developed by Elvira Ebert and had already been pre-validated in four laboratories (Ebert et al., 1999). For the validation study – in contrast to the pre-validation study – some modifications of the test protocol were undertaken, including the use of the "Clostridia rabbit (multi-component) antiserum BRP Batch 1" as a reference to compare the potency of the tested sera. The aim of the validation study was to prove reproducibility within different international laboratories.

Therefore, the participants were supplied with the ELISA kit, including the reference serum, the monoclonal antibody, the test sera, the control sera, the conjugate, skim milk powder as well as the multi-well plates and mylar sealing tapes.

The participants were asked to test each of the four test serum samples four times on four different days (in order to evaluate the inter-day precision).

2.9 Statistical methods

The potency of the test preparations was calculated by analysing individual assays as parallel line assays (Finney, 1978), comparing the response with log concentration. If necessary, a square-root transformation was applied to the response in order to obtain better linearity. For assessment of the intra- and inter-laboratory variation, the geometric coefficient of variation (GCV) (Kirkwood, 1979) was provided for each sample.

All analyses were performed using SAS, Version 8.2 (SAS, 1999-2001).

3 Results

All seven participating laboratories provided their results. The data of one laboratory had to be excluded from the analysis owing to technical problems. Linearity was given at dilutions of 1:16 to 1:128.

The intra-assay precision coefficients of variation were found to be in the range of 10% up to 20%, depending on the laboratory. The test demonstrated good reproducibility between the different participants. Figure 2 shows the calculated potency values for the four test sera and their mean value. All sera indicated a potency above the required 5 IU. The results were very close. A greater variation was found only within the results for the product with the highest potency.

4 Discussion

The vaccines used for the study all clearly passed the potency requirements. This result was in agreement with the information given by the manufacturers. The induced potencies were in the range of 10 international units per ml for the vaccines 1, 3 and 4 (TS 1, TS 3 and TS 4). The Vaccine 2 induced much higher antibody titres as can be seen in the corresponding test serum 2. Like the other products as well, it is a multivalent vaccine containing different antigens. The details given on the package insert indicate a very high content of binding units of *Clostridium perfringens* epsilon toxoid. Therefore it could be concluded that the high content directly correlates with the increased antibody induction in the rabbit sera.

The validation study confirmed the transferability and reproducibility of the competitive ELISA and, for this reason, the applicability of testing vaccine batches for their content of epsilon toxin. A high specificity is given on the basis of the monoclonal antibody: This monoclonal demonstrated protection against epsilon toxin both in the cell test and in a mouse protection test. In the ELISA, the polyclonal rabbit sera must compete against the monoclonal, and the amount of displacement correlates with the efficacy of the test product. The second tool, the clostridia rabbit reference preparation with a defined content allows the calculation of the potency using a parallel line model.

For testing of one vaccine batch, the monograph prescribes two immunisations of ten rabbits four weeks apart. Fourteen days after the second immunisation, the animals are bled and equal blood volumes of each rabbit are blended in a serum pool. Investigations on the reduction of this number of animals unfortunately confirmed the necessity of the ten animals. However, sufficient blood for the ELISA can be obtained by puncture of the ear artery, so that killing of the animals is not necessary.

With the availability of this serological assay the use of the mouse neutralisation test will no longer be justified. Manufacturers as well as control authorities will be able to test the content of epsilon toxin in vaccines by this alternative method. The advantages are summarised as follows:

- Complete abolition of the mouse neutralisation test,
- reduction of time of exposure,
- no exposure with infectious material.

References

Ebert, E., Öppling, V., Werner, E. und Cussler, K. (1998). Entwicklung und Prävalidierung von Alternativmethoden zur Wirksamkeitsprüfung von Clostridium perfringens-Impfstoffen. *ALTEX 15, Supplement*, 59-61.

Ebert, E., Oeppling V., Werner E. and Cussler K. (1999). Development and prevalidation of two different ELISA systems for the potency testing of Clostridium perfringens b- and e-toxoid containing veterinary vaccines. *FEMS Immunology and Medical Microbiology 24*, 299-311.

Finney, D. J. (1978). Statistical methods in biological assay (3rd Edition). London: Charles Griffin.

Kirkwood T. B. L. (1979). Geometric means and measures of dispersion. *Biometrics 35*, 908-909.

Lucken, R., Daas, A. and Esposito-Farese, M.-E. (2001). Collaborative study for the establishment of a European Pharmacopoeia Biological Reference Preparation for Clostridia antiserum for serological potency testing of clostridial vaccines for veterinary use. *Pharmeuropa Spec. Issue Biol. 2001 Feb*; 2000 (2), 65-87.

Pharmeuropa (1997). Clostridium perfringens vaccine for veterinary use. *Pharmeuropa vol. 9, No 4*, 648-651.

Pharmeuropa (2002). Clostridium perfringens vaccine for veterinary use, monograph 0363. *Pharmeuropa 2002 Jul 14.3*, 2250-2251.

SAS 1999 – 2001. SAS Institute Inc., Cary NC.

Acknowledgements

The study was supported by the German Ministry of Education and Research (project 0312636). We would like to thank EDQM for contributing the Clostridia rabbit antiserum Ph. Eur. BRP 1, and we are very grateful to the participants for their participation and co-operation in this study. We also thank Melanie Schindler for performing the tests and preparing the study.

Correspondence to

Ute Rosskopf-Streicher
Paul-Ehrlich-Institut
Paul-Ehrlich-Str. 51-59
D-63225 Langen
Germany
phone: +49-6103-77 7415
fax: +49-6103-77 1254
e-mail: rosut@pei.de