Collaborative Study for the Establishment of Erysipelas ELISA Coating Antigen European Biological Reference Preparation Batch N°1

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1. SUMMARY

The development and validation of suitable alternatives for the replacement of *in vivo* challenge testing in the evaluation of vaccines is an important goal for national authorities and manufacturers involved in the assessment of quality, safety and efficacy of such products. To that end, 13 laboratories from 9 European countries, including 5 manufacturers, 7 authorities and EDQM[3], have taken part in a collaborative study to evaluate the suitability of a candidate reference preparation of erysipelas coating antigen for ELISA as a European Pharmacopoeia Biological Reference Preparation (Ph. Eur. BRP N°1). The new Ph. Eur. BRP is intended for use in a serological assay, which would significantly reduce the suffering of animals in the potency assays of inactivated erysipelas vaccines.

Participants were provided with sufficient study material, including the candidate coating antigen, and a panel of test sera from mice which had been immunised with vaccines representative of products on the European market, in order to evaluate the performance of the coating antigen in an enzyme-linked immunosorbent assay (ELISA) which had previously performed successfully in a pre-validation study [1] and in an international validation study [2].

Results of the collaborative study indicate that the candidate batch of erysipelas ELISA coating antigen is suitable to act as a Ph. Eur. biological reference preparation. The final study report was presented at the 110th session of the Ph. Eur. Commission (June 19-21, 2001) and the material was duly adopted as Erysipelas ELISA Coating Antigen Ph. Eur. BRP N° 1 for use in the enzyme-linked immunosorbent assay in the context of the serological potency assay for inactivated erysipelas vaccines.

2. INTRODUCTION

Swine erysipelas is a world-wide bacterial disease of great economic importance. Vaccination is a well-accepted method of preventing infection. Vaccines are produced with strains of serovar 2, but provide cross-protection against most serovars. Inactivated products (aluminium hydroxide-adsorbed cultures or lysate extracts) are widely used.

Regulations concerning the Quality Control (QC) of inactivated erysipelas vaccines are laid down in the European Pharmacopoeia (Ph. Eur.) monograph *0064* [3]. The potency is tested by a virulent challenge in mice. Briefly, the test vaccine and a reference vaccine are administered to groups of 10 mice, each. Three weeks later all animals (including a control group of ten mice) are challenged with a virulent strain of *E. rhusiopathiae*. Potency is calculated by comparison of the survival rate within the reference and the test groups. From the viewpoint of animal welfare, the severe suffering caused by the challenge procedure stresses the urgent need for an alternative. The monograph is currently under revision with the intention to include alternative potency assays [4].

Recently, we described a serological assay system (ELISA) which has the potential to replace this challenge-based model [1, 2, 5]. On the basis of investigations characterising protective antigen structures of *E. rhusiopathiae*, we developed an enzyme-linked immunosorbent assay to measure protective erysipelas antibodies in mice. 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 of known potency. The ELISA has performed successfully in a pre-validation study to evaluate protocol transfer, protocol performance and protocol refinement [1] and in an international validation study [2].

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[3]Abbreviations: **BRP**: Biological reference preparation, **cBRP**: Candidate biological reference preparation, **CV**: Coefficient of variance, **CVr**: Relative coefficient of variance, **EDQM**: European Directorate for the Quality of Medicines, **ELISA**: Enzyme-linked immunosorbent assay, **E. rhusiopathiae**: Erysipelas rhusiopathiae, **g**: gram, **GCV**: Geometric coefficient of variation, **IgG**: Immunoglobulin G, **IU**: International Unit, **kDa**: Kilodalton, **LD_{50}**: Dose required to cause lethality in 50% of subject animals, **mL**: millilitre, **µL**: microlitre, **N°**: Number, **PEI**: Paul Ehrlich Institut, **Ph. Eur.**: European Pharmacopoeia (Pharmacopée Européenne), **RP**: Relative potency, **SDS-page**: Sodium dodecyl sulphate polyacrylamide gel electrophoresis, **WHO**: World Health Organisation, **QC**: Quality control

However, as the coating antigen for the ELISA was not available in sufficient quantity to act as a Ph. Eur. BRP, the Paul-Ehrlich-Institut (PEI) was asked to prepare a new batch as candidate (c) Ph. Eur. Biological Reference Preparation (BRP).

3. AIM OF THE STUDY

The aim of this collaborative study was to determine the suitability of the candidate reference preparation of erysipelas coating antigen for ELISA as a European Pharmacopoeia Biological Reference Preparation (Ph. Eur. BRP No 1).

4. PARTICIPANTS

Thirteen laboratories from nine European countries including five manufacturers, seven authorities and EDQM participated in the study. Each laboratory was randomly coded to ensure anonymity. A list of the participants is provided in section 11. The order of listing does not necessarily correspond to the codes given to the laboratories.

5. MATERIALS, METHODS AND STUDY DESIGN

5.1. Preparation of coating antigen (candidate Ph. Eur. BRP N°1)

The official German reference strain for the mouse challenge test, *E. rhusiopathiae* strain, Frankfurt XI, serovar N [6] was used to prepare the antigen according to the method of Groschup et al. [7] using EDTA and alkaline treatment. The culture was prepared and kindly provided by INTERVET UK.

SDS-page performed according to the method of Laemmli [8] demonstrated the presence of the major protective protein of *E. rhusiopathiae* with a molecular weight of 66 to 64 kDa as described by others [7, 9].

The following analytical data apply for the new batch:

The freeze-dried erysipelas coating antigen candidate BRP has a protein content of 0.733 mg/mL which represents 87% recovery of the activity before freeze drying. The vials do not contain any stabiliser or other excipient.

