Collaborative Study for Validation of a Serological Potency Assay for Rabies Vaccine (inactivated) for Veterinary Use

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ABSTRACT

European Pharmacopoeia (Ph. Eur.) monograph 0451 on Rabies vaccine (inactivated) for veterinary use describes an in vivo batch potency test that is based on the NIH test. This assay uses a large number of mice and results in a significant degree of suffering. In the interest of replacement, reduction and refinement of animal tests (3R) a serological potency assay for Rabies vaccine (inactivated) for animal use, developed and validated at the Paul-Ehrlich-Institut, has been assessed in a collaborative study organised by the EDQM (European Directorate for the Quality of Medicines & HealthCare). The goal was to demonstrate the wider transferability of the proposed assay and confirm its suitability. The study involved 13 laboratories and assessed 4 different vaccines from the EU market. Results of the study confirm that a limit test using a relatively small number of animals in a serological assay is possible, reproducible and reliable. The optimal number of animals per vaccine is product specific but may roughly be indicated to be between 8 and 10 for the products included in this study. Non-responders should be included in the analysis because they may reflect sub-potent vaccines. However, there may be a need to impose a maximum on the number of non-responders allowed for the reference vaccine as a monitor for assay validity. This assay provides a significant 3R improvement in terms of both the number of animals used and the amount of suffering entailed and provides a more reliable and reproducible assay format than the vaccination challenge assay. It also reduces the time required as compared to the vaccination challenge assay. It has been recommended to the Ph. Eur. group of experts 15V that this assay be included as an alternative to the batch potency assay in the Ph. Eur. monograph 0451.

KEYWORDS

Rabies vaccine (inactivated) for veterinary use, NIH test, 3R alternative, serological assay, batch potency test, European Pharmacopoeia.

1. AIM

The aim of the collaborative study was to demonstrate the wider transferability of the proposed assay and confirm its suitability for the potency assay of inactivated rabies vaccines for veterinary use.

2. INTRODUCTION

The Ph. Eur. monograph 0451 on Rabies vaccine (inactivated) for veterinary use [1] describes in section 3-5 an *in vivo* batch potency test that is based on the NIH test and involves a vaccination challenge assay with test and reference vaccines.

Section 2-4-3 of monograph 0451 allows the replacement of the vaccination challenge assay for batch potency by a validated alternative method and briefly describes a serology based assay.

The vaccination challenge assay is well known to be both heavy on animal use and a very harsh assay for the animals. It is also known to have high variability and can be problematic in terms of reaching all of the validity criteria [2, 3]. However, despite the possibility to use an alternative assay, the vaccination challenge assay is still widely used for batch release of inactivated rabies vaccines for veterinary use as attempts to use the serological assay presently outlined in the Ph. Eur. monograph have not been convincing.

In the interest of applying the 3R principle for humane alternatives to animal tests [4] and the goal of advancing harmonisation of an assay with potentially better reproducibility, the Paul-Ehrlich-Institut (PEI) has developed and validated an alternative serology assay in their laboratory [5]. The published assay involves the immunisation of groups of mice with the test vaccine diluted appropriately or the reference standard vaccine preparation which is adjusted to the minimum potency allowed. 14 days after immunisation blood samples are taken and the sera are tested individually for rabies antibody using a virus neutralisation assay for the detection of antibodies against rabies with a fluorescence detection method. Dilutions of the sera that reduce the number of fluorescent cells by 50 per cent are calculated. The vaccine passed if the antibody titres obtained with the test vaccines were greater than or equal to the antibody titres obtained for the reference standard (P=0.95). Two similar assays, the Rapid Fluorescent Focus Inhibition Test (RFFIT) and a Fluorescent Antibody Virus Neutralisation test (FAVN) are described in the World Organisation of Animal Health (OIE) Manual of Diagnostics Tests and Vaccines in a chapter concerning rabies as standard approved techniques [6]. Dr. Krämer convincingly presented the vaccine batch potency assay outlined in the publication [5] and the validation data from her laboratory at the annual meeting for OMCLs involved in batch release of veterinary vaccines in May 2008, Strasbourg, and to the Biological Standardisation Steering Committee of the European Pharmacopoeia in June 2008, Strasbourg. As a result the Biological Standardisation Steering Committee endorsed a collaborative study to validate the wider transferability of the proposed assay and to confirm its suitability for inactivated rabies vaccines on the European market. A small scale feasibility phase showed that the assay could be successfully transferred to another laboratory and the following collaborative study was then carried out.

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3. PARTICIPANTS

13 laboratories from 10 countries including EU Member States, Canada and the USA participated in the collaborative study. 8 were official control laboratories of Regulatory Authorities and 5 were manufacturers. Participants are listed in alphabetical order by country in section 9 of this report. Throughout the report the participants are referred to by a code number which is unrelated to the order of listing.

4. STUDY OUTLINE

4.1. Principle of the assay

The serological assay used involves the immunisation of groups of 6 mice with approximately 1/5th the recommended dose volume of the test vaccine diluted appropriately, or of the reference standard vaccine preparation which is adjusted to the minimum potency allowed in the Ph. Eur. 14 days after immunisation blood samples are taken and the sera are tested individually for rabies antibody using the described virus neutralisation assay. Briefly, sera are titrated on 96 well microtitre plates and incubated for 1h with rabies virus. After adding BHK cells and incubating for 48h the presence of un-neutralised rabies virus is revealed by immunofluorescence. Dilutions of the sera that reduce the number of fluorescent cells by 50 per cent are calculated.

4.2. Assay design

Laboratories were asked to test the potency of 4 different inactivated rabies vaccines for veterinary use using the candidate serological method. Samples were coded (A-D) so as not to reveal their identities. The vaccines represent a range of products available on the EU market and are produced by different manufacturers. Three independent repetitions of the assay with the 4 test vaccines and the reference preparation were to be performed. The serum neutralisation activity of the test vaccines were assessed for compliance by comparing against activity for the reference vaccine which was set at the minimum allowed potency.

In addition, participants were provided with a panel of 3 prepared test sera labelled High, Medium and Low to be tested in the virus neutralisation assay. The sera panel was to be tested on 3 separate occasions. All assays included a negative serum sample.

In addition to the main study, 1 laboratory accepted to retest each of the test samples using the Ph. Eur. vaccination challenge assay for Rabies vaccine (inactivated) for veterinary use (Ph. Eur. monograph 0451) to confirm the potency of the test samples.

4.3. Materials

4.3.1. Material provided by the EDQM

4.3.1.1. Test Samples

Sample A

A commercially produced current lot of Rabdomun (Essex Animal Health-Pfizer). A liquid presentation rabies vaccine (inactivated) of the Flury LEP strain. 1 mL/ dose. Approved specification >1 IU/dose. Manufacturer's estimated potency at release: 7.3 IU/mL.

Sample B

A commercially produced current lot of Rabigen (Virbac). A liquid presentation rabies vaccine (inactivated) of the PRV strain. 1 mL/ dose. Approved specification >1 IU/dose. Manufacturer's estimated potency at release: 15 IU/mL.

Sample C

A commercially produced expired lot (exp. date 06, 2005) of Biocan R inj. a.u.v. (Bioveta, a.s.). A liquid presentation rabies vaccine (inactivated) of the SAD Vnukovo strain. 1 mL/ dose. Approved specification >2 IU/dose. Manufacturer's estimated potency at release in 2003: 3 IU/mL. Potency as determined at PEI in 2009: 0.25 IU/mL.

Sample D

A commercially produced current lot of Nobivac Rabies (Intervet/Schering-Plough Animal Health). A liquid presentation rabies vaccine (inactivated) of the PRV strain. 1 mL/dose. Approved specification >3 IU/dose. Manufacturer's estimated potency at release: 8 IU/mL.

All test samples were kindly donated by the respective manufacturers.

Each laboratory received 6 vials (2 per test) of each test sample to be stored at 4°C upon receipt.

4.3.1.2. Control Sera (CS)

Control sera were prepared and pre-tested in the laboratory of the co-project leader at PEI.

3 vials of 100 µL were provided for each sera type to be stored at -20 °C upon receipt (1 vial per test).

Serum "low"

Mean estimated activity from 10 assays: 2.4 IU/mL, SD 0.98.

Serum "medium"

Mean estimated activity from 10 assays: 5.8 IU/mL, SD 1.12.

Serum "high"

Mean estimated activity from 10 assays: 28.1 IU/mL, SD 10.04.

Negative sera

Negligible activity.

4.3.1.3. Reference Material

Ph. Eur. BRP batch 4

A freeze dried preparation of 11 IU/vial to be reconstituted in 1 mL phosphate buffered saline pH 7.1. To be stored at -70 °C upon receipt.

Each laboratory received 3 vials.

2nd WHO IS for rabies immunoglobulin (RAI)

30 IU/ampoule

This material was to be reconstituted and diluted in 15 mL of PBS. Once completely resuspended the material should be aliquoted in 150 µL volumes and stored at –70°C until use.

Each laboratory received 1 vial.

4.3.1.4. Specific reagent

Fluorescein labelled antibody

Rabies antibody fluorescein conjugate (FDI, Microtest), to be dissolved in 5 mL Aqua dest. 4 aliquots of 1.25 mL were prepared and stored at –20°C upon receipt.

Each laboratory received 1 vial.

4.3.2. Other required materials supplied by the participant

Rabies is a zoonotic disease and is pathogenic for humans. While working with rabies virus, the local biosafety rules for this material must be strictly applied. Tests have to be performed in laboratories with adequate containment. The staff of the laboratory should be familiar with the handling of rabies virus. Only persons showing a sufficient anti-rabies antibody titre should perform rabies testing.

