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Quality control of inactivated erysipelas vaccines: results of an international collaborative study to establish a new regulatory test

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Abstract

According to the specifications of the European Pharmacopoeia (Ph. Eur.) monograph (Swine Erysipelas Vaccine (Inactivated), Monograph no. 64, European Pharmacopoeia, 3rd edn., 1997) on erysipelas vaccines for veterinary use, batch potency is estimated in a multi-dilution assay after immunisation and infection of mice. Recently, we described a serological assay system (ELISA) which has the potential to replace this challenge-based model (Beckmann R, Cussler K. Wirksamkeitsprüfung von Rotlaufimpfstoffen an der Labormaus. ELISA kontra Infektionsversuch. ALTEX 1994;Suppl. 1:39–45; Rosskopf-Streicher U, Johannes S, Hausleithner D, Gyra H, Cussler K. Suitability of an ELISA for the batch potency test in laboratory mice. Pharmeuropa BIO 1998;1:65–70). The humoral immune response is quantified in pooled sera of ten mice three weeks after immunisation . The results are expressed as relative potency (RP) in comparison to a reference serum. After a pre-validation study had been performed with success (Rosskopf-Streicher U, Johannes S, Wilhelm M, Gyra H, Cussler K. Potency testing of swine erysipelas vaccines by serology — results of a pre-validation study. ALTEX 1999;16:123–8), we initiated an international collaborative study with five European manufacturers and seven regulatory authorities to validate the assay and model. All participants were provided with blind-coded erysipelas vaccines of different potencies, the ELISA kit and test instructions. The participants had to immunise mice, to prepare serum samples and to perform the ELISA. Inter-laboratory reproducibility was reported by the pass/fail criteria of the vaccines under test. Intra-laboratory precision was assessed by comparing repeated measurements on three consecutive days. Day-to-day variation within the laboratories was statistically analysed by comparing pairs of RPs using Lin's concordance correlation coefficient. The results show that the ELISA is indeed a suitable alternative to replace the vaccination-challenge test. Furthermore, this new model reduces the number of animals required for the potency test by ~80%.

Keywords: Erysipelas; Inactivated vaccines; Batch potency test; Challenge model; ELISA; Validation study

1. Introduction

Swine erysipelas is a bacterial disease of great economic importance and world-wide distribution. The causative organism is *Erysipelothrix rhusiopathiae*, a slim gram-positive rod. Most isolates from clinical cases belong to serovars 1 and 2 [5]. Vaccination is a most efficient means to prevent the disease in animals. Inactivated products (aluminium hydroxide-adsorbed cultures or lysate extracts) are widely used [6,7]. They are usually prepared from cultures of serovar 2 and induce cross-protection to the most relevant serovars of *E. rhusiopathiae* [8,9].

In Germany, currently ten monovalent erysipelas vaccines or products combined with other antigens are licensed. The quality control tests for batch release are prescribed in the Ph. Eur. [1]. The potency is tested by a virulent challenge in mice. Briefly, three dilutions of the test vaccine and three dilutions of a standard vaccine are administered to groups of 16 mice, each. Three weeks later all animals (including a control group of ten mice) are challenged with 100–1000 LD_{50} of a virulent strain of *E. rhusiopathiae*. Potency is calculated by

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comparison of the survival rate within the reference and the test groups [1]. US regulations require a similar mouse protection test [10].

In Europe, at least 10 000 mice are annually used to perform this potency test. However, the actual figure may be much higher than the calculated minimum number of animals stipulated by the monograph, since animals are also used for titration of the challenge dose, and additional tests are performed during the vaccine shelf-life [11]. From the viewpoint of animal welfare, the high number of animals needed for each test (at least 106 mice) and the severe suffering caused by the challenge procedure stresses the urgent need for an alternative [12].

However, before a test can be considered for routine use, a proper development regarding the basis of the method and its applicability for the intended purpose must have taken place. Furthermore, the provision of a protocol and the definition of a prediction model for the interpretation and application of the results are the prerequisites for evaluation of the method in a pre-validation or validation study [13].