Content per vial: 0.5 mL freeze-dried (0.367 mg/vial).

Residual humidity, determined by the semi-micro method as outlined in Ph. Eur. Chapter 2.5.12, is 0.04 % (according to WHO specifications for reference materials the expected content should be not more than 3%).

The precision of fill was determined as 0.4962 ± 0.0036 g (mean ± standard deviation n =10 relative standard deviation 0.72%).

1 vial of antigen is sufficient to coat approximately 20 microtitre plates.

The immunogenicity of the preparation was demonstrated in laboratory mice. A detailed report is given in the annex.

5.2. Material provided for the study

The participants received the following reagents together with the cBRP to perform the ELISA:

- A serum panel of 6 different samples prepared by PEI according to the method described in [2]. Briefly, serum from mice vaccinated against erysipelas was collected and pooled three weeks after immunisation of the animals. Composition of the vaccines used can be found in Table 1.
- The reference serum prepared after injecting mice subcutaneously with 5 International Units (IU) of the WHO standard in a volume of 2 mL as outlined in [2]. 5 IU represents 1/10 the pig dose (50 IU) as specified in the Ph. Eur. monograph.
- A negative serum prepared from unvaccinated mice as outlined in [2].
- The conjugate (peroxidase labelled goat-anti-mouse IgG)
- The substrate (tetramethyl-benzidine)
- Multiwell plates (F-form)

Table 1 — *Specification of erysipelas vaccines used for production of the serum panel*

Test Sera	Vaccine	Type of product	Comments
1	1 (1:8)	Serovar 1 and 2 Aluminum hydroxide adjuvanted	Normal range potency
2	1 (1:20)	As above	Normal range potency
3	1 (1:100)	As above	Low potency
4	2	Combined Vaccine (with porcine parvovirus) Erysipelas lysate Aluminum hydroxide adjuvanted	Normal range potency
5	3	Combined Vaccine (with porcine parvovirus) Erysipelas serovar 1 and 2 Aluminum hydroxide adjuvanted	Normal range potency
6	4	Serovar 2 Aluminum hydroxide adjuvanted	Very high potency

5.3. Design of the study

Participants were asked to test the panel of mouse serum samples using the candidate erysipelas coating antigen BRP with the kit provided.

First a multiwell plate was coated with the cBRP antigen. Antibodies against *E. rhusiopathiae* in the test serum bind to this antigen. The bound antibodies were detected using peroxidase-labelled goat-anti-mouse IgG and the reaction was visualised with tetramethyl-benzidine as substrate. Negative serum and conjugate controls were included. A reference serum of known potency was assayed on every plate, and the results obtained with the test sera were compared with those for this preparation.

Each serum was tested in three independent assays with freshly made dilutions on three different days. The test and reference sera were to be determined in triplicate and in seven dilution steps.

5.4. Statistical analysis

The potency of the test preparations were calculated by analysing individual assays applying a four parameter logistic model, taking the response against the log of the dilution. Calculations were performed using the procedure PROC NLIN from SAS, Version 8.1 [10].

Combined potency was calculated for each product across all assays by taking the geometric mean of the results from the individual assays within each laboratory. Overall potency estimates were calculated as geometric mean of laboratory means. For each serum the overall potency estimate was used to calculate potency as % of the overall potency (see Tables 2 and 3).

For assessment of the interlaboratory variation the geometric coefficient of variation (GCV) is provided (see Table 4).

The logarithm of the relative potencies for each test serum were compared across laboratories by means of a mixed linear model with 2 factors: laboratory (fixed) and day within laboratory (random).

6. RESULTS

6.1. Data evaluation

All participating laboratories provided their raw data of the ELISA printouts. In some cases the measurement of one serum, or plate of different laboratories had to be excluded. This was justified by very high % coefficient of variance (CV) within distinct sera on the different days or where a titration was not visible.

The results of Laboratory L had to be excluded completely on the basis of very low extinction values for the reference serum, % CV greater than 20% within two sera and no visible titration of Serum 6. Possible causes may be non-sufficient experience in the ELISA technique or pipetting errors. Also excluded were the data of Laboratory G for Serum 6, where a titration was not given.

Serum 6 originated from a batch of vaccine with an extremely high potency (greater than 450 IU per dose in the multiple-dilution mouse potency test, which requires a minimum of 50 IU per dose for erysipelas vaccines). Accordingly, this sample gave very high extinction values and therefore high relative potencies. All laboratories confirmed this in the ELISA results.

6.2. Statistical analysis

Comparison of the logarithm of the relative potencies showed no statistically relevant laboratory effects for sera 3, 4 and 5 however statistically relevant laboratory effects were observed for sera 1 and 2 (due to Laboratory G) and serum 6 (due to Laboratory I).

6.3. Potency results

Six serum samples had been chosen, including four samples (sera S1, S2, S4 and S5) of a potency in the usual range of 50 - 200 International Units (IU) per dose, one sample (S6) with a very high potency (~ 450 IU per dose) and one sample (S3) with a low potency (~ 30 IU per dose).

Potency estimates for the various sera and their relative potencies are shown in Table 2. The Figures 1 to 6 illustrate the potency results for the individual serum samples in different laboratories. As shown in Table 2 and Figures 1-6 the potency estimates resulting from Laboratory G tend to be higher than those from the other laboratories.