4.3.2.1. Instruments, Material

- Laminar flow
- CO_2 – incubator
- Fluorescence microscope, filter 450-490 nm
- Water bath (37°C)
- Test tube shaker (e.g. Vortex)
- 96 wells microtiter plates, flat bottom (Nunc, Greiner)
- Pipettes, variable (e.g. 10-100µL, 100-1000µL)
- Pipettes, multi-channel (50-200 µL)
- Syringes (1mL), needles (0.45 x 25 mm, 26Gx1) for vaccination, (0.60 x 25 mm, 23Gx1) for taking blood
- Serum tubes (1 mL, Eppendorf, Sarstedt)

4.3.2.2. Reagents, media, cells

- BHK-21 cells, 2.5 x 10^5 cells/mL
- Rabies challenge virus (adapted to BHK-21 cells), diluted to 100 $CCID_{50}$/50µL MEM-Earle (Biochrom AG Cat.No. F0315)
- Cell growth medium with 10% FCS (1l MEM-Earle with 10 mL L-glutamine, 10 mL NEA, 100 mL FCS)
- Dilution medium without FCS
- PBS, pH 7.1
- 80 % Acetone (diluted with H_2O)
- Mouse anesthetic (e.g. Ketamin 10 mg/mL with Xylazin 0.4 mg/mL; application: 0.1 mL intraperitoneal per 10g animal weight)

4.3.2.3. Animals

- NMRI mice, female, 18-20 g; 6 mice per vaccine and reference (30 mice per assay)

4.4. Method

Participants were provided with a common protocol to perform the assay, details of which are provided below.

4.4.1. Sera preparation

For each test: 6 animals per test vaccine and 6 animals per reference standard.

Animals were to be allowed an adaptation period of 2-3 days before immunisation.

4.4.1.1. Preparation of samples for injection

BRP4 standard was dissolved and diluted to 1 IU/mL using PBS (1 mL BRP4 + 10 mL PBS).

Sample A: no dilution

Sample B: no dilution

Sample C: diluted 1:2 using PBS (1 mL vaccine + 1 mL PBS)

Sample D: diluted 1:3 using PBS (1 mL vaccine + 2 mL PBS)

4.4.1.2. Immunisation

6 mice were vaccinated respectively with a test vaccine or the BRP4 standard.

Each mouse was injected intraperitoneally with 0.2 mL of the vaccine or the BRP (prepared as noted above).

4.4.1.3. Blood collection and preparation of sera

Blood was collected 14 days after immunisation by heart puncture after anaesthetising the animals (post-mortem puncture and jugular bleeding were accepted as a possible alternative to heart puncture). In either case the staff members should be well trained and experienced with the method they used. The anaesthetic used should be tested in 2-3 control mice prior to the test.

Serum was extracted after centrifugation (5,500 x *g*) and kept at -20°C until the neutralisation test was carried out.

4.4.2. Neutralisation Test

Each assay required:

- 2 control plates
- 2 plates per test sample
- 2 plates for the reference standard (BRP 4)

4.4.2.1. Plate set up and neutralisation

Prepare*:*

- standard serum RAI 30 IU/mL (IS) diluted to 2 IU/mL
- BHK-21 cells, 2.5 x 10^5 cells/mL
- working dilution of CVS = Rabies challenge virus (adapted to BHK-21 cells) diluted to 100 $CCID_{50}$/50 µL

Virus titration: prepare 10-fold dilution steps (10^{-1}-10^{-4}) from working dilution for the control plate; use 50 µL of each virus dilution (10^0-10^{-4}) per well.

CONTROL PLATE 1 (REFER TO PLATE LAYOUT)

Titration of RAI

Place 80 µL of the medium in wells A1-A6, 50 µL of the medium in wells B1-G6 and 200 µL of the medium in wells H1-H6. Add 20 µL of RAI (2 IU/mL) in wells A1-A6. Transfer 50 µL of each well from row A to H, discard 200 µL from wells H1-H6.

Virus titration

Place 50 µL of the medium in wells A7-E12. Add 50 µL of each virus dilution (10^0-10^{-4}) as indicated.

Negative control

Place 40 µL of the medium in wells G7-G12 and add 10 µL of negative serum in each well.

Cell control

Place 100 µL of the medium in wells H7-H12.

CONTROL PLATE 2 AND SERUM TITRATION PLATES (REFER TO PLATE LAYOUT)

- Place 80 µL of the medium in wells A1-A12.
- Place 50 µL of the medium in wells B1-G12.
- Place 200 µL of the medium in wells H1-H12.
- Perform 2-fold dilution steps with 4 replicates per control serum or test serum: pipette 20 µL of each of the 1st test serum into wells A1-A4, 20 µL of the 2nd test serum into wells A5-A8, etc. Transfer 50 µL of each of the serum dilutions from row A to B to C until H. Discard 200 µL from row H.

Control plate 1

	1	2	3	4	5	6	7	8	9	10	11	12
	RAI						CVS					
A	1:5	1:5	1:5	1:5	1:5	1:5	10^0	10^0	10^0	10^0	10^0	10^0
B	1:10	1:10	1:10	1:10	1:10	1:10	10^{-1}	10^{-1}	10^{-1}	10^{-1}	10^{-1}	10^{-1}
C	1:20	1:20	1:20	1:20	1:20	1:20	10^{-2}	10^{-2}	10^{-2}	10^{-2}	10^{-2}	10^{-2}
D	1:40	1:40	1:40	1:40	1:40	1:40	10^{-3}	10^{-3}	10^{-3}	10^{-3}	10^{-3}	10^{-3}
E	1:80	1:80	1:80	1:80	1:80	1:80	10^{-4}	10^{-4}	10^{-4}	10^{-4}	10^{-4}	10^{-4}
F	1:160	1:160	1:160	1:160	1:160	1:160						
G	1:320	1:320	1:320	1:320	1:320	1:320	NC	NC	NC	NC	NC	NC
H	1:1600	1:1600	1:1600	1:1600	1:1600	1:1600	CC	CC	CC	CC	CC	CC

RAI=immunoglobulin standard, CVS=rabies virus, NC=negative control, CC=cell control

Control plate 2 and serum titration plates

				1	2	3	4	5	6	7	8	9	10	11	12
Medium	Sera/ transfer	Dilution		CS / Low				CS /Medium				CS / High			
80 µL	20 µL	1:5	A												
50 µL	50 µL	1:10	B												
50 µL	50 µL	1:20	C												
50 µL	50 µL	1:40	D												
50 µL	50 µL	1:80	E												
50 µL	50 µL	1:160	F												
50 µL	50 µL	1:320	G												
200 µL	200 µL	1:1600	H												

CS=control sera (high, medium or low)

- Add 50 µL of CVS working dilution per well for all wells in control plate 2 and the serum titration plates and to the RAI and NC wells of control plate 1.
- Incubate the plates for 1h at 37°C in a CO_2 incubator.
- Add 100 µL of BHK-21 cell suspension (2.5 x 10^5 cells/mL) to each well in the serum titration plates and to each of the wells of the control plates.
- Incubate plates at 37°C in a CO_2 incubator for 48 h ± 1h.

4.4.2.2. Staining of infected cells (day 2) (48 h ± 1h)

- Discard medium and wash MTP once by adding 100 µL of PBS in each well.
- Discard PBS, add 100 µL of acetone (80% at room temperature) per well.
- Incubate MTP for 30 min at room temperature.
- Discard acetone and dry MTP in the air.
- Dilute an aliquot of the rabies conjugate 1:100 with PBS (e.g. 1.2 mL conjugate +118.8 mL PBS).
- Add 90 µL of conjugate per well and incubate at 37°C for 30 min.
- Discard conjugate, wash twice with 150 µL of PBS and once with 150 µL of distilled water.
- Dry MTP in the air.

4.4.2.3. Test evaluation

Read the plates under UV-microscope (100-fold magnification) and note the results in the test protocol:

Virus is neutralised → Negative immunofluorescence (sign –)

Virus is not neutralised → Positive immunofluorescence (sign +)

The test is valid if:

- all cell controls (CC) are free from fluorescing cells;
- the titre of the CVS used is between 1.5 and 2.5 $\log_{10}$ ID_{50}/50 µL.

A vaccine complies if a one-sided limit test (Wilcoxon-Mann-Whitney's exact test) shows that antibody titres obtained with the test vaccines are greater than the antibody titres obtained for the standard (P=0.95).

5. RESULTS

13 laboratories submitted results from assays. In this report they are referred to by their code numbers (1 to 13) allocated at random and not necessarily corresponding to the order of listing in the list of participants. 11 of the 13 laboratories carried out 3 fully independent assays using 6 mice per assay and per vaccine, as requested. Laboratory 10 reported results from 1 assay only and laboratory 13 performed 3 assays using 6 mice per assay per vaccine but all the sera were evaluated in the same experiment. Statistical evaluation was performed at the EDQM.

5.1. Serum activities by serology

The virus neutralisation ratios were submitted to statistical calculations using the CombiStats software [7]. The dose/response curves were estimated using the probit model [8]. In cases where no slope could be estimated, the Spearman/Kaerber method was used. The relative activity per serum was calculated against the 2nd International Standard for RAI. An example sheet is provided in Appendix 1. Activities below the quantification limit were set to 0 IU/mL and these are designated as "non-responders" in the remainder of this report. Activities above the quantification limit were set to 999 IU/mL which is an arbitrarily chosen value higher than the highest measurable activity with the chosen design. The exact value is irrelevant since subsequent analyses are based on rank-orders. As it is more convenient to work with activities on an arithmetic scale they were transformed

to logarithms after addition of 1 IU/mL to avoid problems with non-responders. All transformed titres are therefore in the range 0 (for non-responders) to 3 (for full responders). A complete overview is given in Table 1 and a graphical rendering of the data is provided in Figure 1.