On the basis of investigations characterising protective antigen structures of *E. rhusiopathiae* [14,15], we developed an enzyme-linked immunosorbent assay (ELISA) to measure erysipelas antibodies in mice [2,3]. After a successful pre-validation study to evaluate protocol transfer, protocol performance and protocol refinement [4], the model to replace the challenge test in the potency test of erysipelas vaccines reached the validation stage. Seven laboratories from regulatory authorities and five manufacturers from seven countries participated in a formal inter-laboratory trial. The results of this investigation clearly demonstrate the suitability of the serological alternative for routine testing of licensed erysipelas vaccines.

2. Material and methods

2.1. Study design

Twelve laboratories were invited to participate in the validation study. Each laboratory was assigned with a capital letter. The participants had to immunise mice with the erysipelas vaccines coded V1–V9. Three weeks after immunisation, mice were bled under narcosis using the method established at this laboratory (see Table 2). Afterwards the ELISA was performed using pooled sera from each group of mice (S1–S9). The laboratories were requested to repeat the ELISA three times in order to assess intra-laboratory precision.

2.2. Laboratory animals, immunisation and serum preparation

It was recommended to use female NMRI mice of SPF status with a body weight of 17–20 g at the time of immunisation. Animals were housed under the usual conditions of the participating laboratories.

Each of ten mice/product received a single subcutaneous injection of 0.2 ml of vaccine (1/10 of the pig dose). Blood samples were drawn three weeks after vaccination under narcosis (see Table 1). After centrifugation equal volumes from each mouse sample were pooled to produce the serum pools S1–S9.

Mouse reference serum was prepared by injecting mice subcutaneously with 5 International Units (IU) of the International WHO Standard [16] in a volume of 0.2 ml. Aliquots of 500 μl were freeze-dried (provided from the organising laboratory).

Unvaccinated mice were bled to provide the negative control serum. The serum was freeze-dried in a volume of 250 μl (provided from the organising laboratory).

Table 1
Mice strains, bleeding procedure and narcosis used by the different laboratories

Laboratory	Mouse strain	Bleeding	Narcosis
A	NMRI	Retro orbital puncture	Mix of hypnorm and dormicum i.p.
C	NMRI	Cardiac puncture	Isoflurane
D	NMRI	Retro orbital puncture	Ether
E	TO	Cardiac puncture	Isoflurane
F	NMRI	Cardiac puncture	Ether
G	NMRI	Cardiac puncture	Methylflurane
H	NMRI	Cardiac puncture	'Dolethal' i.p.
I	NMRI	Cardiac puncture	Methylflurane
J	NMRI	Retro orbital puncture	Ether
L	NMRI	Cardiac puncture	Isoflurane

Table 2
Specifications of the vaccine samples V1–V9

Vaccine	Kind of product[a]	Remarks
V 1	C, L, A	
V 2	M, A	
V 3	C, A	
V 4	M, L	
V 5	C, A	
V 6	M, A	
V 7	M, A	= V 6, antigen reduced
V 8	M, A	= V 7, antigen reduced
V 9	M, L	

[a] M, monovalent vaccine; C, combined vaccine with an additional antigen component; A, aluminium-adsorbed vaccine; L, lysate vaccine.

2.3. Vaccines

Nine inactivated erysipelas vaccines, code-labelled as V1–V9 were used. The vaccines differed in composition (adjuvant, monovalent vs. multiple antigens) and the method of preparation (see Table 2). The V7 and V8 were prepared from product V6 with graded antigen content in the complete vaccine base. Thus, these three samples differed only in the amount of antigen.

The vaccines were representative for the spectrum of products on the German market.

2.4. Preparation of coating antigen

The official German reference strain for the mouse challenge test, *E. rhusiopathiae* strain, Frankfurt XI, serovar N [17] was used to prepare the antigen according to the method of Groschup [14] using EDTA and alkaline treatment:

1. Thawing of the wet bacteria.
2. Centrifugation of the wet bacteria carried out at 14 000 × *g* for 25 min at 4°C.
3. Decantation of the supernatant. Dissolving the pellet (5 g) in 50 ml 10 mM Tris–HCl buffer pH 7.2, containing 1 mM EDTA. Incubation on a water bath at 37°C for 30 min.
4. Centrifugation at 18 000 × *g* for 25 min at 4°C.
5. Decantation of the supernatant. Dissolving the pellet using 0.01 M NaOH to a volume of 100 ml per tube. Transferring of the preparation into a beaker. Recording the pH.
6. Incubation of the suspension in cold store room (4°C) for 18 h with constant stirring.
7. Neutralisation of the suspension with 2 M HCL, recording the pH.
8. Centrifugation at 8200 × *g* for 25 min at 4°C.
9. Sterile-filtration of the supernatant (0.45 μm filter).