Table 3 summarises the relative potencies and in Figure 7 the ranking of the serum samples S1-S6 is illustrated. The mean potency including all laboratories is given in brackets. As mentioned above the highest RP values were reached by Laboratory G. The five sera S1, S2, S4, S5 and S6 have values higher than 1 and are therefore above the requirement of a potency equal or better than 1. Serum S3 with a mean potency of 0.40 failed within all laboratories gaining values lower than 1. The participants demonstrated overall agreement in their results.

6.4. Intra-laboratory repeatability

In Figure 8 the relative coefficients of variance (CVr) for the six samples (S1-S6) at a serum dilution of 1:80 are shown. With values not exceeding 10% for most determinations a good intra-laboratory repeatability could be demonstrated.

Table 3 summarises the relative potencies and shows that for serum 3 the potency calculated was below 1 at each laboratory while for all other products the calculated potencies were above 1 indicating a potency at least as high as the reference.

7. DISCUSSION

As a general principle, potency assays for inactivated bacterial vaccines 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 mainly based on vaccination-challenge assays [13]. Currently, the batch potency assay for swine erysipelas is based upon a lethal challenge procedure in mice [3]. Such assays involving great suffering should be given the highest priority with respect to validation of alternative methods [14, 15]. However, in order to develop effective and credible alternative methods, it is essential to understand the mechanisms of protection.

As described previously [7, 9] the structural proteins of *E. rhusiopathiae* in the range of 66-64 kDa are responsible for the induction of protection. An ELISA using a coating antigen containing these proteins should therefore be suitable to detect protective antibodies.

It can be assumed that the ELISA batch potency assay used in this study is capable of reflecting the immunogenicity of inactivated erysipelas vaccines in the laboratory mouse. Differences in the antigen content of vaccines prepared artificially using the same vaccine base measured *in vivo* were also reflected in the ELISA [1, 2]. The ELISA method for detection of erysipelas antibodies was optimised in previous studies in which adaptation and standardisation of the protocol was undertaken [1, 2].

It was the objective of this study to evaluate a new batch of coating antigen which had been prepared in sufficient quantity to serve as a Ph. Eur. reference material that would allow manufacturers,

Table 2 — *Relative potencies of test sera (three independent measurements per laboratory)*

Serum	Lab.	Geom mean of 3 assays	Potency as % of OM	Potencies Day 1	Day 2	Day 3
S1	A	6.29	106.7	6.45	6.96	5.54
	B	4.87	82.7		4.51	5.26
	C	4.92	83.4	5.11	4.78	4.87
	D	4.76	80.7	4.76	4.76	
	E	4.89	83.0	5.62	4.21	4.96
	F	5.08	86.2	2.32	6.68	8.48
	G	10.81	183.4	13.13	9.27	10.39
	H	6.44	109.2	4.59	7.50	7.75
	I	6.54	111.0	5.57	8.20	6.13
	J	6.22	105.6	5.24	6.78	6.79
	K	5.46	92.6	5.80	5.34	5.26
	M	6.38	108.2		6.24	6.52
Over all mean (OM)		**5.90**				
S2	A	2.09	95.4	1.93	2.33	2.03
	B	1.95	88.9		1.85	2.05
	C	1.83	83.4	1.79	1.84	1.85
	D	1.59	72.4	1.79	1.40	
	E	1.76	80.4	2.02	1.57	1.73
	F	2.42	110.2	1.88	2.23	3.35
	G	3.64	166.2	4.42	3.16	3.46
	H	2.37	108.2	1.84	2.79	2.59
	I	2.81	128.3	2.66	3.39	2.46
	J	2.12	97.0	1.83	2.24	2.34
	K	1.85	84.5	1.91	1.77	1.87
	M	2.55	116.6		2.35	2.77
Over all mean (OM)		**2.19**				
S3	A	0.37	92.7	0.42	0.35	0.34
	B	0.30	76.1		0.42	0.22
	C	0.35	89.4	0.39	0.41	0.28
	D	0.38	96.4	0.39	0.38	
	E	0.34	85.7	0.37	0.32	0.33
	F	0.45	112.5	0.38	0.44	0.54
	G	0.52	130.2	0.49	0.48	0.58
	H	0.44	111.2	0.37	0.51	0.46
	I	0.48	122.2	0.52	0.55	0.39
	J	0.39	99.4	0.36	0.40	0.42
	K	0.33	84.6	0.34	0.33	0.34
	M	0.45	114.6		0.46	0.45
Over all mean (OM)		**0.40**				
S4	A	12.15	92.0	11.11	12.75	12.68
	B	16.82	127.4		12.99	21.77
	C	8.54	64.6	9.18	8.16	8.30
	D	13.42	101.6	16.73	10.81	13.37
	E	10.94	82.8	10.46	10.85	11.52
	F	13.41	101.6	10.53	9.43	24.31
	G	21.42	162.2	34.44	17.56	16.24
	H	12.49	94.6	14.06	12.93	10.72
	I	12.24	92.7	13.38	11.39	12.04
	J	12.37	93.7	11.85	11.47	13.93
	K	13.76	104.2	15.41	12.50	13.53
	M	14.67	111.1	20.38	12.07	12.84
Over all mean (OM)		**13.20**				

Table 2 (cont'd) — *Relative potencies of test sera (three independent measurements per laboratory)*

