Control of *Clostridium perfringens* Vaccines using an Indirect Competitive ELISA for the Epsilon Toxin Component

Examination of the Assay by a Collaborative Study

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SUMMARY

Investigations on the replacement of the mouse neutralisation test for proving vaccine batches of *Clostridium (C.) perfringens* toxoid vaccines were performed since several years. The European Pharmacopoeia (Ph. Eur.) monograph *Clostridium perfringens* vaccines for veterinary use (0363) is prescribing a potency test by immunisation of rabbits and checking the induction of specific antibodies against the toxins in a mouse neutralisation test. Since the monograph was revised, immunochemical methods are favoured to detect directly specific antibodies in the rabbit sera.

An indirect competitive ELISA using a monoclonal antibody was established at the Paul-Ehrlich-Institut for the detection of antibodies against the epsilon toxin component of *C. perfringens*. It was revised using the Clostridia rabbit antiserum Ph. Eur. Biological Reference Preparation (BRP) Batch 1 as reference serum. With a defined content of 11 International Units (IU) of *C. perfringens* epsilon antitoxin this reference serum enables the calculation of the potency of rabbit sera under test.

For the collaborative study vaccine products of different composition licensed for the German and European markets were used. Seven international laboratories were included. Aim was to make a prediction on the transferability and precision of the test method.

The results showing a satisfactory intermediate precision and transferability of the test confirmed the applicability of the ELISA method for the batch control of *C. perfringens* vaccines. Therefore a replacement of the mouse neutralisation test is available.

KEYWORDS

*C. perfringens* type B and D; *C. perfringens* toxoid vaccines; enterotoxaemia; pulpy kidney disease; competitive ELISA; replacement animal test; *in vitro* assay; mouse neutralisation test; Clostridia rabbit antiserum Ph. Eur. BRP; monoclonal antibody; parallel line assay.

ABBREVIATIONS

BRP: Biological Reference Preparation; C.: *Clostridium*; CI: Confidence interval; CPE: Cytopathic effect; CV: Coefficient of variation; EDQM: European Directorate for the Quality of Medicines; FPLC: Fast performance liquid chromatography; Int. ref: Internal reference; GCV: Geometric coefficient of variation; IU: International unit; LD<sub>50</sub>: 50 percent lethal dose; OD: Optical density; OMCL: Official medicines control laboratory; mab: Monoclonal antibody; PBS: Phosphate buffered saline; MNT: Mouse neutralisation test; SD: Standard deviation; SNT: Serum neutralisation test.

1. INTRODUCTION

Epsilon toxin is one lethal major toxin formed by bacteria of the *Clostridium (C.) perfringens* B and D types. Together with *C. perfringens* type C, they are the most common types encountered in veterinary medicine. The most susceptible species to infection are sheep, cattle and pigs. The toxin induces dysentery in lambs, necrotic enterotoxaemia in calves and piglets as well as pulpy kidney disease in sheep [1]. For active immunisation, toxoid vaccines are commonly used. In Germany, four multivalent vaccines containing epsilon toxoid are licensed. The requirements for quality, safety and efficacy of these vaccines are laid down in the European Pharmacopoeia (Ph. Eur.) monograph 0363: “*Clostridium perfringens* vaccine for veterinary use” [2].

The potency of these vaccines is measured in terms of their ability to induce specific antitoxins against epsilon toxin in the sera of vaccinated rabbits. The potency of the pooled serum obtained from the vaccinated rabbits is determined by comparing the quantity necessary to protect mice or other suitable animals against the toxic effects of a fixed dose of *C. perfringens* epsilon toxin with the quantity of a reference preparation necessary to give the same protection [3]. The pooled sera of rabbits immunised with *C. perfringens* types B and D vaccines must contain not less than 5 IU antitoxin per millilitre.

Investigations on the replacement of the mouse neutralisation test (MNT) were performed previously [4,5,6]. At the Paul-Ehrlich-Institut an ELISA based on a monoclonal antibody (mab) against *C. perfringens* epsilon toxin for quantification of antibody induction in vaccinated rabbits was developed and pre-validated [7]. The hybridoma cells for production of the highly specific antibody 5B7 were produced at the Paul-Ehrlich-Institut. The mab demonstrated neutralisation of the lethal effect of epsilon toxin in mice as well as toxin neutralisation in a serum neutralisation test with MDCK cells, where no cytopathogenic effect was visible [8].

