

Potency Testing of Swine Erysipelas Vaccines by Serology – Results of a Pre-validation Study

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Summary

The European Pharmacopoeia (Ph. Eur.) monograph on Swine Erysipelas Vaccine (inactivated) (Ph. Eur., 1997) requires the potency of each batch to be demonstrated in a mouse protection test. In this parallel-line bioassay, mice are challenged with a virulent strain of Erysipelothrix (E.) rhusiopathiae after immunisation with different doses of either the standard preparation or of the test vaccine. More than one hundred animals are necessary for the routine testing of a single batch. In previous studies (Beckmann und Cußler, 1994; Roskopf-Streicher et al., 1998), we have shown that an indirect enzyme-linked immunosorbent assay (ELISA) may be used to quantify the humoral response of mice. This method can replace the challenge model for the purposes of potency testing in the following manner: Ten mice are immunised subcutaneously with 1/10 of the vaccine dose required for pig vaccination. After three weeks, the mice are bled under anaesthesia. Serum samples are pooled and the antibody content is compared to that of a reference serum.

In view of animal welfare the advantages of the alternative model are obvious: A highly reduced number of animals and the replacement of challenge exposure. This includes the discontinuation of the control group with a mortality rate of 100%.

A pre-validation study was initiated to evaluate the performance of the serological method. Eight laboratories used the test kits to evaluate pooled serum samples from vaccinated mice. Both the reagents and the test protocol were shown to be satisfactory. The intra- and inter-laboratory reproducibility that was achieved indicates that the method is a strong candidate for validation.

Zusammenfassung: Prüfung der Wirksamkeit von Rotlaufimpfstoffen durch Serologie - Ergebnisse einer Prävalidierung
Die Anforderungen an die Qualität der Rotlaufimpfstoffe sind im Deutschen Arzneibuch, 10. Ausgabe (DAB 10), festgelegt. Die Wirksamkeit muß derzeit in einem Tierversuch, der eine Belastungsinfektion an Mäusen einschließt, nachgewiesen werden. Für die Prüfung einer Impfstoffcharge sind mindestens 106 Tiere nötig. Das Ergebnis (Sterberate) dient zur Berechnung der Schutzkraft in Internationalen Einheiten (I.E.). In einem Entwurf zur Neufassung der Monographie von

Rotlaufimpfstoffen wird folgende Methode für die Chargenprüfung vorgestellt: Jeweils einer Gruppe von 10 Mäusen werden 1/10 der Zieltierdosis der Testvakzine bzw. des Referenzimpfstoffes verabreicht. Eine ungeimpfte Kontrollgruppe wird mitgeführt. Drei Wochen nach der Immunisierung werden die Tiere einer Belastungsinfektion mit einer virulenten Kultur von Erysipelothrix rhusiopathiae unterzogen. Die ungeimpften Tiere müssen innerhalb von 2 bis 5 Tagen an dieser Infektion sterben. Aus der Sterberate der geimpften Tiere läßt sich die Wertigkeit des Impfstoffes errechnen.

Die Infektionsversuche sind mit schweren Belastungen für die Tiere verbunden. In der Regel verenden mehr als 50% der Mäuse. Der Tierversuch erfordert den Umgang mit infektiösem Material und ist mit hohen Kosten verbunden. Zudem wird die Stabilität des Internationalen Standardpräparats in jüngster Zeit angezweifelt. Eine Ersatzmethode ist daher dringend erforderlich. Da die Immunität gegenüber der Rotlaufinfektion überwiegend humoral bedingt ist, können serologische Nachweissysteme herangezogen werden.

In einem Vorläuferprojekt wurde ein ELISA entwickelt (Gyra et al., 1994; Beckmann et al., 1994) und auf seine Einsetzbarkeit für die Chargenprüfung getestet. Es ist das Ziel dieses Anschlußprojektes, den ELISA zu optimieren und in einem Ringversuch zu validieren, um eine Akzeptanz als Ersatzmethode zum Belastungsversuch zu erreichen.