Serum	Lab.	Geom mean of 3 assays	Potency as % of OM	Potencies Day 1	Day 2	Day 3
S5	A	2.87	98.6	2.92	2.69	3.01
	B	3.19	109.6		2.42	4.19
	C	2.23	76.7	2.05	2.27	2.39
	D	2.42	83.2	1.83	2.09	3.70
	E	2.82	97.1	3.14	2.25	3.18
	F	3.07	105.5	2.21	3.30	3.95
	G	3.77	129.6	4.97	3.10	3.47
	H	2.71	93.0	2.66	2.73	2.73
	I	4.34	149.2	5.76	3.82	3.72
	J	2.63	90.6	2.58	2.34	3.02
	K	2.55	87.8	2.70	2.50	2.47
	M	2.89	99.2	3.75	2.34	2.74
Over all mean (OM)		**2.91**				
S6	A	21.02	106.1	18.18	21.63	23.63
	B	26.70	134.7		18.94	37.64
	C	15.51	78.2	12.51	16.64	17.91
	D	11.88	60.0	17.39	10.15	9.50
	E	16.27	82.1	19.49	14.03	15.77
	F	23.74	119.8	14.26	30.80	30.48
	H	19.73	99.6	15.92	24.46	
	I	33.18	167.4	38.45	33.35	28.48
	J	21.50	108.5	20.56	19.70	24.52
	K	18.99	95.8	19.52	22.32	15.72
	M	17.36	87.6	17.16	20.42	14.93
Over all mean (OM)		**19.82**				

Table 3 — *Relative potencies (geometric mean)*

Lab.	Test serum 1	2	3	4	5	6
A	6.3	2.1	0.4	12.2	2.9	21.0
B	4.9	1.9	0.3	16.8	3.2	26.7
C	4.9	1.8	0.4	8.5	2.2	15.5
D	4.8	1.6	0.4	13.4	2.4	11.9
E	4.9	1.8	0.3	10.9	2.8	16.3
F	5.1	2.4	0.4	13.4	3.1	23.7
G	10.8	3.6	0.5	21.4	3.8	
H	6.4	2.4	0.4	12.5	2.7	19.7
I	6.5	2.8	0.5	12.2	4.3	33.2
J	6.2	2.1	0.4	12.4	2.6	21.5
K	5.5	1.9	0.3	13.8	2.6	19.0
M	6.4	2.6	0.5	14.7	2.9	17.4

Table 4 — *Summary of the overall potencies*

Serum	Mean potency*	95%-confidence interval	Inter-laboratory GCV (%)
1	5.90	5.10 – 6.82	25.8
2	2.19	1.89 – 2.54	26.1
3	0.40	0.36 – 0.44	18.0
4	13.20	11.45 – 15.22	25.1
5	2.91	2.59 – 3.27	20.3
6	19.82	16.44 – 23.90	32.2

* geometric mean

authorities and other interested parties to estimate the potency of erysipelas vaccines using serology instead of challenge. All participating laboratories were experienced in ELISA techniques, however to differing degrees. Several laboratories had already participated in the pre-validation and/or validation study.

Serum antibody levels were estimated by comparing curves of test and reference serum giving a value expressed as relative potency (RP). The reference has an arbitrary value of 1, which represents the pass mark for the vaccines being tested.

Overall, good agreement was demonstrated in the study concerning the ranking of the serum samples (Figure 7). All laboratories detected the serum sample of insufficient potency with RP values below 1 and the remaining samples in the appropriate ranking order with RP values above 1.

Additional investigations have been performed with regard to the protectivity of the BRP in mice. It could be demonstrated, that mice immunised with the antigen preparation were protected against an erysipelas challenge of more than 100 LD_{50}. For details see the annex**.**

8. CONCLUSION

The results of this study clearly show that the cBRP is suitable to be used as a reference material. It is therefore proposed that this batch of erysipelas coating antigen becomes Erysipelas ELISA Coating Antigen Ph. Eur. BRP Batch N° 1.

It is also proposed, based on the results of this and previous studies, that the existing Ph. Eur. monograph for inactivated erysipelas vaccines should be modified to replace the lethal challenge test by an ELISA test, using the newly established BRP as standard reference material.

9. ACKNOWLEDGEMENTS

The organisers express their sincere thanks to the project leader, Dr. K. Cussler (PEI), scientific advisor Ute Rosskopf-Streicher (PEI) and all the participants for their contributions to this study. The help of K. Redhead (Intervet, UK) who initialised the preparation of the bacterial culture of strain Frankfurt XI, serovar N through Intervet UK is highly acknowledged. The contribution of Arnold Daas EDQM statistician is also gratefully acknowledged.

The study was run by the EDQM with the support of the Council of Europe and the European Commission under the aegis of the Biological Standardisation Programme (internal project number BSP038) with Dr. C. Milne acting as project monitor.

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11. PARTICIPANTS (in alphabetical order)