The protein content determined by the Pierce BCA Protein assay [18] was 530 μg per ml. SDS–page performed according to the method of Laemmli [19] demonstrated the presence of the major protective proteins of *E. rhusiopathiae* with molecular weights of 66–64 and 40–35 kDa as described by others [14,20].

2.5. Indirect ELISA

The indirect ELISA is an easy to perform assay type, where in the first step the antigen is coated to a microtitre plate:

1. Multiwell plates (no. 655001, GREINER GmbH, Germany).
2. Coating: 1:100 diluted antigen, 100 μl/well, incubated overnight at 4°C.
3. Blocking: PBS/skimmed milk 5%, 150 μl/well, incubated for 1 h at 37°C.

4a. Sera: 1:20 diluted reference serum (5 IU) and test sera (serial dilution factor 2), 100 μl/well (PBS/skimmed milk 5%).

4b. Test control: 1:20 diluted, 100 μl/well, six fold (position H1–H6).

4a/b. Incubation for 1 h at 37°C.

5a. Conjugate: 1:10000 diluted goat-anti-mouse IgG (H and L), peroxidase-labelled (DIANOVA, Hamburg, Germany).

5b. Conjugate control: PBS/skimmed milk 5%, 100 μl/well, sixfold. Incubation.

5a/b. Incubation for 1 h at 37°C.

6. Substrate: tetramethyl-benzidine [TMB] (SIGMA, Deisenhofen, Germany) working solution, 100 μl/well, incubation in the dark for 5 min at room temperature.
7. Stop buffer: 1 M H_2SO_4, 50 μl/well.
8. Reading: at 450 nm.

3. Statistical methods

The serum antibody levels were estimated by comparing parallel parts of the curves of each test serum and the reference serum. The values were expressed as relative potencies (RP) [21]. The reference serum was given the arbitrary value of 1. This value represents the pass–fail criterion for the vaccines under test. Vaccines inducing antibody levels with a value ≥ 1 pass the test. Vaccines inducing lower potencies than 1 fail the test.

The results of the relative potency test were compared using a fixed effects linear model, which takes into account the factors test serum, laboratory and day of measurement. The reproducibility (inter-laboratory variation) and the repeatability (intra-laboratory variation) were addressed by comparing pairs of relative potencies. Lin's concordance correlation coefficient $\hat{\rho}_c$ [22] was used to quantify the degree of deviation from the total agreement, namely the 45° line through the origin ($\hat{\rho}_c = 1$).

Table 3
Serological results expressed as the mean of relative potencies (three measurements) of each laboratory for the nine vaccine samples[a]

Lab.	V1/S1	V2/S2	V3/S3	V4*/S4	V5/S5	V6/S6	V7/S7	V8*/S8	V9 #/S9
A	20.51	3.16	13.18	0.30	7.97	3.78	2.95	0.16	1.58
C	11.98	7.19	56.08	0.49	5.43	12.53	3.27	<.0	1.47
D	24.17	4.95	28.38	0.45	3.10	6.09	0.43	0.47	0.53
E	13.20	2.83	5.51	<1.0	3.96	13.00	2.58	<1.0	<1.0
F	28.97	3.16	4.32	0.49	5.40	15.52	1.86	<1.0	0.69
G	6.03	2.59	11.87	0.49	5.47	12.02	5.02	0.99	0.58
H	14.62	1.85	7.44	0.41	8.22	11.26	3.07	0.39	0.48
I	6.41	2.64	5.41	0.75	6.15	5.05	1.42	0.14	0.98
J	6.01	3.51	24.49	0.35	4.53	7.04	1.02	0.22	0.53
L	7.69	2.92	6.09	0.48	9.86	7.24	3.25	0.29	0.63

Pass = RP ≥ 1
Fail = RP < 1

[a] The vaccines V4* and V8* were products of low quality with a potency of less than 50 IU per pig dose. The corresponding sera S4 and S8 confirmed the weak potency by failing in the test with RP values lower than 1 (<1 was used when the value was below minimum optical density of 0.050). The potency of V9 # (product with a non-conventional adjuvant) estimated in vivo could not be confirmed by serology. Calculated were values below 1 with the exception of the results of laboratory A and C.