In Anlehnung an den Entwurf der überarbeiteten Monographie schlagen wir folgende Methode zur Chargenprüfung vor: 10 Mäusen wird 1/10 der Schweinedosis des zu testenden Impfstoffes s.c. appliziert. Anstelle der Infektion wird das Blut der Tiere 3 Wochen später unter Narkose gewonnen. Anschließend werden die Seren gepoolt und im ELISA auf ihren Antikörpergehalt geprüft. Dabei wird die Probe mit einem Referenzserum mit bekanntem Antikörpergehalt verglichen.

Das Referenzserum im Test wurde von uns durch Immunisierung von Mäusen mit 5 internationalen Einheiten des Rotlaufstandards (Rf 2) gewonnen. Die Auswertung erfolgte durch ein Software Programm, welches die relative Wirksamkeit des Testimpfstoffes zur Referenz bewertet.

Ziel dieser Prävalidierungsstudie war es, festzustellen, ob die ELISA Ergebnisse in verschiedenen Laboratorien reproduzierbar sind. Anwendbarkeit der Referenzmaterialien und des Testprotokolls sollten ebenfalls beurteilt werden.

Keywords: 3R, refinement, reduction, Erysipelas vaccines, batch potency test, mouse model, ELISA, pre-validation study

1 Introduction

Swine erysipelas is a bacterial disease of world-wide importance. Immunisation is very effective in preventing this infection. Vaccines have already been used for 50 years and are safe, efficient and easy to produce.

The requirements for the quality control of erysipelas vaccines are prescribed by the Ph. Eur. (1997). The potency has to be demonstrated using an animal challenge model. At least three dilutions of the International Standard and of the test vaccine are used to immunise separate groups of mice and an additional group of ten unvaccinated mice is also required. Three weeks after vaccination, all mice are challenged with a virulent culture of *E. rhusiopathiae*. The unvaccinated control animals have to die within five days of infection in order for the test to be valid. The potency of the test product is evaluated by comparing the survival rates (protection dose) of mice vaccinated with the Standard vaccine with those of mice given the test vaccine. The value obtained is expressed in International Units (IU).

At least 106 animals are required to test one batch of vaccine. This potency test uses a large number of animals and it is also an extremely severe procedure. Therefore, the highest priority should be given to the development and validation of alternatives (Hendriksen et al., 1998).

The mechanism by which erysipelas vaccines induce a protective immune response is clearly related to humoral immunity (Roth, 1982a). Thus serological methods which measure protective antibodies against *E. rhusiopathiae* are promising candidates to replace the challenge procedure. Recently we developed an ELISA, which is suitable for this purpose (Roskopf et al., 1998). The establishment of this *in vitro* method is based on data gathered in a pre-validation study involving eight laboratories of various types, e. g. Control Laboratories, manufacturers and academic institutions. Each of the participating laboratories was provided with the test kit and protocol.

2 Animals, material and methods

2.1 Laboratory animals

Female NMRI mice were purchased from Charles River, Kisslegg, Germany. The mice weighed 18-20g at the

time of immunisation. Animals were housed under conventional conditions in Makrolon cages (size 3) and were fed commercial pellets; water was available *ad libitum*.

2.2 Vaccines

Nine erysipelas vaccines were used, including both monovalent and combined products (see table 1). All vaccines contained serovar 2, while two products also contained serovar 1. Combined vaccines included a porcine parvovirus component. Three vaccines (V1, V2 and V3) were diluted with complete vaccine base by the manufacturer and only differed in their antigen content. Two vaccines (V6 and V7) were diluted with saline to obtain products of lower quality.

2.3 Procedure of immunisation

Each vaccine was administered to 50 mice (see 2.1.) in order to enable sufficient amounts of serum to be collected for the study. Mice received one subcutaneous vaccination of 1/10 of the pig dose. Three weeks later, the animals were bled under anaesthesia. Blood samples were incubated at room temperature for one hour and centrifuged for 2 minutes at 6000 g (Sarstedt micro tubes No 41.150.005) to separate out the blood clots. The sera from each group were pooled, aliquotted, coded and stored at -20° C.