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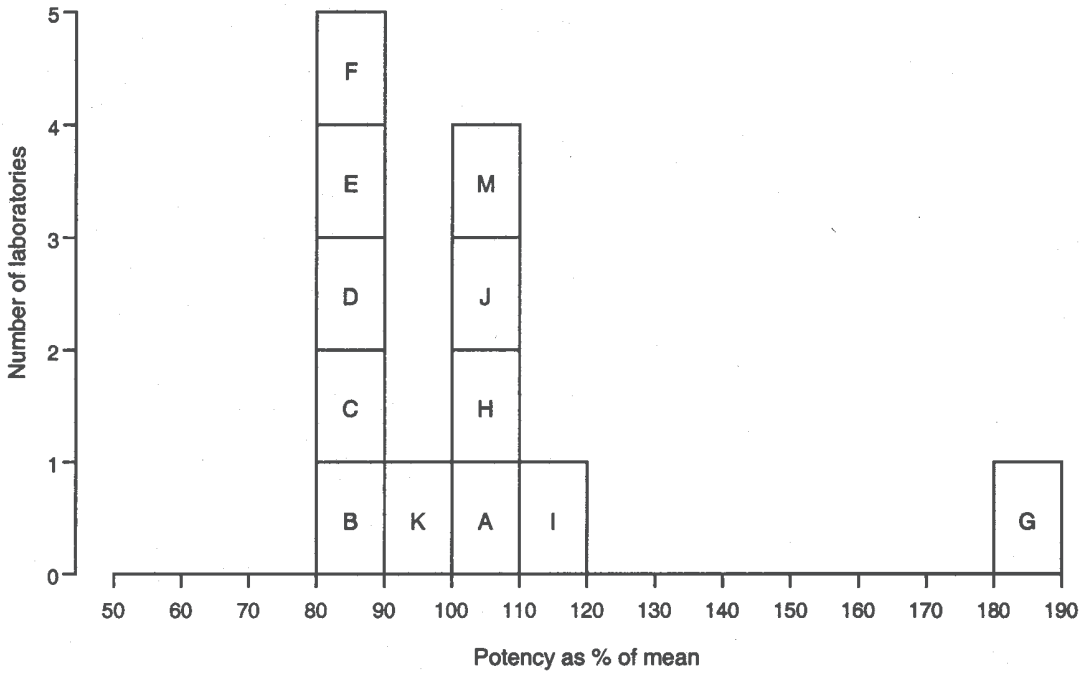

Figure 1 — *Serum 1 potency as a percentage of the overall mean, for each laboratory*

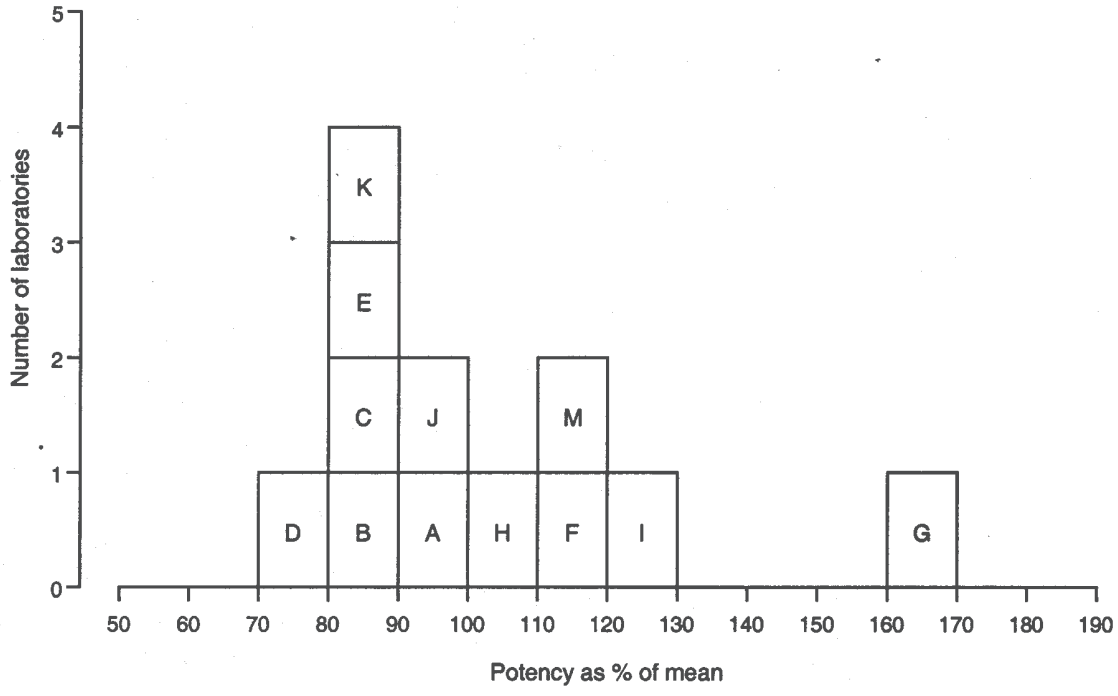

Figure 2 — *Serum 2 potency as a percentage of the overall mean, for each laboratory*