4. Results

4.1. Data evaluation

All participating laboratories provided the raw data of their ELISA outprints. Two participants (B and K) had to be excluded on the basis of deviation from the prescribed protocol.

4.2. Reproducibility

The nine vaccine samples represented a broad spectrum of potencies, including potencies higher than 50 IU which should 'pass' (samples V1, V2, V3, V5, V6 and V7) and samples with lower potencies (samples V4 and V8) which should 'fail' the test. All serological results confirmed the pass criteria with exception of the result for V7 from Laboratory D. Samples V4 and V8 induced low antibody titres and therefore were 'failed' by all laboratories. The results are presented in Table 3.

A discrepancy appeared in the serological data for Vaccine 9. Two laboratories (A and C) confirmed the in vivo result (> 50 IU) with a pass for this product, whereas the data of the other laboratories indicated a 'fail' for this sample.

The study also included samples with graded antigen contents of the same antigen preparation (diluted in complete vaccine base). V6 had the highest content (110 IU, determined in the mouse potency test), V7 with 62 IU and V8 with 13 IU per pig dose. This gradation was confirmed in the serological results of all laboratories (see Fig. 1). Only Lab D deviated, with a fail result for V7.

4.3. Intra-laboratory precision

To evaluate the precision within the laboratories, each serum was assayed on 3 consecutive days. The mean value of the optical density for each serum pool and dilution step (seven dilutions), the standard deviation (SD) and the relative coefficient of variation (CVr) were evaluated.

The formula $CVr = (SD/mean/(\sqrt{3})) \cdot 100$ [23] was used to calculate the CVr values.

A very good repeatability of the test was demonstrated for all laboratories. As an example the CVr results (mean value) of the serum dilution 160 of all sera and the reference serum shown in Fig. 2.

In nearly all multiple pair comparisons of the three measurements using Lin's concordance correlation co-

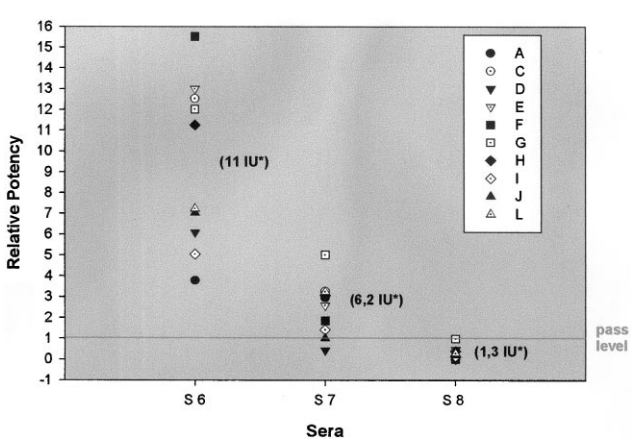

Fig. 1. Ranking of the sera S6, S7 and S8 reflecting the gradation of the vaccine samples V6, V7 and V8. * 1/10 of the pig dose, measured by the manufacturer using the Ph. Eur. test.

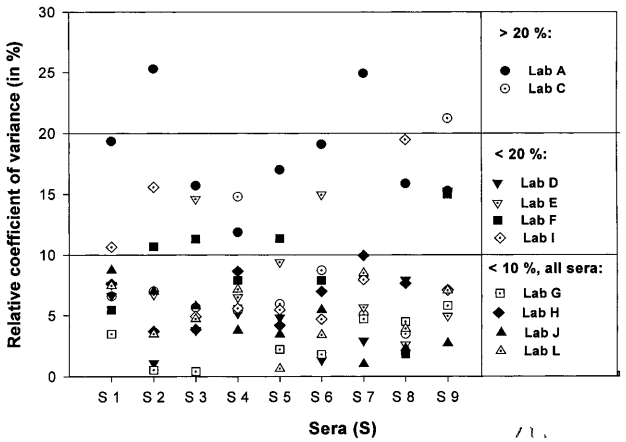

Fig. 2. The relative coefficients of variance (CVr) for the nine samples (S1–S9) at a serum dilution of 1:160 are shown. The most CVr values were in the range up to 10%, some were in the range 10–20%, while two laboratories (A and C) achieved values above 20% for certain sera.