Meanwhile, the “Clostridia rabbit (multi-component) antiserum BRP Batch 1” became available at the European Directorate for the Quality of Medicines (EDQM). The reference serum was included and now the method offers the possibility for replacement of the MNT [9].

2. AIM OF THE STUDY

The collaborative study was performed to assess the transferability and reproducibility of an ELISA based on a monoclonal antibody (mab) for quantification of rabbit anti-*C. perfringens* epsilon toxin antibodies.

3. PARTICIPANTS

Seven laboratories of different Official medicines control laboratories (OMCL) took part in the study (see section 9).
4. STUDY DESIGN, MATERIALS AND METHODS

4.1. Study design
The participants were supplied with the ELISA kit including the reference serum, the monoclonal antibody, the test and control sera, the conjugate, skim milk powder as well as the multi-well plates. They were asked to test each of the four test serum samples four times on four different days. The test sera were obtained by immunisation of rabbits with four *Clostridium perfringens* epsilon toxin vaccines of different compositions and produced by different methods. All vaccines were combination products, licensed for the German and European market. The batches used all fulfilled the requirement of at least 5 IU per ml.

4.2. Materials
The participants were supplied with the test protocol and the corresponding ELISA kit.

The following reagents were provided:
- Reference serum: 2 vials.
- Clostridia rabbit (multi-component) antiserum: 1 vial.
- BRP Batch 1, assigned activity: 11.0 IU *Clostridium perfringens* epsilon antitoxin per vial (EDQM catalogue reference C24244000).
- Monoclonal antibody 5B7: 1 vial.
- The hybridoma cells of the mab 5B7 against epsilon toxin were produced at the Paul-Ehrlich-Institut and cultured in *vitro* by using the miniPERM system from Heraeus. The supernatant was tested 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 1:6400 of the hybridoma supernatant. The protective effect was also proven in a mouse challenge test. The supernatant was diluted at 1:10 and mixed separately with 10 different toxin concentrations each. The mice were protected even against the highest amount of toxin (270-fold LD50) [7,8]. Afterwards, the culture supernatant was purified by Fast Performance Liquid Chromatography (FPLC).
- Antigen (Epsilon toxin): 2 vials.
- *Clostridium perfringens* type D (NCTC 0834604) was used for the culture preparation of the epsilon toxin. The culture supernatant was concentrated and dialysed against phosphate buffered saline (PBS). Aliquots of 650 µl were freeze-dried and stored at 2-8°C. The culture supernatant was kindly provided by Dr. Frauke Roth (University of Göttingen, Germany).
- Test sera (TS 1 to TS 4): 4 × 2 vials.
- Immunisation and blood sampling for the production of test sera were performed as follows. The immunisation was performed as described under Potency in the monograph “*Clostridium perfringens* vaccine for veterinary use” [2]. For each product ten rabbits of the race “White New Zealand” with a body weight of 1,800 gram aged between 3 and 6 months were used for testing. After an acclimatisation period, the animals received one dose as stated on the label, and after four weeks a second dose. Two weeks after the second injection, the rabbits were bled by cardiac puncture (with regard to the collaborative study to have enough samples for repeated testing). Blood samples (of individual rabbits) were incubated at room temperature for at least one hour and centrifuged for 15 minutes at 6,000 g to separate blood clots. Equal volumes of individual sera of one vaccine group were added to produce one serum pool. Later on, the serum was freeze-dried in aliquots of 1 ml. The vials were stored at 2-8°C.
- Negative serum: 2 vials.
- Rabbit serum, freeze-dried, 1 ml per vial.
- Positive serum (Int. Ref.): 3 vials.
- Rabbit serum aEps1a, freeze-dried, 0.5 ml per vial.
- Conjugate, anti-mouse IgG: 2 vials.
- Peroxidase labelled goat anti-mouse-IgG (H+L) (Dianova).
- Skim milk powder: 150 g (Difco).
- ELISA-plates: 12 plates.
- Multiwell plates (No. 655001, F-form, medium binding capacity, Greiner, Germany).
- Mylar sealing tapes: 12 tapes (TECAN, Germany).