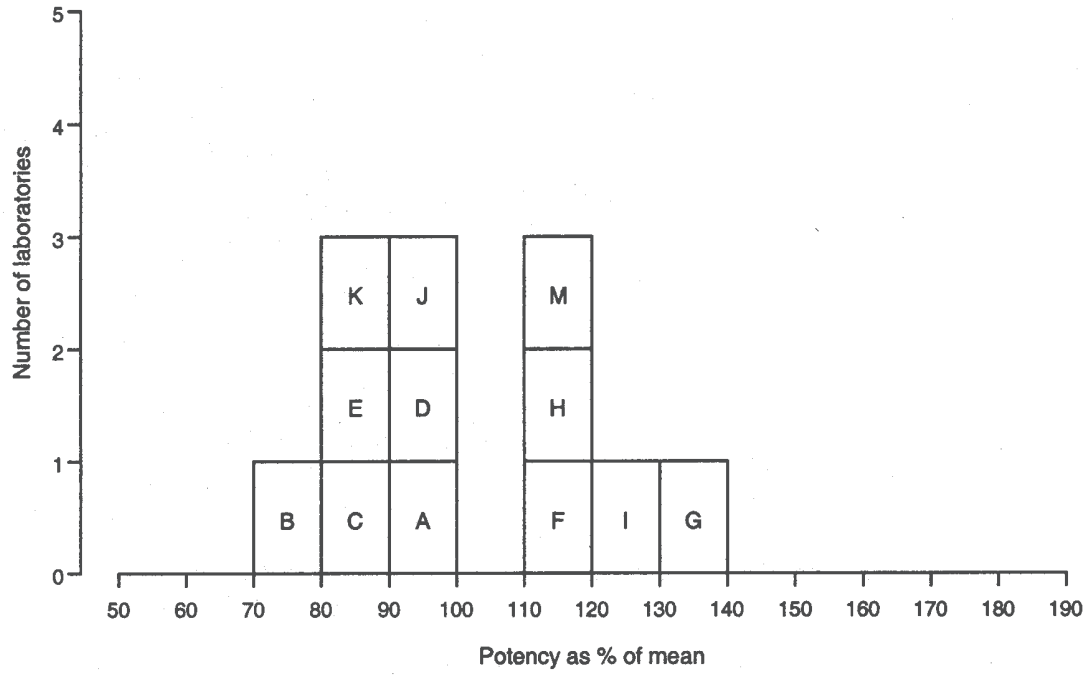

Figure 3 — *Serum 3 potency as a percentage of the overall mean, for each laboratory*

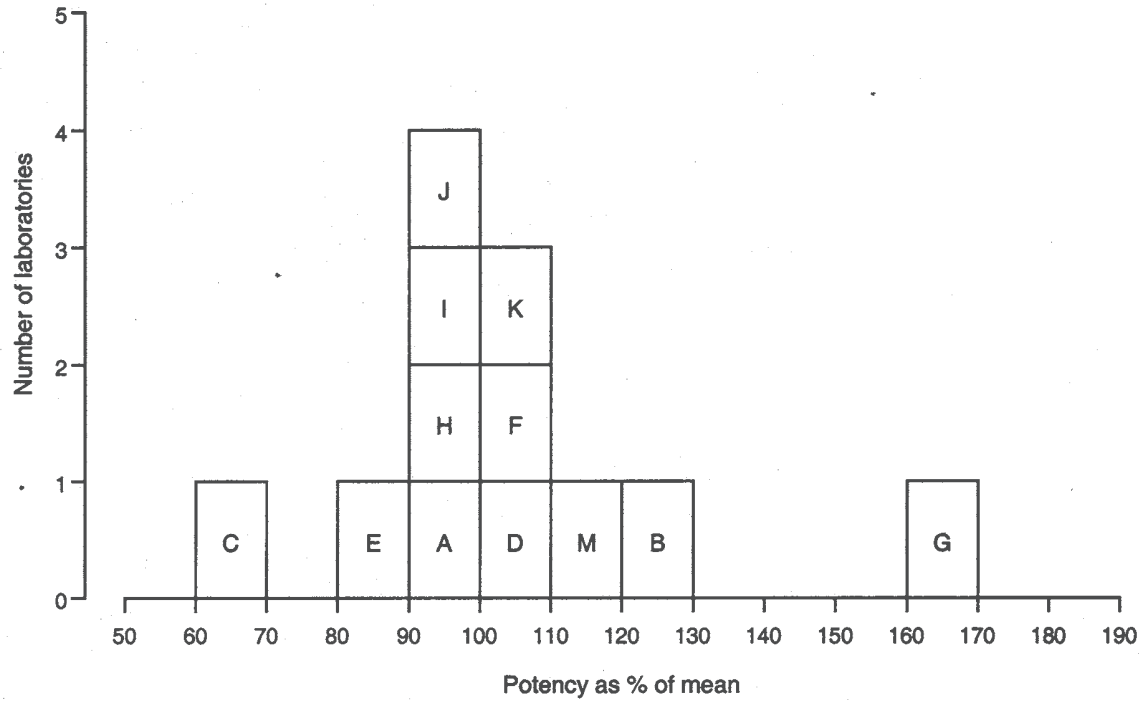

Figure 4 — *Serum 4 potency as a percentage of the overall mean, for each laboratory*

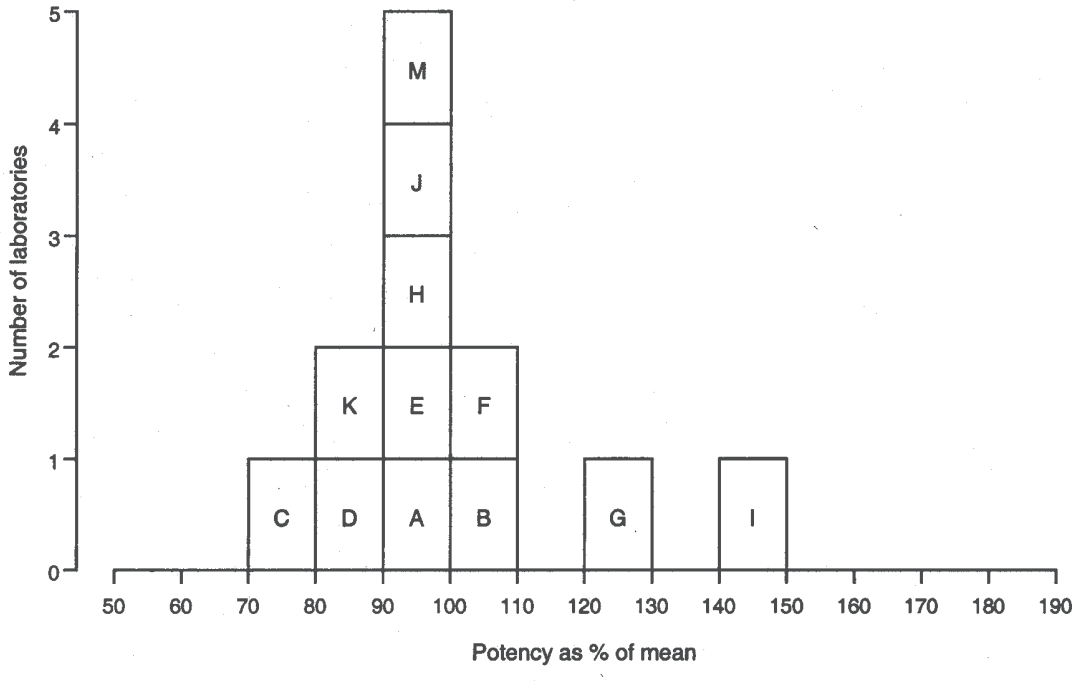

Figure 5 — *Serum 5 potency as a percentage of the overall mean, for each laboratory*