efficient, the precision was in the range good to very good ($\hat{\rho}_c \geq 0.86$), while moderate agreement was reached between different days in laboratories A ($\hat{\rho}_c = 0.75$), E ($\hat{\rho}_c = 0.70$) and F ($\hat{\rho}_c = 0.63$) (Table 4).

4.4. Analysis of variance

Two highly significant ($P < 0.001$) factors, namely test serum and laboratory, caused a variation in the logarithm of relative potencies, as shown by an analysis of variance. 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 effect ($P = 0.978$) at $\alpha = 5\%$. A significant influence of the serum had to be expected as the ELISA is intended to detect different antibody levels within the different serum samples.

5. Discussion

As a general principle, potency tests have been designed to measure the ability of a vaccine to induce protection against subsequent virulent challenge. Traditionally, inactivated vaccines for mammals are tested for batch potency in laboratory animal models which are based mainly on vaccination-challenge tests [24]. Currently, the batch potency test for swine erysipelas is based upon a multi-dilution lethal challenge procedure in mice [1,10]. Such tests involve many animals, in addition the great suffering should give the highest priority with respect to validate alternative methods [12]. However, in order to develop effective and credible alternative methods, it is essential to understand the mechanisms of protection [13]. As described earlier [14,15,25,26] the structural proteins of *E. rhusiopathiae* in the range of 66–64 kDa are responsible for the induction of protection. Therefore, our ELISA using a coating antigen with high amounts of P64 [27] should be suitable to detect protective antibodies.

It can be assumed that the ELISA batch potency test is capable of reflecting the immunogenicity of inactivated erysipelas vaccines in the target species, as enzyme immunoassays based on the same coating antigen and the same test principle have already been performed in mice and pigs [3,5,28]. Furthermore, the serological immune response in pigs has been investigated using ELISA and compared with the outcome of subsequent challenge [5,27,28].

The ELISA had been optimised in previous experiments. The inter-laboratory transferability of the assay was assessed in a pre-validation study. Participating laboratories were provided with all test reagents and performed the test according to the standard protocol. A good inter-laboratory precision and reproducibility was achieved in this study [4] and a further adaption and standardisation of the protocol was undertaken.

The objective of the validation stage was to evaluate the animal test itself (immunisation and bleeding of mice) and to look for its influence on the results of the new procedure. In addition, it was necessary to establish whether the alternative is suitable to assay all products, including combined vaccines of various compositions from different manufacturers. It should be capable to distinguish batches with satisfactory potency (> 50 IU in a pig dose) from those being unsatisfactory. Furthermore, the reliability and reproducibility of the ELISA should be confirmed.

A total of ten animals per group was chosen to test a vaccine sample, because previous investigations with

Table 4
Statistical analysis of the intra-laboratory precision expressed as Lin's concordance correlation coefficient $\hat{\rho}_c$ for pairs of relative potencies in each laboratory for measurements of 3 consecutive days

	Lab.									
	A	C	D	E	F	G	H	I	J	L
Days 1–2	0.97	0.99	0.98	0.70	0.88	0.98	0.93	0.99	0.98	0.93
Days 2–3	0.89	0.86	0.95	0.88	0.87	0.99	0.98	0.90	0.96	0.87
Days 1–3	0.75	0.90	0.97	0.99	0.63	0.99	0.98	0.91	0.99	0.98

groups of five, eight, ten and 15 mice demonstrated a considerable variation in individual immune response which prohibited a further reduction of animal numbers [29].