2.4 Reference serum

Mouse reference serum was prepared by injecting each of 500 mice (see 2.1.) subcutaneously with 5 IU of the International Standard in a volume of 0.2 ml. The procedures for bleeding and serum preparation

were as described in 2.3.

2.5 Negative serum

The negative control serum was prepared using unvaccinated mice (see 2.1.). The procedures for bleeding and serum preparation were as described in 2.3.

2.6 Preparation of coating antigen

The *E. rhusiopathiae* mouse challenge strain, Frankfurt XI, serovar N was used to prepare the coating antigen (Moos, 1983). It was extracted with EDTA and alkaline treatment according to the method of Groschup et al. (1991). Briefly, wet bacteria were suspended in buffer (Tris HCl) containing EDTA (1mM) and incubated for half an hour. After centrifugation, the cell pellet was resuspended in 0.01M NaOH and incubated with constant stirring for eighteen hours. Following neutralisation with 2M HCl, the cells were removed by centrifugation. The supernatant was concentrated by filtration (Bottle top filter, 45 µm, Nalgene) and aliquotted into vials. Each vial contained 530 µg of protein. Antigenic components were separated by SDS-polyacrylamide gel electrophoresis according to the method of Laemmli (1970). Proteins were visualised with Coomassie blue. The major protective proteins of *E. rhusiopathiae* have molecular weights of 64 to 66 kDa and 40 to 35 kDa (Groschup et al., 1991; Lachmann and Deicher, 1986).

2.7 Study design

Eight laboratories participated in the pre-validation study. The participants are mentioned at the end of the report (Annex). The order of listing does not correspond

Table 1: Specifications of the inactivated erysipelas vaccines used in the pre-validation study

Vaccine (V) Serum (S*)	Mono (M) Combined (C)	Adsorbate (A) Lysate (L)	Comment
V1 (S1)	M	A	
V2 (S2)	M	A	Erysipelas antigen reduced (dilution of V1)
V3 (S3)	M	A	Erysipelas antigen reduced again (dilution of V2)
V4 (S4)	C	L	Parvoccomponent
V5 (S5)	C	A	Parvoccomponent
V5 (S5)	M	L	diluted with saline
V7 (S7)	M	A	diluted with saline
V8 (S8)	M	A	
V9 (S9)	M	A	

S* = serum pool S obtained by immunisation with the corresponding vaccine

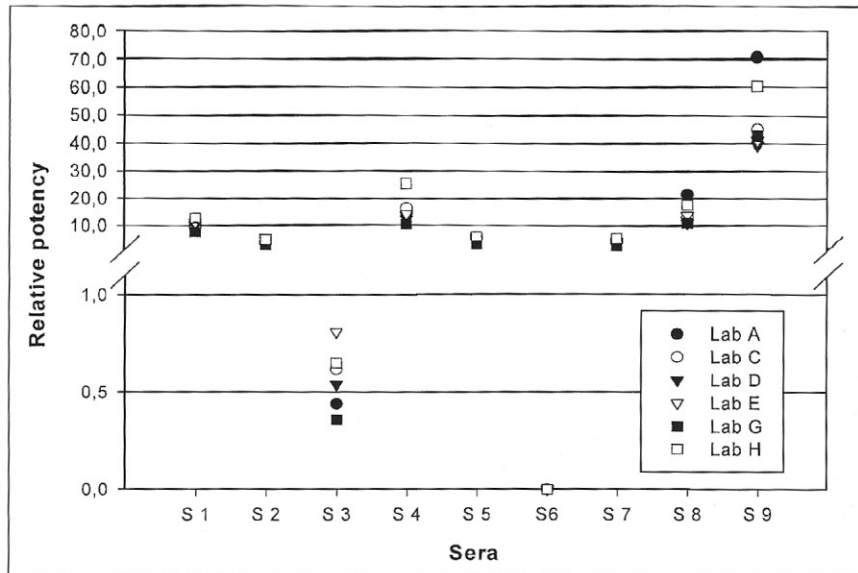


Figure 1: Distribution of the relative potency for each serum. Shown are the mean values of 3 measurements for each laboratory.