4.3. Methods

4.3.1. ELISA methodology
A standardised ELISA indirect competitive method was evaluated in this study. The epsilon toxin is used as antigen for coating the multi-well plates. In a second step, the serum samples are titrated and pre-incubated. The purified mab 5B7 against epsilon toxin is added afterwards for competition with the polyclonal sera. In a further step, peroxidase labelled goat-anti-mouse IgG binds to the mabs. The reaction is visualised by addition of hydrogenperoxide/tetramethyl-benzidine as substrate. Controls included on each plate were a positive and negative serum and conjugate control. The sera under test were compared with the clostridia rabbit antiserum, BRP Batch 1, also titrated on each plate. The standard operating procedure used in the collaborative study can be provided upon request to the EDQM.

4.3.2. Statistical analysis
The potency of the test preparations were calculated by analysing individual assays as parallel line assays [10] and by comparing the response with log concentration. If necessary, a square-root transformation was applied to the response in order to obtain better linearity. Potencies were estimated by fitting parallel lines through all samples included in an assay simultaneously. Combined potency within a laboratory was calculated for each product (sample) across all assays using the (unweighted) geometric mean of the results from the individual assays within the laboratory. For each product, overall potency estimates were calculated as (unweighted) geometric mean of laboratory means. For assessment of the intra- and inter-laboratory variation, the geometric coefficient of variation (GCV) [11] was provided for each sample (overall as well as within each laboratory).

All analyses were performed using SAS, Version 8.2 [12].

5. RESULTS
For samples TS 1 and TS 2, results of a total of 28 assays, and for samples TS 3 and TS 4, results of a total of 30 assays from 7 laboratories were available. The total data set consisted of 4,872 readings (OD-values). Eighteen (0.37 per cent) of the readings had to be classified as pipetting or reading errors. Due to technical problems the data from laboratory C resulted in unreliable potency estimates. Therefore, laboratory C was excluded from further analysis on potencies. The individual potencies are listed in Table 1.
In order to achieve linearity, some of the assays had to be transformed using a square-root transformation. Responses at the extremes falling outside the linear range of the dose-response curves were omitted. In most cases, dilutions included in the analysis were in the range of 1:16 to 1:128. Tests of validity (linearity & parallelism) were performed at the 1 per cent significance level. Most problems with linearity or parallelism were caused by the internal control aEPS1a. Parallelism was rejected for one assay (lab, F, day 3, plate 1) and linearity was rejected in two assays (lab A, day 2, plate 2 and lab F, day 4, plate 1). Following a visual inspection of the data, the deviations did not seem to invalidate the results (the observed deviation from the regression lines revealed a very low variability). It was thus decided to include these assays in the analysis, despite the formal failure to prove validity.

Summary information for the different test substances is provided in Table 2 and Figures 1 and 2 which show that vaccines used for the collaborative study all induced antibody titres above the required 5 IU per ml. This is in accordance with the batch protocols, where all products fulfilled the release requirements.

Table 1 - Individual potencies expressed in IU per ml

<table>
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<tr>
<th>Lab</th>
<th>Day</th>
<th>Plate 1</th>
<th>Plate 2</th>
<th>Int. Ref.</th>
<th>Plate 1</th>
<th>Plate 2</th>
<th>Int. Ref.</th>
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<td>4.67</td>
<td>7.58</td>
<td>8.14</td>
<td>3.54</td>
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</table>

Relative potencies, assuming an assigned value of 11 IU/ml for the reference.
Int. Ref.: internal reference.

In accordance with the batch protocols, where all products fulfilled the release requirements.