The use of a defined mouse strain seamed to be a critical parameter. The NMRI strain was prescribed for testing, because this outbred strain had been used during the development of the test. However, two laboratories used differing mouse strains. Laboratory K had to be excluded because the use of strain CF1 resulted in markedly deviating data (data not shown). Whereas the outbred strain TO used by laboratory E obviously had no influence on the test results. Subsequent investigations performed in two of the participating institutions indeed substantiated that the influence of the animal strain used for immunisation may be considerable (data not shown).

The rationale to use one tenth of the pig dose for immunisation of mice is based on the requirements for erysipelas vaccines [1], where the minimum antigen content of a pig dose is specified as 50 IU [16]. That quantity provides sufficient protection in pigs (data not shown). 5 IU administered to mice give a protection rate of more than 90% after exposure to a lethal challenge (100–1000 LD_{50}) of *E. rhusiopathiae*.

All laboratories were experienced in ELISA techniques, however to differing degrees. Nevertheless, intra-laboratory (intermediate) precision achieved by the participants (pair-wise comparisons) reached a $\hat{\rho}_c$ of ≥ 0.86. A moderate agreement was reached between days 1–3 in laboratory A ($\hat{\rho}_c = 0.75$), days 1–2 in laboratory E ($\hat{\rho}_c = 0.70$) and days 1–3 in laboratory F ($\hat{\rho}_c = 0.63$) (see Table 4). Overall, excellent agreement was demonstrated in the study concerning the pass–fail criteria for the vaccine samples (Table 3). Two weak samples (V4 and V8), which didn't pass the mouse potency test induced antibody levels lower than the reference in all laboratories (RP values < 1). The samples V1, V2, V3, V5, V6 and V7 with a high potency all met the requirements with RPs > 1. Only laboratory D showed a deviation for V7 where the vaccine failed in the test (Table 3).

However, conflicting results were received for sample V9. In lab A and lab C the sera achieved RP values > 1, but in all other laboratories these sera failed as having values < 1. The sample had passed the Ph. Eur. mouse challenge test, showing an activity of only slightly more than 50 IU. In addition this was the only vaccine, which contained an adjuvant different from $Al(OH)_3$). It may be that the use of such a non-conventional adjuvant has a major influence on the development of the humoral immune response. Further studies in mice with vaccines containing other adjuvants are in progress to investigate this issue.

Three samples of the same vaccine base, but with a gradation of the antigen content (V6, V7 and V8) were included in the study. The results of all laboratories reflect the differences in the antigen content by graded antibody levels. Fig. 1 shows the in vitro data in comparison with the IU determined in the challenge model. Both samples with a potency > 5 IU in the mouse-challenge test also met the required RP of ≥ 1 in the alternative test. The low content of 1.3 IU of product V8 was confirmed with a mean RP value much lower than 1. Thus, the results of the serological method are in agreement with the data of the mouse-challenge tests (potency expressed in IU). Furthermore, this provides evidence that the ELISA is able to detect vaccine batches with insufficient potency.

Today, positive steps are being taken to promote the harmonisation of requirements for biological products through regional and/or international bodies, such as the pharmacopoeias, WHO or OIE. Due to the strong scientific basis and the involvement of control authorities from Europe and USA as well as some of the largest vaccine manufacturers, it is expected that the ELISA will be used to monitor the potency of erysipelas vaccines and therefore is a potential candidate to promote harmonisation and to become one of the first internationally accepted alternative tests in vaccine quality control.

6. Conclusions

During the pre-validation and validation stage, the transferability of the ELISA was demonstrated using the parameters: precision, repeatability, reproducibility and robustness. The validation study confirmed the practicability of the proposed method for a wide range of inactivated erysipelas vaccines. The blind trial included the immunisation procedure, the bleeding and sampling procedure and the ELISA performance. The intra- and inter-laboratory precision achieved lay in the range of good to very good.

It can be concluded, that the assay is able to detect vaccine samples of inferior quality. However, a possible influence of a deviating mouse strain and a non-conventional adjuvant on ELISA results could be observed in the study. Therefore it is recommended, that each manufacturer should start an in-house validation study to demonstrate the suitability of the ELISA for their specific product(s) and to prepare and calibrate an in-house reference vaccine or reference serum using a vaccine batch that has been shown to be effective in the pig challenge test.

Based on the results of this study, the existing pharmacopoeia monograph should be modified.

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