to the codes denoted throughout this report by capital letters (Laboratory A-H). Serum samples were coded (S1-S9) and were tested blind by the participants. Each laboratory was provided with the ELISA kit (sera, coating antigen, conjugate, substrate, buffer and microtitre plates) and the Standard Operating Procedure (SOP). The ELISA was performed as described by Rosskopf-Streicher et al. (1998). The plates were coated with antigen and then one reference and 3 test sera were applied to each plate. A negative serum control and conjugate control were also included on each plate.

The ELISA was carried out with nine test sera (see table 1) and repeated three times (to assess intra-laboratory variation). Test and reference sera were diluted in twofold steps (11 dilutions). In addition, each serum was diluted 1:1000 and plated into 22 wells (to evaluate intra-assay precision).

2.8 Data calculation

The data were expressed as Relative Potencies (RP) which compare the potency of the test preparation with that of a reference preparation (calculations performed using the Relative Potency Calculation Software, USDA) (Wilbur, 1993). The reference serum has the arbitrary value of 1. This value represents the pass mark for the test vaccines. Sera with a value ≥ 1 have the same or a higher potency than the reference serum

and pass the requirements. Sera with potencies lower than 1 fail in the test.

2.9 Statistical methods

Sources of variation in the relative potency results were assessed using a fixed effects linear model which took into account the serum tested, the laboratory which carried out the test and the day on which the test was conducted.

The variability between laboratories (reproducibility) and the day-to-day variation within a laboratory (repeatability) were calculated by comparing pairs of relative potencies. Lin's concordance correlation coefficient $\hat{\rho}_c$ (Lin, 1992) is an appropriate index to use in order to quantify the degree to which pairs deviate from total agreement, i.e. the 45° line through the origin ($\hat{\rho}_c = 1$).

3 Results

3.1 Data returned and data excluded

Results were received from all laboratories. The results from two laboratories (B and F) were not evaluated either because data were submitted too late, or because the values for the blanks and negative serum controls were not within the specified range (negative serum: optical density 0,060 and conjugate control: optical density 0,025). The only data that could be used from laboratory F was that relating to intra-assay precision.

3.2 Inter-laboratory reproducibility

Figure 1 shows the results of measurements performed on the various sera (mean value of day 1-3) by each laboratory. As described under 2.9, a vaccine will pass the test, when the serum RP is ≥ 1 . If the RP is below 1, a vaccine will fail the test and will not meet the potency requirements. All laboratories assessed sera S1, S2, S4, S5, S7, S8 and S9 as indicating a „pass“ and S3 and S6 as indicating a „fail“ in the test.

Table 2 illustrates the potency ranking of the vaccines according to the serological results. Number 1 indicates the product that induced the highest immune response and No 9 the product that induced the lowest response. There is good agreement between the results from different laboratories.

3.3 In vivo-in vitro comparison

The results for sera S1-S3 in the serological test system showed a gradation that related to the ranking of the respective vaccines (V1-V3) in the mouse-challenge-test (as assessed by the manufacturer). Vaccine 3 was quantified at 28 IU (2.8 IU per

Table 2: Ranking of the sera with a score of 1 to 9. 1 corresponds to the product inducing the highest immune response and 9 to that inducing the lowest response. Five sera were ranked in the same position by each laboratory. Four sera showed differences in only one of the assigned rankings.