In order to assess the intra- and inter-laboratory variation due to the serological method itself, 3 centrally provided control sera were included in each assay. All laboratories provided data from 3 independent assays as requested, except for laboratory 13 which provided only 1 set of data. The log transformed activities are listed in Table 2 and a graphical representation of the mean value per laboratory is given in Figure 2. The overall mean of the sera is 0.44 IU/mL, 0.85 IU/mL and 1.38 IU/mL for the low, medium and high control sera respectively, with respective standard deviations of 0.13 IU/mL, 0.18 IU/mL and 0.23 IU/mL. This implies that there is an overall satisfactory separation between the 3 sera and that the vast majority of the individual results stay within a 2-fold range around the overall mean.

5.2. Potency of the vaccines

The potency of the test vaccines A, B, C and D was assessed by comparison of the induced serum activities with those induced by reference preparation BRP Batch 4 (assigned potency 11 IU/vial). The dilution scheme was carefully designed for optimal discrimination (pass/fail) at the critical level of the labelled potency. The BRP had to be diluted to 1 IU/mL and 0.2 mL had to be injected into the mice, which is the dose that was expected to induce measurable activities at the low end of the dose-response regression. The test vaccines had to be administered at a dose of 0.2 IU on the basis of the labelled potency. For vaccines A and B, which both had a labelled potency of 1 IU/mL this implied 0.2 mL of undiluted vaccine. The rationale is that if the test vaccines induce higher activities than the BRP at this dose, the conclusion is justified that the injected dose was actually more than 0.2 IU and therefore the original sample contained at least 1 IU/mL. For vaccines C and D, which had labelled potencies of 2 IU/mL and 3 IU/mL respectively, this implied a 1:2 and 1:3 dilution prior to injection of 0.2 mL into the mice. The rationale is similar: if higher activities are induced than the BRP, the injected dose was actually more than 0.2 IU and therefore the diluted samples contained more than 1 IU/mL. It then follows that the undiluted samples contained at least 2 IU/mL and 3 IU/mL respectively.

The activities were compared with the one-sided exact test of Wilcoxon-Mann-Whitney [9] using the CombiStats software. This test is a non-parametric test based on rank-orders and is robust against non-normality and outliers. If the test is significant ($P<0.05$) and the mean response of the test vaccine is higher than the mean response of the BRP, the vaccine is said to pass the release test. Otherwise it fails or additional testing would have to be performed in practice. It should be noted that a significant p-value alone is not sufficient as it may also indicate that the test-vaccine is significantly less potent than the required limit. This is because the software decides on the direction of the one-sidedness of the test on the basis of the mean response. An example sheet is provided in Appendix 2. An overview of the resulting p-values is given in Table 3a for the individual assays (6 mice per vaccine) and in Table 3b for the pooled results per laboratory (18 mice).

As part of the study, 1 laboratory carried out the challenge assay in mice to confirm the expected potencies. They were found to be 9.2 IU/vial, 3.9 IU/vial, 2.1 IU/vial and 19.2 IU/vial for vaccines A, B, C and D respectively. Before the study the project leader also determined the potency of vaccine C using the *in vivo* assay, with an averaged result of 0.25 IU/mL from 2 assays.

Vaccine C was included in this study because it was known to be sub-potent and was therefore expected to fail the test. The other vaccines were expected to pass the test. As can be seen from Table 3a this is confirmed in most of the assays with correct decisions in 90%, 90%, 97% and 74% of the assays for the 4 test vaccines respectively. When the results of the 18 mice per laboratory are pooled the number of correct decisions is 100% for vaccines A and B. Laboratory 2 has a repeatable problem with the correct assessment of vaccine C. Laboratory 5 has a repeatable problem with the correct assessment of vaccine D. As the pass/fail decision is very clear and reproducible in all other laboratories it would be worthwhile to investigate why these 2 laboratories deviated so largely from the overall outcome.

5.3. Inclusion or exclusion of non-responders

It is known that some animals tend to show no immune reaction, even for relatively high doses. To overcome this problem it had been suggested to exclude non-responders from the statistical analyses. The resulting p-values upon exclusion of non-responders are also shown in Tables 3a and 3b. However, as can be seen from Table 1 the number of non-responders tends to depend on the potency of the vaccines. The rate of non-responders is 5.9% for the BRP, 3.6% for vaccine A, 2.3% for vaccine B, 7.2% for vaccine C and 2.7% for vaccine D. The rate for the sub-potent vaccine C is markedly higher than for the high potency vaccines A, B and D. This observation indicates that exclusion of non-responders can bias the outcome of the assay as the non-responsiveness may not be entirely intrinsic to the animal, but may reflect a sub-potent vaccine. Moreover, as can be seen from Tables 3a and 3b exclusion of non-responders does not improve the number of correct pass/fail decisions. To reflect the true potency non responders should therefore be included in the analysis. However, there may be a need to impose a maximum on the number of allowed non-responders for the reference vaccine, e.g. not more than 1 non-responder in a set of 6, or not more than 2 in a set of 8, in order to monitor assay validity. An example of how calculations could be performed to determine the number is provided in appendix 3.

5.4. Number of animals required

As can be expected, the number of correct pass/fail decisions increases with the number of animals included in the assay. A number of 6 animals per vaccine may be sufficient for vaccines that are well above the labelled potency, but for vaccines closer to this limit a higher number of animals may be required. As can be seen from Table 3b the number of correct pass/fail decisions becomes in many laboratories almost 100% when the results of 18 mice are pooled. The optimal number of animals will probably be less than 18. In order to get an idea about the optimal number of animals for the vaccines included in this study, the results were submitted to bootstrap resampling for sample sizes between 3 and 12 drawn from the set of 18 mice, with replacement and ignoring inter-assay variation. The estimated rate of “pass” decisions is listed in Table 4. Each percentage is the result of 100.000 bootstrap simulations. Ideally the percentages should be 100% for vaccines A, B and D and should be 0% for vaccine C. It can be seen that a quite satisfactory discriminative power can in many cases already be achieved with 8-10 animals. The use of only 6 animals may be sufficient in some cases, but in general it seems recommendable to use at least 8 animals. Using more than 10 animals does not noticeably increase the power of the test anymore.

6. DISCUSSION

The Ph. Eur. monograph 0451 on Rabies vaccine (inactivated) for veterinary use [1] describes in section 3-5 an *in vivo* batch potency test that is based on the NIH test and involves a vaccination challenge assay with test and reference vaccines. A single *in vivo* assay for a given test vaccine requires the use of at least 100 animals. Due to the variable nature of the assay and the difficulties encountered meeting the validity criteria it is in addition often necessary to repeat the assay [2,3]. Section 2-4-3 of the monograph 0451 allows the replacement of the vaccination challenge assay for batch potency by a validated alternative method and briefly describes a serology based assay. Despite the possibility of using a serological assay until present this has not been put into practice.

B. Krämer and colleagues at PEI have developed and validated a serological potency assay for Rabies vaccine (inactivated) for veterinary use which greatly reduces the number of animals required and refines considerably the amount of suffering entailed [5]. The aim of this collaborative study was to demonstrate the wider transferability of the proposed assay and confirm its suitability for the potency assay of inactivated rabies vaccines for veterinary use with the ultimate goal of encouraging its use as a replacement method for the vaccination challenge assay for batch potency. The study involved 13 laboratories who tested 4 different vaccines from the European market with different strain composition. One of these samples was known to be of insufficient potency. Centrally provided control sera of different activity was also provided to help evaluate the assay *in vitro* aspects of the method.

Results using commonly provided control sera of high, medium and low potency (Table 2 and Figure 2) demonstrate good intra- and inter-laboratory variation of the *in vitro* component of the method as evidenced by the overall satisfactory separation between the 3 sera and the vast majority of the individual results which stay within a 2-fold range around the overall mean. The traditional vaccination challenge assay is known to be highly variable [2, 3] and as a reflection of this specifications in the Ph. Eur. monograph 0451 are set with confidence limits of not less than 25 per cent and not more than 400 per cent of the estimated potency. This study suggests that the proposed method would provide a significant improvement with respect to assay repeatability and reproducibility. It should also be noted that the vaccination challenge assay requires at least 4 weeks for completion not including the assay preparation and data analysis. The proposed assay could be performed in just under 3 weeks thus providing a reduction in the time required to complete the analysis.

Potency of the test vaccines were compared to the reference standard (BRP4) using a non-parametric limit test based on rank-orders which is robust against non-normality and outliers. Three vaccines of sufficient potency (A, B and D) and 1 of insufficient potency (C) were evaluated against the BRP. As noted in Tables 3a and 3b the vast majority of individual assays (6 mice) resulted in the appropriate outcome. When results per laboratory were pooled (18 mice), 100 per cent of correct results were found for vaccines A and B. For vaccines C and D all laboratories but 1 found the correct result. Laboratory 2 had a repeatable problem with vaccine C and Laboratory 5 had a repeatable problem with vaccine D. It is unclear why these 2 laboratories had discrepant results in these specific cases. Although the method was new to all users the technique seems to have been well transferred as evidenced by the results for the other vaccines in those labs. There may have been unaccounted for problems with dilution for individual assays however this is difficult to confirm. Participants were all requested to use female NMRI mice and no one reported a deviation from this strategy. However as always with animal experiments it remains possible that the source of strains used could be a contributing factor to observed differences.