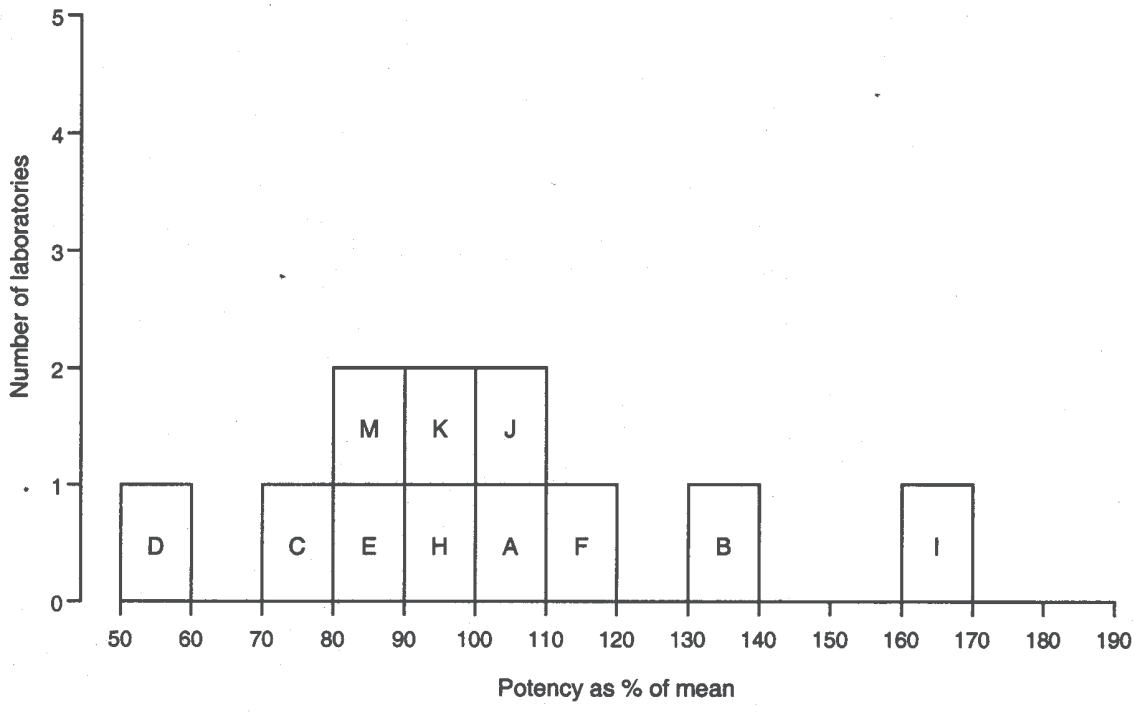

Figure 6 — *Serum 6 potency as a percentage of the overall mean, for each laboratory*

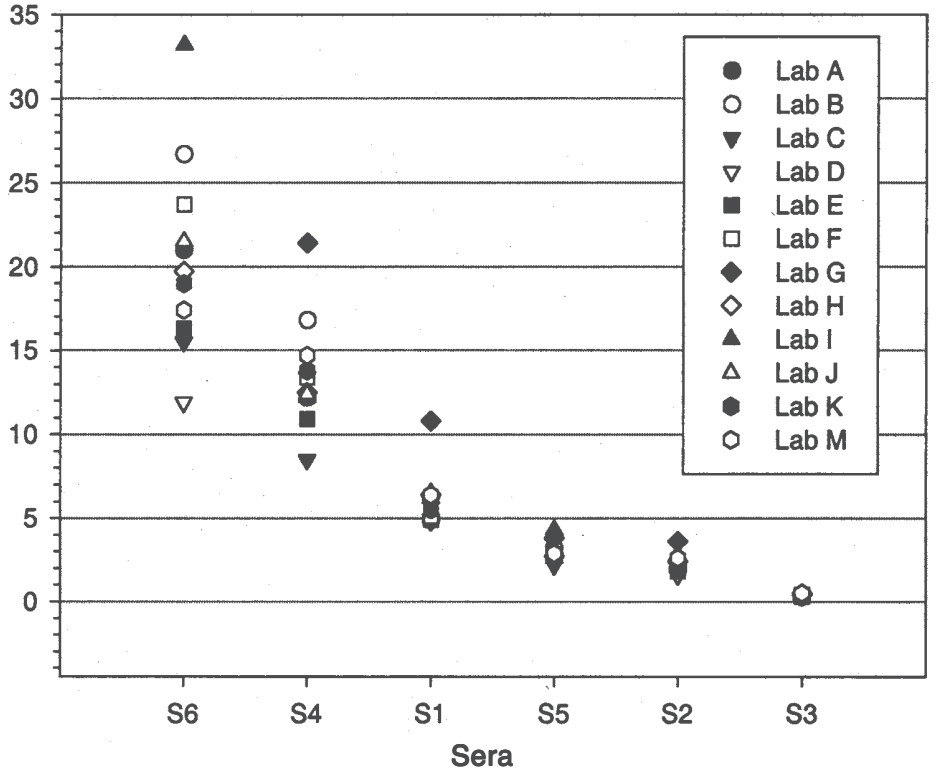

Figure 7 — *Relative potencies for each of the sera as determined in the different laboratories (A-K and M). The y axis represents the relative potencies (mean of 3 calculations)*