Lab.	S1	S2	S3	S4	S5	S6	S7	S8	S9
A	4	7	8	3	5	9	6	2	1
C	4	7	8	2	5	9	6	3	1
D	4	6	8	2	5	9	7	3	1
E	4	6	8	2	5	9	7	3	1
G	4	6	8	3	5	9	7	2	1
H	4	7	8	3	5	9	6	2	1

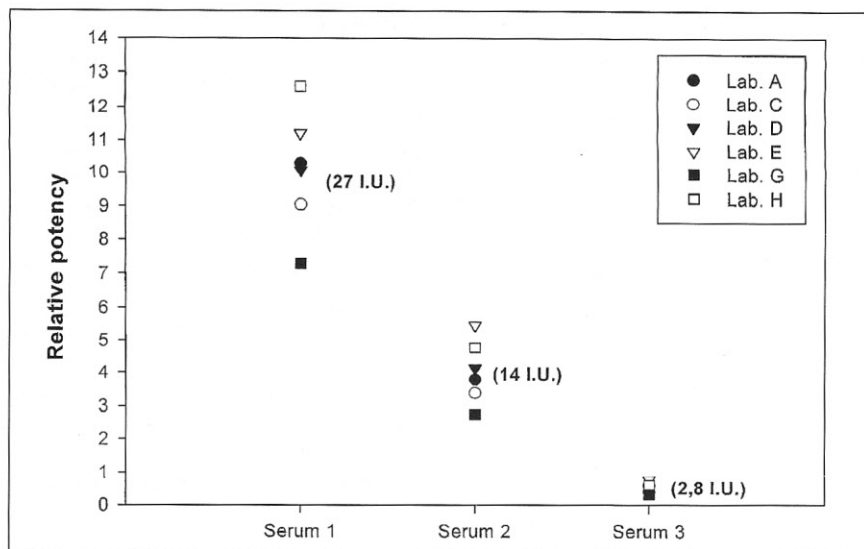


Figure 2: Three vaccines, which differed only in their antigen content, showed a gradation of results in the challenge test (values in brackets, 1/10 of the pig dose) as well as in the serological test. The vaccine that failed the animal test had an RP value of < 1 and therefore also failed in the serological test.

mouse dose). All laboratories reached the same conclusion in assigning an RP value below 1 for Serum 3.

Thus, the vaccine was demonstrated to have failed the potency requirements in both systems. The increasing antibody content of Serum 2 and Serum 1 was measurable. These data correlated with the increasing antigen content of vaccine 2 and vaccine 1. Vaccines 1 and 2, in the same way as sera 1 and 2, were shown to have potencies > 50 IU expressed in RP values as a multiple of 1.

This gradation in results provides the first demonstration of the correlation between the *in vivo* and the serological me-

thod (see fig. 2) and demonstrates the importance using the complete vaccine base for dilution.

Vaccines diluted with saline (V6 and V7) showed a high deviation from the IU gained in the animal test and those results obtained by serology. Antibodies induced by V6 (diluted from 260 IU per pig dose to 50 IU per pig dose) were extremely low and a calculation of the RP value was not possible. In contrast, V7 (diluted from 140 IU to a pig dose of 14 IU) showed RP values in the range of 3 to 4.

3.4 Intra-assay precision

In order to evaluate the precision within laboratories, each of twelve sera was diluted 1:1000 and repeatedly tested 22fold on one plate. Afterwards the mean value (MV) of the optical density, standard deviation (SD) and the coefficient of variation (CV) had been calculated for each serum (Formula: $CV = SD \times 100/MV$). So we gained twelve Cvs from the labs.

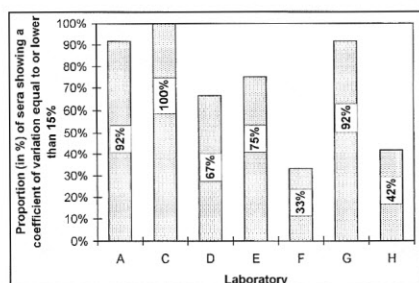


Figure 3: For the Intra-assay precision we compared the variation coefficients of twelve sera. Each serum had been diluted 1:1000 and tested in the ELISA 22fold by all laboratories. The mean value of the optical density, standard deviation and the coefficient of variation (CV) had been calculated for each serum separately. The columns represent the portion of sera (out of 12) with a CV lower than 15% in per cent.

The columns in figure 3 show the portion (in per cent) of sera (out of 12) with a CV equal or lower than 15% per laboratory.