In the strategy used in this study 1/5th the recommended dose volume of the test vaccine diluted appropriately, or of the reference standard vaccine preparation which is adjusted to the minimum potency allowed in the Ph. Eur. was administered to the mice. The Ph. Eur. monograph 0451 minimum requirement for potency of an inactivated rabies vaccine for veterinary use is 1 IU/dose. The reference material (BRP4) was thus diluted to 1 IU/mL (on the basis of a 1 mL dose) and 0.2 mL was injected into each mouse. For test vaccines A and B the approved specification is 1 IU/mL so 0.2 mL of the vaccines were administered undiluted. However some of the vaccines tested had approved specifications above 1 IU/mL (2 IU/mL for vaccine C and 3 IU/mL for vaccine D). As the activity of the test vaccines was to be compared to the reference (at 1 IU/mL) the test vaccines were diluted appropriately (1:2 or 1:3 respectively) before administration for a valid comparison. As evidenced by the results, this strategy worked effectively in the given circumstances. Application of this strategy for a given vaccine should be validated on an individual basis. It may also be interesting when developing a standard strategy for a vaccine with a specification higher than 1 IU/mL to consider applying the reference vaccine at the approved cut off dose (e.g. 2 IU/mL or 3 IU/mL) and then administering the test vaccine undiluted. This variation in the approach would have to be appropriately validated for the individual case.

In the assay set up of this study, 6 animals were used per sample. While this resulted in the majority of individual assays achieving the anticipated result it can be expected that the number of correct pass/fail decisions increases with the number of animals used in the assay. An analysis of all the available data using a simulation to determine the results for sample sizes between 3 and 12 was performed and the results are provided in Table 4. It appears that satisfactory results with sufficient discriminating power can be obtained in many cases with 8 -10 animals. While the use of 6 animals may be sufficient in some cases and particularly if the vaccines are well above the labelled potency, in general at least 8 animals would be recommended. Depending on the vaccine 10 animals may be required however more than 10 does not appear to provide significant advantages. In any case the use of 8-10 animals per test group would significantly reduce the number of animals required per assay to 16-20 (reference and test vaccine/assay) as compared to 100 for the vaccination challenge assay. The final choice of group size should be established based on evidence collected during the validation for individual vaccines.

In any animal experiment it may be expected to encounter some animals that are non-responders i.e. that show no immune reaction even at very high doses. It had been suggested that animals which show no response in this assay could be considered non-responders and excluded from the final analysis. To evaluate the impact of this proposal results were calculated both with and without the presumed non-responders. The results are shown in Tables 3a and 3b. The number of animals without measurable response appears to depend on the potency of the vaccine. The number is lower for vaccines A, B and D which have potencies above the cut off and higher for vaccine C which has reduced potency. This higher number of animals which lack a measurable response in the case of vaccine C is presumably due to the presence of both ‘real’ non-responders and a lack of

response related to sub-potency of the vaccine. As such the exclusion of non-responders could bias the assay outcome. It is also noted that the exclusion of non-responders does not improve the number of correct pass fail decisions. It is therefore unadvisable to remove non-responders from the final calculations. Nevertheless it may be useful to impose a maximum on the number of allowed non-responders for the reference vaccine in order to keep surveillance on the validity of the assays once assay conditions are established and validated e.g. not more than 1 non-responder in a set of 6 or not more than 2 in a set of 8 (example calculations provided in appendix 3).

The assay outlined in the collaborative study is a limit test which is able to discriminate vaccine samples that have a potency value above the approved minimum. As noted in section 5 and throughout the discussion, individual validation for specific vaccines can fine tune the discriminatory power to specific situations by choosing the most suitable conditions in terms of reference dose, dilutions used and animal number. A survey of typical vaccine batches from EU manufacturers suggests that in general the vaccines are formulated with a comfortable margin above the minimum allowed potency. As such this assay could be an effective tool as a batch potency test in confirming the compliance with the specifications. It provides a real advantage in terms of animal welfare and assay consistency. However, in cases where the batch potency of the test vaccine is at the limit of acceptable potency values, significantly more animals would have to be used in order to determine if the batch were just above, or just below the line. Alternatively in such cases the vaccination challenge assay might be used to assess the potency, although the vaccination challenge assay has also its limitations, particularly with respect to its precision.

Although the final result of the assay is a limit test which does not involve a final 'potency value' for the test vaccine, the relative activity per serum is calculated against a common standard for rabies immunoglobulin (2nd IS RAI) and the assay includes the calculation of the overall mean of the sera from the test and reference preparations. These values if charted over time can be used to help monitor both the assay consistency (with respect to the values obtained for the BRP) and production consistency (by observing the values obtained for successive batches of the test vaccine).

7. CONCLUSIONS AND RECOMMENDATIONS

The study has shown that a limit test using a relatively small number of animals in a serological assay as compared to the full vaccination challenge *in vivo* potency test is possible, reproducible and reliable. The optimal number of animals per vaccine is product specific but may roughly be indicated to be between 8 and 10 for the products included in this study. Non-responders should be included in the analysis because they may reflect sub-potent vaccines. However, there may be a need to impose a maximum on the number of non-responders allowed for the reference vaccine as a monitor for assay validity. In this study it was necessary to fix a given dose for the reference that would be used to compare with all test vaccines but the optimal dose for reference and test vaccine should be determined during the in-house validation of the method to further improve its discriminative power.

This assay provides a significant 3R improvement for the batch potency testing of Rabies vaccine (inactivated) for veterinary use in terms of both the number of animals used and the amount of suffering entailed and as evidenced by the study provides a more reliable and reproducible assay format than the vaccination challenge assay. It also reduces the time required as compared to the vaccination challenge assay.

The Ph. Eur. already allows for replacement of prescribed assays by 3R alternatives provided sufficient validation has been carried out. This collaborative study is a major step in the validation of such an assay and all users are strongly encouraged to take the remaining necessary steps to validate and implement the assay in their own specific situations without delay.

It is also strongly recommended that details of this assay be taken up by the group of experts 15V of the Ph. Eur. for inclusion as an alternative assay to the batch potency test in monograph 0451. This would be an important step to facilitate the regulatory implementation of the assay.

Additionally, international partners are encouraged to review the data and consider acceptance of the assay in their own systems as this is an important contributing factor to successful implementation of alternative methods in light of the global nature of the veterinary vaccine industry.

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10. ABBREVIATIONS

3R: Replacement, reduction, refinement of animal tests, BHK: Baby hamster kidney cell line, BRP: Biological Reference Preparation, BSP: Biological Standardisation Programme, $CCID_{50}$: Cell culture infectious dose 50%, CS: Control serum, CVS: Challenge virus strain, EDQM: European Directorate for the Quality of Medicines & HealthCare, EU: European Union, FAVN: Fluorescent Antibody Virus Neutralization, FCS: Fœtal calf serum, FDI: Fujirebio Diagnostics, Inc., IS: International standard, IU: International unit, IVI: Institute of Virology and Immunoprophylaxis, LEP: Low egg passage, MEM: Minimum Essential Medium, MTP: Microtitre plate, NEA: Non-essential amino acids, NIH: National Institute of Health, NMRI: Naval Medical Research Institute, OIE: World Organisation for Animal Health (Office International des Epizooties), OMCL: Official Medicines Control Laboratory, PBS: Phosphate-buffered saline, PEI: Paul-Ehrlich-Institut, Ph. Eur.: Pharmacopée Européenne / European Pharmacopoeia, PRV: Pseudo-rabies virus, RAI: Rabies immunoglobulin, RFFIT: Rapid Fluorescent Focus Inhibition Test, SAD: Street–Alabama–Dufferin, SD: Standard deviation, USA: United States of America, UV: Ultraviolet

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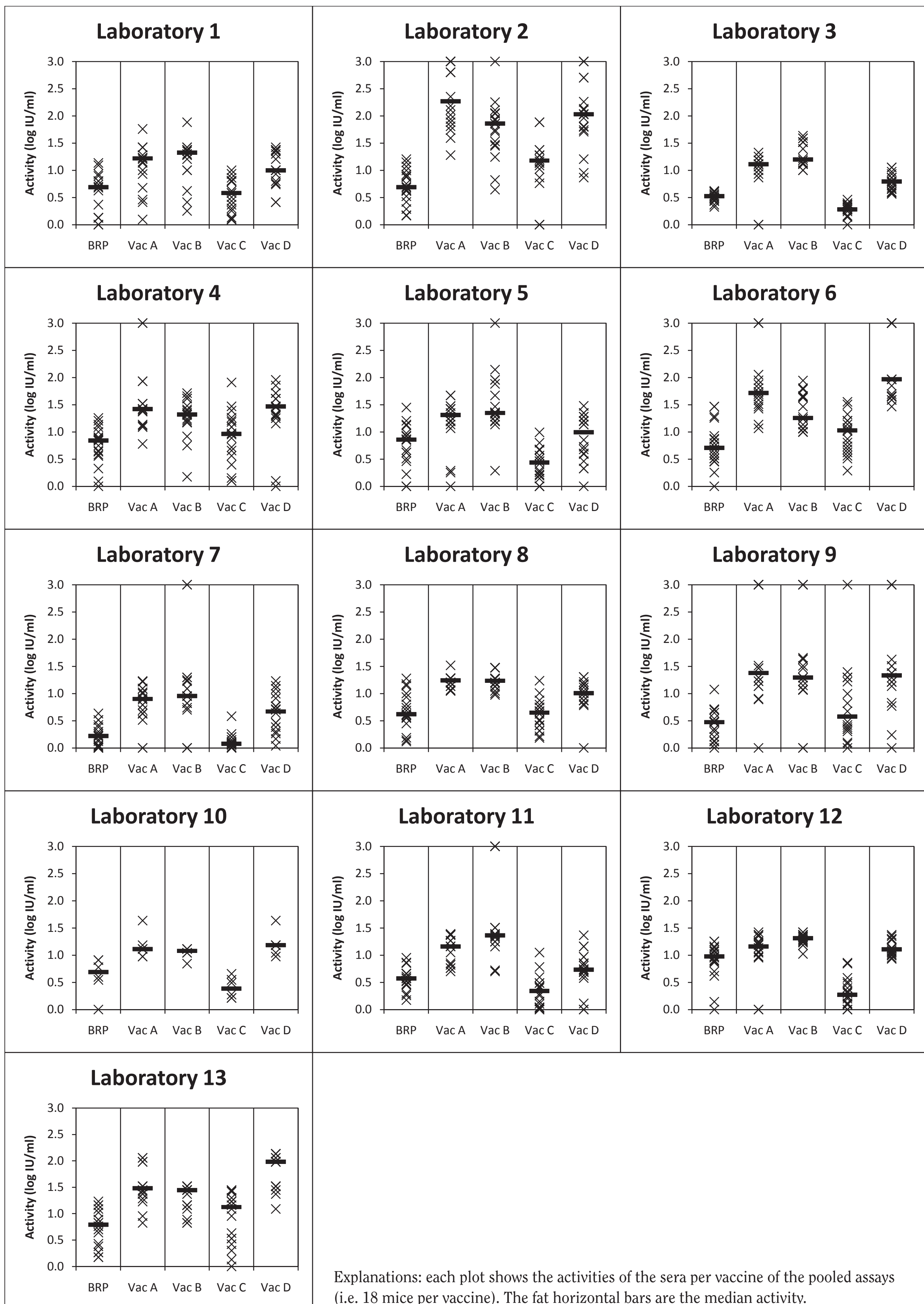
Table 1 – *Estimated activities per serum (log IU/mL)*