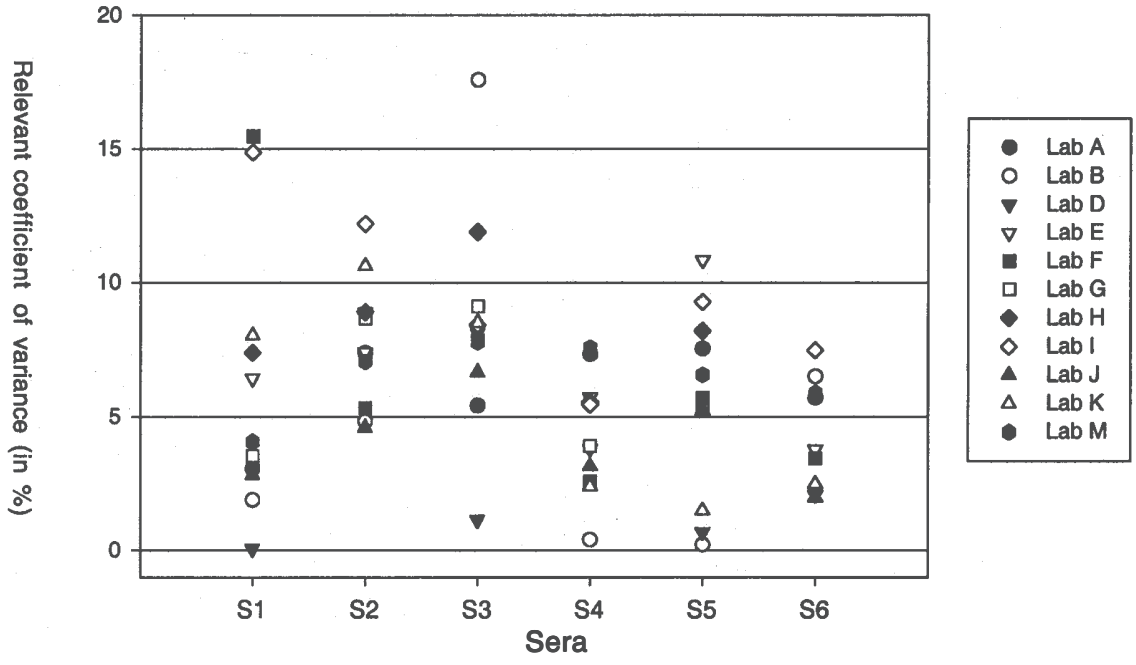

Figure 8 — *Relative coefficient of variance of the different sera samples at a dilution of 1:80*

ANNEX

IMMUNISATION-CHALLENGE TEST IN LABORATORY MICE TO DEMONSTRATE THE PROTECTIVE ACTIVITY OF THE ELISA COATING ANTIGEN CBRP

1. PURPOSE OF THE STUDY

The ELISA is intended to detect antibodies in mouse serum. These antibody titres are used as a correlate for protectivity against a virulent challenge. Thus, the antigen itself must induce protection in immunised animals.

2. ANIMALS AND METHODS

The antigen preparation was mixed with aluminium hydroxide gel (Fa. Behring) and stored for 8 days at 4° C to allow absorbtion.

Two groups of 10 laboratory mice (outbread strain NMRI, three weeks old) were immunised subcutaneously with 0.274 mg of antigen in a volume of 0.5 mL. One group received the same treatment as a booster three weeks later. Two additional animal groups served as untreated controls.

All animals were challenged three weeks after immunisation or two weeks after booster immunisation with a culture of *E. rhusiopathiae* strain FFM XI (approx. 100 LD_{50}) which is the German Reference Strain for the batch potency test of inactivated erysipelas vaccines. Table 1 summarises the test design.

Table 1 — *Immunisation-challenge test in laboratory mice*

Animal group	**No of animals**	**Treatment Immunisation**	**Challenge**
Group 1	10	Day 0	Day 21
Group 2	10	-	
Group 3	10	Day 0 + Day 21	Day 35
Group 4	9	-	

After challenge animals were monitored twice a day over a period of eight days. Mice demonstrating severe clinical signs of erysipelas infection were humanely killed.

3. RESULTS

A single immunisation of the antigen resulted in a protection rate of 90% against a severe challenge. The booster immunisation increased the protectivity to 100%. All control animals died or were humanely killed during a five day period.

The results are summarised in Table 2.

Table 2 — *Protection of laboratory mice immunised with the coating antigen*

Animal group		**Survival rate**
Single immunisation	(Group 1)	90% (9/10)
Control group	(Group 2)	0% (0/10)
Double immunisation	(Group 3)	100% (10/10)
Control group	(Group 4)	0% (0/9)

4. CONCLUSION

The ELISA coating antigen is highly protective after immunisation with aluminium hydroxide adjuvant. After booster immunisation the protectivity reached 100% in this study. Thus it can be concluded that the ELISA coating antigen cBRP is able to detect protective erysipelas antibodies in mouse serum samples.