3.5 Statistical analysis

An analysis of variance reveals that the source of variation in the relative potencies is due to the highly significant ($p < 0.001$) factors of test serum and laboratory. The factor relating to the day of testing, which is nested within the factor for the laboratory in the linear model, does not show any significant influence ($p = 0.058$) at $\alpha = 5\%$. The serum effect has to be significant, because the value of the ELISA lies in its ability to detect different antibody levels in sera. Multiple comparisons between all pairs of laboratories, using simulation-based simultaneous 95% confidence intervals, show a tendency to significant differences between those pairs which have laboratory A or laboratory H in common (see also table 3).

Multiple pairwise comparisons using Lin's concordance correlation coefficient demonstrate the reproducibility to be in the range acceptable to very good ($\hat{\rho}_c \approx 0.78$, table 3) and the repeatability to be in the range good to very good ($\hat{\rho}_c \approx 0.85$, table 4). The sole exception is the moderate agreement between days 2 and 3 in laboratory A, which was apparently due to results obtained for sera 8 and 9 ($\hat{\rho}_c = 0.62$, table 4).

4 Discussion

Vaccines prepared from *E. rhusiopathiae* serovar 2 have been successfully used to prevent swine erysipelas for more than 50 years. The vaccines induce protection in mice and swine challenged with different serovars of *E. rhusiopathiae*, including the most common isolates of serovar 1 and 2 (Takahashi et al., 1984).

The potency requirements for erysipelas vaccines are part of the Ph. Eur. (1997). The monograph requires a minimum an-

Table 3: Inter-laboratory (lab-to-lab) variation showing Lin's $\hat{\rho}_c$ for pairs of mean relative potencies from three consecutive days in each laboratory.

Laboratory	A	C	D	E	G
C	0.86				
D	0.78	0.98			
E	0.81	0.99	0.99		
G	0.83	0.98	0.99	0.98	
H	0.96	0.91	0.83	0.87	0.85

tigen content of 50 IU as determined in a mouse protection test. By definition, one IU of the International Standard protect 50% of the animals within a population of mice. More than 100 mice are needed in order to test the potency of a single batch of vaccine. At least half of the animals succumb from erysipelas during the test. Therefore, there is an urgent need for an alternative test system that avoids the challenge procedure and uses fewer animals.

Recently, a serological test system based on an antigen extract, which includes the protective antigen fraction of *E. rhusiopathiae*, has been developed (Rosskopf et al., 1998). However, the relevance of such new test systems as indicators of efficacy in pigs has to be demonstrated. Unfortunately, the relevance for the target species of the established laboratory animal challenge test has never been demonstrated. The IU value has been defined only in the mouse system. To bridge this gap, a challenge test has been carried out in swine using different doses of the standard vaccine. An injection of 50 IU protected pigs against virulent challenge (challenge dose: 10^6 cfu/0,1 ml of serovar 1 and 10^7 cfu/0,1 ml of serovar 2) (unpublished results).

A potency test should be able to confirm that the potency of a test vaccine batch is at least equivalent to the potency of a batch with demonstrated efficacy in pigs. Immunisation of mice with an antigen dose of 5 IU (1/10 of a pig protection dose) seems suitable for this purpose. A serum pool obtained from mice vaccinated with 5 IU is therefore a representative reference for calculation of the relative potency of a test product.

An alternative method must be fully developed and properly validated before it can be considered for regulatory use. However, apart from general guidelines on toxicological studies, little has been published on the validation of alternative methods in vaccine quality control. Recently, a workshop on validation studies for alternative methods for the potency testing of vaccines was held (Hendriksen et al., 1998). The criteria indicating the readiness of a test for inter-laboratory validation were defined:

- Test development in the laboratory of origin
- Pre-validation (informal inter-laboratory study)

Table 4: Intra-laboratory (day-to-day) variation showing Lin's $\hat{\rho}_c$ for pairs of relative potencies in each laboratory