Lab	Mouse	BRP Batch 4			Vaccine A			Vaccine B			Vaccine C			Vaccine D		
		Assay 1	Assay 2	Assay 3	Assay 1	Assay 2	Assay 3	Assay 1	Assay 2	Assay 3	Assay 1	Assay 2	Assay 3	Assay 1	Assay 2	Assay 3
1	1	0.740	0.368	1.090	1.207	1.137	1.160	1.278	0.621	1.373	0.368	0.507	0.583	0.802	1.349	1.373
	2	0.802	0.000	0.895	0.679	1.422	1.230	1.349	1.278	1.373	0.999	0.933	0.131	0.413	1.207	1.302
	3	0.000	0.932	0.699	1.422	1.422	1.760	1.422	0.413	1.373	0.621	0.810	0.257	1.422	0.999	1.373
	4	0.802	0.679	0.131	0.413	0.461	0.097	0.999	0.999	1.373	0.092	0.106	0.583	0.740	0.999	0.760
	5	0.802	0.125	0.000	1.422	0.999	1.230	0.254	1.884	1.302	0.802	0.862	0.435	0.932	0.413	1.373
	6	0.125	1.137	0.627	1.278	0.932	1.230	1.207	1.422	1.373	0.621	0.792	0.301	0.999	0.999	1.373
2	1	0.626	0.755	0.617	1.884	3.000	2.801	3.000	0.823	1.449	0.866	1.090	1.199	1.810	1.739	1.795
	2	0.175	0.524	0.921	3.000	2.111	2.801	1.472	1.534	2.104	1.278	1.022	1.274	3.000	0.954	2.131
	3	0.679	0.645	0.164	3.000	1.739	2.017	1.244	1.742	1.711	0.000	1.373	1.133	3.000	2.111	1.712
	4	1.147	0.894	0.995	3.000	1.592	1.941	0.645	2.053	1.859	0.000	1.373	0.000	3.000	2.036	2.029
	5	0.417	0.699	1.071	1.810	2.185	2.801	1.919	2.252	1.859	1.884	0.760	1.274	0.866	1.961	2.704
	6	0.289	0.875	1.206	1.278	3.000	2.355	1.919	2.040	1.940	1.884	1.160	1.274	1.207	2.260	2.704
3	1	0.602	0.437	0.461	1.113	1.183	0.932	1.520	1.183	0.999	0.277	0.277	0.461	0.863	0.607	0.866
	2	0.602	0.437	0.511	1.183	1.113	0.999	1.520	1.113	1.137	0.000	0.354	0.327	0.974	0.773	0.932
	3	0.494	0.538	0.327	1.183	1.183	1.067	1.520	1.183	0.999	0.277	0.354	0.368	0.863	0.652	0.866
	4	0.494	0.538	0.565	1.325	1.113	0.866	1.395	1.637	1.588	0.138	0.160	0.168	0.789	0.652	0.679
	5	0.602	0.538	0.621	1.254	0.000	0.000	1.218	1.113	1.588	0.398	0.243	0.289	1.053	0.719	0.802
	6	0.602	0.437	0.368	1.113	1.113	0.999	1.520	1.113	1.137	0.314	0.277	0.368	1.053	0.587	0.565
4	1	0.836	0.634	1.137	1.397	1.090	1.422	1.239	1.387	0.917	0.398	0.954	0.560	0.000	1.287	1.611
	2	1.256	0.000	0.999	1.933	1.519	3.000	1.659	1.713	1.614	0.977	1.090	0.718	1.470	1.452	1.852
	3	0.905	0.881	1.207	0.781	1.519	1.422	1.179	1.170	0.177	1.470	0.152	1.263	0.102	1.713	1.611
	4	0.655	1.090	0.327	1.113	1.519	1.137	1.329	1.310	1.210	1.183	0.888	1.215	1.470	1.309	1.254
	5	0.559	0.583	0.866	1.933	1.373	1.422	0.751	1.354	1.416	0.102	1.090	1.391	1.958	1.153	1.611
	6	0.851	0.747	0.092	3.000	1.373	1.422	1.179	1.480	1.614	0.659	1.908	0.103	1.325	1.713	1.611
5	1	0.665	0.621	0.866	1.477	1.422	0.289	1.671	3.000	1.884	0.000	0.802	0.415	1.479	1.137	1.278
	2	0.848	0.000	1.207	1.477	1.349	0.254	1.360	1.349	1.137	0.000	0.145	0.000	0.726	1.349	1.278
	3	0.975	0.621	0.000	1.671	1.067	1.207	1.332	1.958	1.278	0.216	0.461	0.255	0.855	0.327	1.137
	4	0.917	0.511	0.222	1.671	1.422	1.137	1.195	1.958	1.207	0.991	0.679	0.462	0.604	0.327	1.207
	5	1.118	0.866	1.137	0.000	1.422	1.278	1.466	1.349	0.289	0.668	0.565	0.289	0.593	0.000	0.461
	6	1.449	0.932	0.461	1.195	1.422	1.207	2.144	1.349	1.278	0.668	0.511	0.195	1.196	1.349	0.679
6	1	0.454	0.508	1.466	1.509	1.137	1.807	1.783	1.067	1.058	0.871	1.325	0.736	1.654	1.662	3.000
	2	0.513	0.794	0.619	1.652	1.067	1.697	1.944	1.662	0.998	0.624	1.552	1.204	1.848	1.588	1.697
	3	0.000	0.461	0.737	1.950	1.736	3.000	1.629	0.999	1.465	1.075	0.289	1.204	1.968	3.000	1.466
	4	1.286	0.863	0.253	1.430	3.000	3.000	1.644	1.137	1.240	1.141	0.562	1.204	1.645	3.000	3.000
	5	0.874	0.571	1.466	2.052	1.588	1.697	1.644	1.067	1.240	0.680	1.039	0.805	1.968	3.000	3.000
	6	1.258	0.680	0.927	1.876	1.736	1.466	1.277	0.999	1.807	1.507	1.015	0.508	1.968	1.588	3.000
7	1	0.261	0.232	0.000	0.000	0.634	1.233	0.000	0.823	3.000	0.071	0.115	0.066	0.383	0.712	0.039
	2	0.633	0.383	0.123	1.090	1.220	0.516	1.090	1.251	1.298	0.000	0.081	0.068	0.700	0.168	0.461
	3	0.423	0.208	0.515	1.022	0.998	0.914	0.954	0.000	1.298	0.203	0.201	0.257	1.088	0.460	1.144
	4	0.232	0.000	0.515	0.888	1.220	0.694	0.954	3.000	0.000	0.051	0.097	0.583	0.760	0.998	0.913
	5	0.000	0.051	0.034	1.090	0.790	0.629	1.090	0.699	0.740	0.071	0.071	0.095	0.768	0.301	1.232
	6	0.301	0.154	0.016	1.022	0.790	0.000	0.954	0.823	1.227	0.000	0.020	0.146	0.642	0.267	0.461
8	1	0.598	0.621	0.935	1.048	1.137	1.278	1.126	1.475	1.207	0.669	0.743	0.518	1.050	1.005	1.215
	2	0.548	0.999	0.687	1.197	1.207	1.278	1.265	1.005	1.207	0.212	0.743	1.008	1.305	1.161	1.008
	3	0.187	0.565	1.280	1.048	1.207	1.278	0.975	1.475	1.278	0.182	0.743	0.628	1.114	0.808	1.008
	4	0.598	0.740	0.451	1.285	1.137	1.207	1.191	1.475	1.137	0.314	0.796	0.875	1.305	0.869	0.874
	5	0.143	0.802	1.146	1.520	1.067	1.278	1.050	1.475	1.278	0.498	1.237	0.419	0.783	0.000	0.874
	6	0.117	0.621	1.173	1.285	1.278	1.278	1.126	1.475	1.278	0.317	0.416	0.679	1.177	0.944	0.874
9	1	0.699	0.490	0.646	1.467	3.000	3.000	0.000	1.075	3.000	0.422	1.297	0.099	1.377	3.000	3.000
	2	0.477	0.472	0.535	0.904	1.292	1.229	1.658	1.294	1.066	0.383	3.000	0.675	1.135	1.291	1.216
	3	0.301	0.348	0.698	3.000	3.000	3.000	1.147	1.294	3.000	0.824	0.074	0.998	0.000	1.505	0.239
	4	0.131	1.075	0.000	1.135	0.000	1.229	1.511	1.294	1.228	1.397	1.297	0.306	1.377	1.627	0.770
	5	0.477	0.116	0.061	1.467	1.292	0.891	0.000	1.631	1.066	0.477	1.297	0.348	0.829	1.505	1.216
	6	0.344	0.714	0.206	1.135	1.515	3.000	1.464	1.631	3.000	0.346	0.000	1.219	1.377	3.000	1.216
10	1	0.602			0.977			0.845			0.547			1.637		
	2	0.910			1.113			1.113			0.659			1.183		
	3	0.000			1.637			1.045			0.494			1.045		
	4	0.547			1.183			1.045			0.277			1.183		
	5	0.910			0.977			1.113			0.212			1.183		
	6	0.781			1.113			1.113			0.212			0.977		
11	1	0.468	0.174	0.608	0.852	0.699	1.161	1.507	1.251	3.000	0.257	0.000	0.000	0.578	0.112	0.968
	2	0.534	0.260	0.663	1.378	0.760	1.370	1.248	1.322	1.158	0.288	0.150	0.487	0.000	0.723	0.000
	3	0.517	0.292	0.663	1.265	0.760	1.370	1.376	1.251	1.350	0.418	0.106	0.487	0.696	0.844	0.968
	4	0.578	0.657	0.663	1.265	1.394	1.161	1.507	3.000	0.723	0.036	0.042	0.785	0.854	0.805	1.160
	5	0.853	0.451	0.952	1.051	0.823	1.370	3.000	0.699	1.350	0.022	0.430	0.396	0.629	0.686	1.160
	6	0.569	0.468	0.865	1.265	0.823	1.161	1.507	1.394	3.000	1.051	0.423	0.598	0.696	0.743	1.365
12	1	1.067	0.977	0.954	1.422	0.977	1.373	1.349	1.183	1.373	0.088	0.119	0.199	0.999	1.048	1.302
	2	0.866	1.254	1.090	1.137	1.183	1.230	1.349	1.254	1.373	0.000	0.102	0.477	1.349	1.124	0.954
	3	0.145	0.000	1.160	1.067	0.000	1.373	1.278	1.254	1.302	0.324	0.000	0.232	1.067	1.282	1.090
	4	0.621	1.254	0.888	1.349	1.254	1.373	1.422	1.183	1.022	0.508	0.398	0.583	0.932	1.124	1.373
	5	1.067	0.910	0.699	1.067	0.977	0.000	1.278	1.325	1.230	0.862	0.845	0.335	1.137	1.124	1.022
	6	1.137	0.977	1.022	1.207	1.045	0.954	1.422	1.325	1.373	0.000	0.243	0.301	1.067	0.991	1.302
13	1	0.823	1.022	0.699	1.286	1.519	1.983	1.160	1.446	1.519	1.160	0.421	0.000	2.132	1.446	1.519
	2	0.888	0.823	1.090	1.375	1.519	1.373	1.160	0.823	0.823	0.131	0.301	0.529	2.057	1.983	1.983
	3	0.428	0.640	1.160	1.982	2.057	1.230	0.888	1.519	1.519	1.446	1.415	1.302	1.519	1.519	1.519
	4	0.383	1.160	0.176	1.375	1.519	1.446	1.446	1.373	1.519	0.954	1.230	0.529	1.373	2.132	1.090
	5	0.888	0.760	0.265	0.954	1.519	1.519	1.446	1.446	1.519	1.230	1.438	1.230	1.983	2.132	1.519
	6	1.230	0.265	0.176	0.823	1.422	2.057	1.446	1.090	1.160	1.230	0.630	1.090	3.000	2.132	2.057