Table 2 - Summary data on potencies (expressed in IU per ml)

<table>
<thead>
<tr>
<th>Sample</th>
<th>Lab</th>
<th>Potency</th>
<th>% of total</th>
<th>95% CI</th>
<th>GCV</th>
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<td>4.65 - 6.50</td>
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<td>127.25</td>
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<td>D</td>
<td>6.20</td>
<td>107.07</td>
<td>5.80 - 6.62</td>
<td>8.22</td>
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<td>E</td>
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<td>F</td>
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<td>4.86 - 5.63</td>
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<td>G</td>
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<td>92.31</td>
<td>4.01 - 7.11</td>
<td>40.70</td>
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Total 5.79 5.03 - 6.65 14.18

In order to achieve linearity, some of the assays had to be transformed using a square-root transformation. Responses at the extremes falling outside the linear range of the dose-response curves were omitted. In most cases, dilutions included in the analysis were in the range of 1:16 to 1:128. Tests of validity (linearity & parallelism) were performed at the 1 per cent significance level. Most problems with linearity or parallelism were caused by the internal control aEPS1a. Parallelism was rejected for one assay (lab, F, day 3, plate 1) and linearity was rejected in two assays (lab A, day 2, plate 2 and lab F, day 4, plate 1). Following a visual inspection of the data, the deviations did not seem to invalidate the results (the observed deviation from the regression lines revealed a very low variability). It was thus decided to include these assays in the analysis, despite the formal failure to prove validity.

Summary information for the different test substances is provided in Table 2 and Figures 1 and 2 which show that vaccines used for the collaborative study all induced antibody titres above the required 5 IU per ml. This is in accordance with the batch protocols, where all products fulfilled the release requirements.

For each test serum, the potency relative to the BRP reference serum (C242440) is given. The corresponding 95 per cent confidence interval (CI) and the estimated intra- and inter-laboratory variability, measured by the geometric coefficient of variation (GCV) are given.
Control of *Clostridium perfringens* Vaccines

Figure 1 - Distribution of the potency values relative to the BRP reference serum

Figures 2a-e show the estimated potencies in relation to the overall mean for that sample.
The intermediate precision of the extinction values for each combination of laboratory, test sample and dilution step, the pertinent mean, standard deviation (SD) and coefficient of variation (CV) were calculated. The data confirmed a good test precision (data not shown in this report).

6. DISCUSSION

In view of animal welfare and the large number of mice used in the mouse neutralisation test, the need for testing the potency of clostridial vaccines in an alternative way was given. In the report of Weisser and Hechler, one manufacturer estimated the number of mice required for potency testing of one batch to be 400 [3].

Previous projects investigated the possibility of a serological test system [7]. The present study confirmed the transferability of the test, no problems occurred during performing the assays. The reagents used seemed to be stable despite the shipment and dry ice storage (monoclonal antibody, conjugate and clostridia reference serum).

The availability of the antigen (C. perfringens epsilon toxin) and of a highly specific monoclonal antibody represented two essential components for the development of a serological test. Since 2000, a multi-componen clostridia rabbit antiserum with a defined potency of 11 IU of C. perfringens epsilon antitoxin per vial (and ml) was available as EDQM reference preparation [9]. By means of this reference, sera of rabbits immunised with the vaccine under test could be directly investigated for the quantification of antibodies against C. perfringens epsilon toxin.

The vaccines used in the validation study all fulfilled the requirements of the Ph. Eur. monograph on potency as shown in Figure 1. The products showed potencies mostly in the range of 10 IU, one product with a very high potency in the range of 38 IU/ml. Unfortunately, no product of less potency than the required 5 IU could be included in the validation study. Therefore, it has to be taken into account that manufacturers are advised to compare serum samples from rabbits immunised with low potency products (below the 5 IU) against the EDQM reference rabbit serum.

The use of an internal positive control serum does not seem to be necessary for future tests. This serum was produced in vitro and therefore the animals do not need to be sacrificed afterwards. As ELISA is a rapid, sensitive, specific, quantitative and a reproducible test method, which requires no expensive reagents or equipment and does not require the laboratory staff to handle infectious material this method should be preferred to the MNT.

To help manufacturers and OMCs to implement ELISA, it is foreseen to qualify and distribute the necessary mabs through the EDQM. Consequently, a rapid move away from MNT to serological testing by ELISA for batch release of C. perfringens vaccines should occur in the near future.

8. REFERENCES


Control of *Clostridium perfringens* Vaccines


9. PARTICIPANTS (LISTED IN ALPHABETICAL ORDER BY SURNAME)

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