Laboratory	A	C	D	E	G	H
day1 - day2	0.85	0.87	0.97	0.97	0.97	0.99
day2 - day3	0.62	0.88	0.90	0.98	0.99	0.97
day1 - day3	0.89	0.99	0.94	0.98	0.99	0.96

- Validation (formal inter-laboratory study)
- Assessment of study and proposals
- Progression towards regulatory acceptance

Pre-validation includes three main phases: protocol refinement, protocol transfer, and protocol performance (Curren et al., 1995). The informal inter-laboratory study carried out at the pre-validation stage involves assessing the inter-laboratory transferability of the method, undertaking any optimisation and standardisation of the protocol which may be considered neces-

sary, and identifying any unexpected problems with the test procedures. The objective of the pre-validation stage is to ensure that the method tested fulfils the criteria for inclusion in a formal validation study.

The requirements for pre-validation and the results of the erysipelas serological method are shown in table 5.

The aim of the pre-validation study was to prove the reproducibility in different laboratories of a serological method for the batch potency testing of erysipelas vaccines. To perform this study it was necessary to use a reference serum prepared by a

Table 5: Overview of criteria operating before and during the pre-validation study. The definitions refer to the guideline of VICH and the report and recommendations of ECVAM workshop 31 [Hendriksen et al., 1998 and VICH, 1997].

Criteria	Prevalidation	Comment
1. Protocol	+	Important for work according to GLP or GMP
1.1. Protocol transfer and performance	+	All laboratories were able to perform the test according to the protocol
1.2. Protocol refinement	+	The study lead to an improvement of the protocol
2. Precision		Expresses the closeness of agreement between a series of measurements of the same sample under prescribed conditions. Criteria: 2.1.-2.3.
2.1. Repeatability	+	Expresses the precision under the same conditions over a short interval of time (intra-assay precision).
2.2. Intermediate precision	+	Expresses variations within laboratories: different days, different analysts, different equipment, etc.
2.3. Reproducibility	+	Expresses the precision between laboratories, for the standardisation of a method
3. Linearity	+	The ability to obtain test results which are directly proportional to the concentration of analyte in the sample
4. Range	+	The interval between the upper and lower concentration of analyte in a sample has a suitable level of precision, accuracy and linearity. The range between 0,5 and 5 is important for the assessment of the RP using our method.
5. Preparation and providing of reference material	+	Coating antigen Reference serum Test sera

vaccine of known potency (5 IU). In future (European Pharmacopoeia Commission, 1999) companies should prepare in-house reference vaccines. However, due to our experience the use of an in-house reference serum would also be possible and reduce the number of animals necessary for the test performance.

The assay performed well when used according to the SOP. Minor changes to the protocol were suggested by the study participants. The reproducibility of results for the test sera, both within and between the laboratories, was demonstrated. There was good agreement between all participants regarding the potency ranking of the different sera (table 2).

Three vaccines with varying antigen content were evaluated by the manufacturer in a mouse challenge test (fig. 2, values in brackets). All laboratories were able to identify the batch of low quality and the gradation of the antigen content was reproducible. The other approach, which was to produce batches of inadequate potency by diluting the product with saline, failed. The two vaccines (V6 and V7) did not reveal the theoretically expected results. This demonstrates the importance of diluting a vaccine with the complete vaccine base (see 3.3.) in order to obtain results, which correlate with those from the *in vivo* model. In view of the revision of the Pharm. Eur. monograph (1999) this fact should be respected for the formulation of product specific reference material. The serological method is suitable for discrimination between vaccines inducing different levels of antibodies.

In conclusion, the ELISA for the detection of protective antibodies against *E. rhusiopathiae* fulfilled the requirements for a pre-validation study. Therefore, the test is suitable for in-process controls and for showing the consistency of the production process (cases where an internationally validated system is not necessary). Such a pre-screen for non-regulatory checks already offers the possibility to save many animals. However, a formal validation study will also be initiated so as to obtain international acceptance and the introduction into the Pharmacopoeia requirements of a serological method for the potency testing of swine erysipelas vaccines.

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