Explanations: grey cells indicate non-responders.



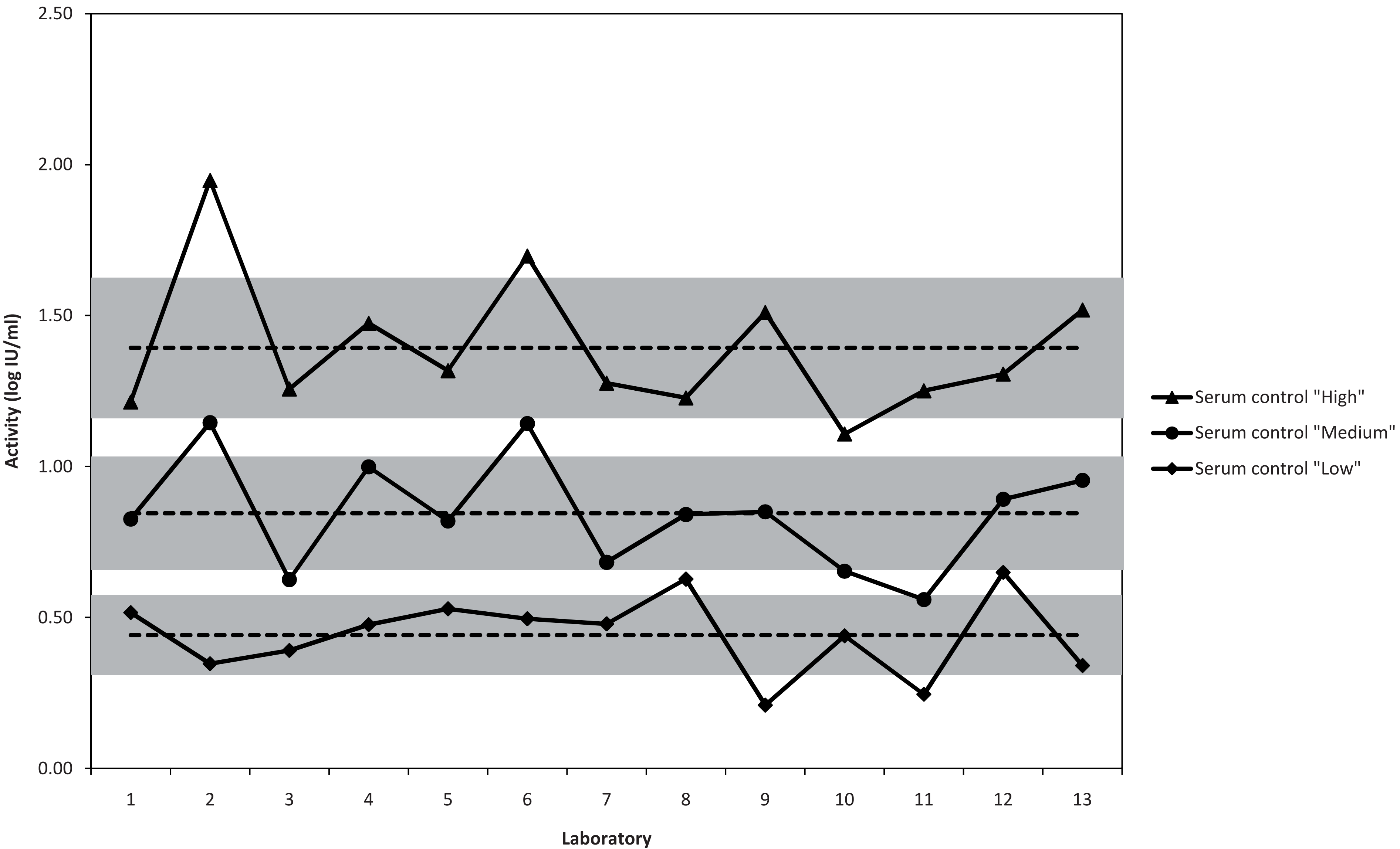
Explanations: each plot shows the activities of the sera per vaccine of the pooled assays (i.e. 18 mice per vaccine). The fat horizontal bars are the median activity.

Figure 1 – *Scatter plots of serum activities per vaccine*

Table 2 – Estimated activities of the control sera (log IU/mL)

Lab	Assay	Low		Medium		High	
		Titre	Mean	Titre	Mean	Titre	Mean
1	1	0.41	0.52	0.79	0.83	1.28	1.21
	2	0.55		0.86		1.13	
	3	0.58		0.82		1.23	
2	1	0.36	0.35	1.00	1.14	>q.l.	1.95
	2	0.38		1.23		2.04	
	3	0.29		1.20		1.86	
3	1	0.45	0.39	0.60	0.63	1.52	1.26
	2	0.40		0.66		1.11	
	3	0.33		0.62		1.14	
4	1	0.44	0.48	0.91	1.00	1.47	1.47
	2	0.53		1.03		1.34	
	3	0.45		1.06		1.61	
5	1	0.55	0.53	0.92	0.82	1.67	1.32
	2	0.62		0.80		1.35	
	3	0.41		0.74		0.93	
6	1a	0.61	0.50	1.37	1.14	1.81	1.70
	1b	0.45		1.13		1.86	
	2	0.41		1.00		1.66	
	3	0.51		1.07		1.46	
7	1	0.43	0.48	0.77	0.68	1.28	1.28
	2	0.54		0.71		>q.l.	
	3	0.47		0.57		>q.l.	
8	1	0.66	0.63	0.91	0.84	1.33	1.23
	2	0.60		0.81		1.08	
	3	0.62		0.80		1.28	
9	1	0.20	0.21	0.70	0.85	1.47	1.51
	2	0.18		0.84		>q.l.	
	3	0.24		1.01		1.55	
10	1	0.44	0.44	0.65	0.65	1.11	1.11
11	1	0.26	0.25	0.57	0.56	1.27	1.25
	2	0.23		0.38		1.32	
	3	0.25		0.73		1.16	
12	1	0.80	0.65	1.00	0.89	1.35	1.31
	2	0.50		0.85		1.20	
	3	0.64		0.82		1.37	
13	1	0.34	0.34	0.95	0.95	1.52	1.52
Mean			0.44		0.85		1.39
Standard deviation			0.13		0.18		0.23

Explanations: >q.l. = above quantification limit. These results are not included in the mean.



Explanations: the dashed lines indicate the overall mean of the 13 laboratories. The grey areas cover 1 standard deviation from the overall mean.

Figure 2 – *Mean activities of the control sera per laboratory*

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Table 3a – *P-values of the one-sided exact test of Wilcoxon-Mann-Whitney per assay*

		Including all mice				Removing non-responders			
Lab	Assay	Vac A	Vac B	Vac C	Vac D	Vac A	Vac B	Vac C	Vac D
1	1	0.062	0.012	0.476	0.083	0.117	0.024	0.476	0.083
	2	0.029	0.047	0.350	0.030	0.056	0.089	0.535	0.061
	3	0.019	0.001	>0.500	0.004	0.039	0.002	>0.500	0.009
2	1	0.001	0.004	0.227	0.002	0.001	0.004	0.010	0.002
	2	0.001	0.004	0.004	0.001	0.001	0.004	0.004	0.001
	3	0.001	0.001	0.064	0.001	0.001	0.001	0.009	0.001
	4	0.001	0.004	0.111	0.002	0.001	0.004	0.024	0.002
3	1	0.001	0.001	>0.500	0.001	0.001	0.001	>0.500	0.001
	2	0.017	0.001	>0.500	0.001	0.002	0.001	>0.500	0.001
	3	0.030	0.001	>0.500	0.003	0.002	0.001	>0.500	0.003
4	1	0.019	0.042	>0.500	0.184	0.019	0.042	>0.500	0.056
	2	0.002	0.001	0.064	0.001	0.004	0.002	0.121	0.002
	3	0.003	0.063	0.242	0.001	0.003	0.063	0.242	0.001
5	1	0.040	0.008	>0.500	0.294	0.004	0.008	>0.500	0.294
	2	0.001	0.001	>0.500	0.361	0.002	0.002	>0.500	0.317
	3	0.130	0.031	>0.500	0.073	0.229	0.061	>0.500	0.134
6	1a	0.001	0.001	0.469	0.001	0.001	0.001	0.469	0.001
	1b	0.001	0.002	0.242	0.001	0.002	0.004	0.396	0.002
	2	0.001	0.001	0.120	0.001	0.001	0.001	0.120	0.001
	3	0.003	0.111	>0.500	0.003	0.003	0.111	0.509	0.003
7	1	0.025	0.023	>0.500	0.004	0.004	0.004	>0.500	0.009
	2	0.001	0.002	>0.500	0.021	0.002	0.004	>0.500	0.041
	3	0.025	0.023	0.228	0.041	0.004	0.004	>0.500	0.080
8	1	0.001	0.001	0.391	0.001	0.001	0.001	0.391	0.001
	2	0.001	0.001	0.277	0.015	0.001	0.001	0.277	0.015
	3	0.024	0.056	>0.500	0.450	0.024	0.056	>0.500	0.450
9	1	0.001	0.058	0.155	0.029	0.001	0.005	0.155	0.002
	2	0.028	0.002	0.194	0.001	0.002	0.002	0.058	0.001
	3	0.001	0.001	0.155	0.006	0.002	0.002	0.268	0.013
10	1	0.001	0.003	>0.500	0.001	0.002	0.006	>0.500	0.002
11	1	0.002	0.001	>0.500	>0.500	0.002	0.001	>0.500	0.032
	2	0.001	0.001	>0.500	0.032	0.001	0.001	>0.500	0.032
	3	0.001	0.004	>0.500	0.002	0.001	0.004	>0.500	0.002
12	1	0.018	0.001	>0.500	0.128	0.000	0.000	>0.050	0.001
	2	0.421	0.029	>0.500	0.110	0.018	0.001	>0.500	0.128
	3	0.095	0.005	>0.500	0.053	0.015	0.005	>0.500	0.053
13	1	0.013	0.011	0.081	0.001	0.013	0.011	0.081	0.001
	2	0.001	0.009	0.350	0.001	0.001	0.009	0.350	0.001
	3	0.001	0.005	0.256	0.003	0.001	0.005	0.100	0.003

Table 3b – *P-values of the one-sided exact test of Wilcoxon-Mann-Whitney of pooled assays*

	Including all mice				Removing non-responders			
Lab	Vac A	Vac B	Vac C	Vac D	Vac A	Vac B	Vac C	Vac D
1	0.000	0.000	>0.500	0.000	0.001	0.000	>0.500	0.001
2	0.000	0.000	0.003	0.000	0.000	0.000	0.000	0.000
3	0.000	0.000	>0.500	0.000	0.000	0.000	>0.500	0.000
4	0.000	0.000	0.126	0.000	0.000	0.000	0.184	0.000
5	0.001	0.000	>0.500	0.152	0.000	0.000	>0.500	0.198
6	0.000	0.000	0.062	0.000	0.000	0.000	0.096	0.000
7	0.000	0.000	>0.500	0.000	0.000	0.000	>0.500	0.001
8	0.000	0.000	>0.500	0.000	0.000	0.000	>0.500	0.000
9	0.000	0.000	0.079	0.000	0.000	0.000	0.062	0.000
11	0.000	0.000	>0.500	0.014	0.000	0.000	>0.500	0.001
12	0.014	0.000	>0.500	0.006	0.002	0.000	>0.500	0.011
13	0.000	0.000	0.056	0.000	0.000	0.000	0.025	0.000

Explanations: grey cells indicate vaccines that fail the release test. Cells with a value of >0.500 indicate that the mean response of the test was lower than the mean response of the standard.

Table 4 – *Estimated probabilities to pass the vaccines using different numbers of mice*

Lab	Vaccine	N=3	N=4	N=5	N=6	N=7	N=8	N=9	N=10	N=11	N=12
1	A	42%	44%	62%	69%	76%	82%	86%	89%	92%	94%
	B	54%	55%	76%	81%	86%	92%	94%	96%	97%	98%
	C	4%	3%	4%	4%	4%	4%	4%	4%	4%	4%
	D	37%	43%	61%	69%	77%	83%	87%	90%	92%	94%
2	A	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%
	B	71%	72%	91%	94%	97%	99%	99%	100%	100%	100%
	C	29%	26%	30%	40%	46%	50%	53%	55%	61%	64%
	D	79%	85%	98%	99%	100%	100%	100%	100%	100%	100%
3	A	70%	63%	56%	86%	82%	94%	93%	91%	97%	96%
	B	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%
	C	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
	D	78%	86%	97%	99%	100%	100%	100%	100%	100%	100%
4	A	68%	77%	92%	96%	98%	99%	100%	100%	100%	100%
	B	53%	55%	71%	80%	87%	91%	93%	95%	97%	98%
	C	11%	9%	14%	15%	17%	19%	20%	21%	24%	24%
	D	65%	60%	63%	84%	83%	91%	93%	94%	97%	97%
5	A	40%	37%	46%	58%	61%	70%	73%	78%	81%	84%
	B	65%	66%	80%	88%	92%	96%	97%	98%	99%	99%
	C	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
	D	11%	9%	12%	14%	15%	17%	18%	20%	20%	21%
6	A	80%	89%	98%	99%	100%	100%	100%	100%	100%	100%
	B	52%	56%	81%	85%	91%	95%	96%	98%	99%	99%
	C	13%	11%	16%	18%	20%	23%	23%	25%	28%	29%
	D	96%	100%	100%	100%	100%	100%	100%	100%	100%	100%
7	A	66%	61%	62%	85%	83%	93%	93%	94%	97%	97%
	B	58%	48%	49%	74%	69%	86%	83%	85%	91%	90%
	C	2%	1%	1%	1%	1%	1%	1%	1%	0%	0%
	D	41%	45%	66%	73%	80%	87%	89%	92%	94%	96%
8	A	68%	69%	83%	91%	94%	97%	98%	99%	99%	100%
	B	65%	67%	86%	91%	95%	97%	98%	99%	99%	100%
	C	4%	3%	3%	3%	3%	3%	3%	3%	3%	2%
	D	35%	31%	42%	50%	56%	63%	66%	71%	75%	78%
9	A	80%	78%	76%	95%	94%	98%	99%	99%	100%	100%
	B	65%	61%	57%	84%	82%	91%	93%	91%	97%	96%
	C	13%	11%	17%	19%	21%	24%	26%	28%	30%	32%
	D	67%	63%	70%	86%	87%	94%	95%	96%	98%	98%
10	A	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%
	B	70%	82%	94%	96%	98%	99%	100%	100%	100%	100%
	C	1%	0%	0%	0%	0%	0%	0%	0%	0%	0%
	D	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%
11	A	70%	77%	94%	96%	98%	99%	100%	100%	100%	100%
	B	88%	94%	99%	100%	100%	100%	100%	100%	100%	100%
	C	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
	D	26%	23%	27%	34%	39%	45%	46%	50%	54%	57%
12	A	19%	21%	27%	33%	38%	43%	47%	51%	54%	57%
	B	71%	82%	95%	98%	99%	100%	100%	100%	100%	100%
	C	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
	D	21%	23%	34%	40%	46%	52%	56%	61%	64%	68%
13	A	76%	83%	96%	98%	99%	100%	100%	100%	100%	100%
	B	48%	64%	81%	89%	94%	96%	98%	99%	99%	100%
	C	15%	14%	19%	23%	25%	29%	31%	33%	35%	37%
	D	92%	97%	100%	100%	100%	100%	100%	100%	100%	100%

Explanations: the percentages give the estimated probability that a vaccine would pass the release test in a given laboratory using N mice per preparation. Each percentage is based on 100.000 bootstrap resamples of size N with replacement from the pool of sera per laboratory and vaccine, ignoring inter-assay variation..

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Appendix 1

CombiStats Version 4.0. Monday, 11 January 2010, 11:42:28 [+01:00]. Page 1 of 2

Study	BSP105 - Rabies serology
Laboratory	01
Assay number	2
Vaccine	A

Remarks:

Standard	
Designation	2nd IS (RAI)
Ass. pot.	2 IU/ml
Predilution	1ml/5ml
Inoculation	0.050ml/well
Doses	(1)
1/1	0/6
1/2	0/6
1/4	0/6
1/8	4/6
1/16	6/6
1/32	6/6
1/64	6/6
1/320	6/6

Sample 1	
Serum	Mouse 1
Ass. pot.	? IU/ml
Predilution	1ml/5ml
Inoculation	0.050ml/well
Doses	(1)
1/1	0/4
1/2	0/4
1/4	0/4
1/8	0/4
1/16	0/4
1/32	0/4
1/64	4/4
1/320	4/4

Sample 2	
Serum	Mouse 2
Ass. pot.	? IU/ml
Predilution	1ml/5ml
Inoculation	0.050ml/well
Doses	(1)
1/1	0/4
1/2	0/4
1/4	0/4
1/8	0/4
1/16	0/4
1/32	0/4
1/64	0/4
1/320	4/4

Sample 3	
Serum	Mouse 3
Ass. pot.	? IU/ml
Predilution	1ml/5ml
Inoculation	0.050ml/well
Doses	(1)
1/1	0/4
1/2	0/4
1/4	0/4
1/8	0/4
1/16	0/4
1/32	0/4
1/64	0/4
1/320	4/4

Sample 4	
Serum	Mouse 4
Ass. pot.	? IU/ml
Predilution	1ml/5ml
Inoculation	0.050ml/well
Doses	(1)
1/1	0/4
1/2	0/4
1/4	0/4
1/8	3/4
1/16	4/4
1/32	4/4
1/64	4/4
1/320	4/4

Sample 5	
Serum	Mouse 5
Ass. pot.	? IU/ml
Predilution	1ml/5ml
Inoculation	0.050ml/well
Doses	(1)
1/1	0/4
1/2	0/4
1/4	0/4
1/8	0/4
1/16	0/4
1/32	2/4
1/64	4/4
1/320	4/4

Sample 6	
Serum	Mouse 6
Ass. pot.	? IU/ml
Predilution	1ml/5ml
Inoculation	0.050ml/well
Doses	(1)
1/1	0/4
1/2	0/4
1/4	0/4
1/8	0/4
1/16	0/4
1/32	3/4
1/64	4/4
1/320	4/4

Model: Quantal responses
Design: Completely randomised
Transformation: y' = y
Theoretical variance: 1

Common slope(factor) = -1.44269 (fixed, p = 0.998)
Correlation | r |: 1.00058 (Weighted)

Sample 1			
Serum	Mouse 1		
(IU/ml)	Lower limit	Estimate	Upper limit
Potency	9.77755	12.6992	16.4940
Rel. to Ass.	?	?	?
Rel. to Est.	77.0%	100.0%	129.9%

Sample 2			
Serum	Mouse 2		
(IU/ml)	Lower limit	Estimate	Upper limit
Potency	19.5551	25.3985	32.9879
Rel. to Ass.	?	?	?
Rel. to Est.	77.0%	100.0%	129.9%

Sample 3			
Serum	Mouse 3		
(IU/ml)	Lower limit	Estimate	Upper limit
Potency	19.5551	25.3985	32.9879
Rel. to Ass.	?	?	?
Rel. to Est.	77.0%	100.0%	129.9%

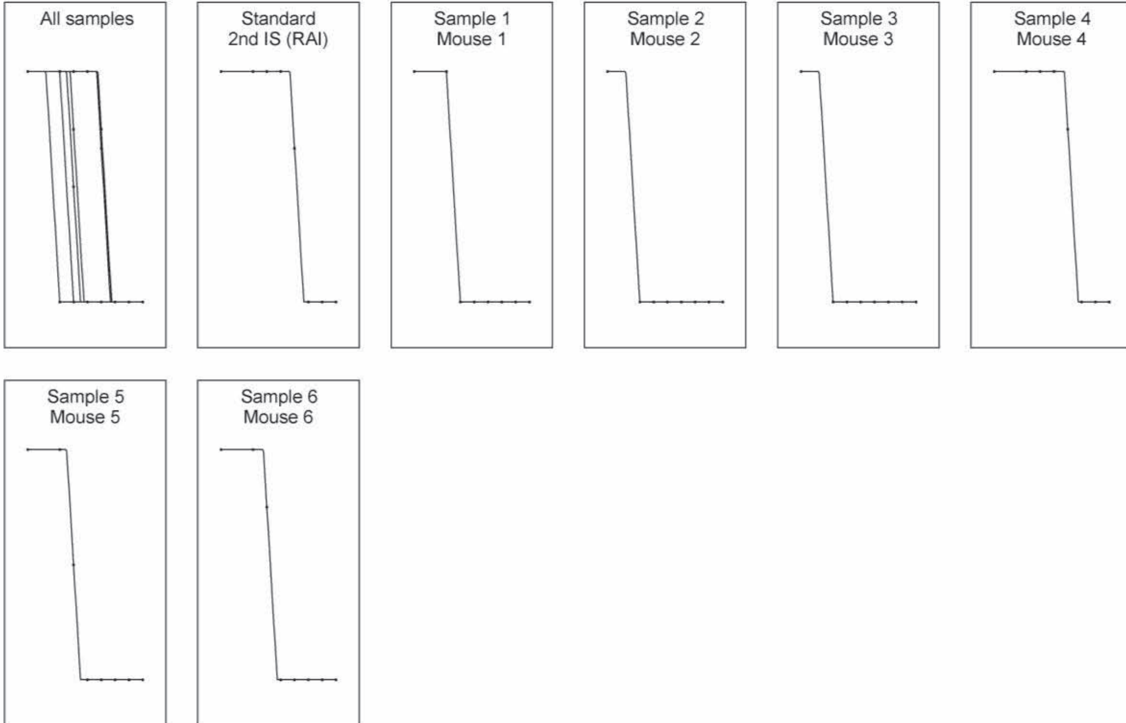
Sample 4			
Serum	Mouse 4		
(IU/ml)	Lower limit	Estimate	Upper limit
Potency	1.27360	1.88775	2.79805
Rel. to Ass.	?	?	?
Rel. to Est.	67.5%	100.0%	148.2%

CombiStats Version 4.0. Monday, 11 January 2010, 11:42:28 [+01:00]. Page 2 of 2

Study	BSP105 - Rabies serology
Laboratory	01
Assay number	2
Vaccine	A

Sample 5			
Serum	Mouse 5		
(IU/ml)	Lower limit	Estimate	Upper limit
Potency	5.84947	8.97970	13.7850
Rel. to Ass.	?	?	?
Rel. to Est.	65.1%	100.0%	153.5%

Sample 6			
Serum	Mouse 6		
(IU/ml)	Lower limit	Estimate	Upper limit
Potency	5.09439	7.55099	11.1922
Rel. to Ass.	?	?	?
Rel. to Est.	67.5%	100.0%	148.2%



Executed by: Calculated by: Approved by:

Filename: ...\Lab 01 Assay 02 Vac A.epa. ID: EDQM/Stat/COE

Appendix 2

CombiStats Version 4.0. Wednesday, 13 January 2010, 16:25:01 [+01:00]. Page 1 of 1

Study	BSP105 - Rabies serology
Laboratory	01
Assay	2

Remarks:

Standard	
Id.	BRP4
Ass. pot.	11 IU/vial
Pre-dil.	1 vial/11 ml
Doses	0.2 ml
(1)	0.368
(2)	0.000
(3)	0.932
(4)	0.679
(5)	0.125
(6)	1.137
	0.540

Sample 1	
Id.	Vaccine A
Ass. pot.	? IU/dose
Pre-dil.	1 dose/ml
Doses	0.2 ml
(1)	1.137
(2)	1.422
(3)	1.422
(4)	0.461
(5)	0.999
(6)	0.932
	1.062

Sample 2	
Id.	Vaccine B
Ass. pot.	? IU/dose
Pre-dil.	1 dose/ml
Doses	0.2 ml
(1)	0.621
(2)	1.278
(3)	0.413
(4)	0.999
(5)	1.884
(6)	1.422
	1.103

Sample 3	
Id.	Vaccine C
Ass. pot.	? IU/dose
Pre-dil.	1 dose/2 ml
Doses	0.2 ml
(1)	0.507
(2)	0.933
(3)	0.810
(4)	0.106
(5)	0.862
(6)	0.792
	0.668

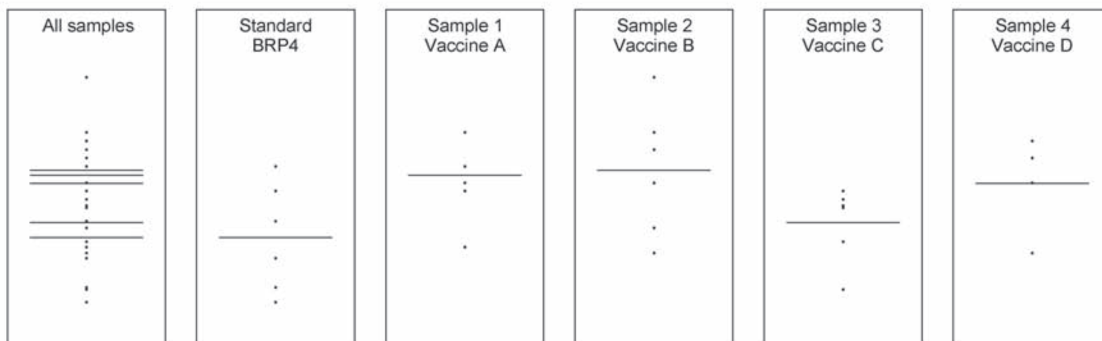
Sample 4	
Id.	Vaccine D
Ass. pot.	? IU/dose
Pre-dil.	1 dose/3 ml
Doses	0.2 ml
(1)	1.349
(2)	1.207
(3)	0.999
(4)	0.999
(5)	0.413
(6)	0.999
	0.994

Sample 1	
Id.	Vaccine A
Limit tested	1.00000 IU / dose
Probability	0.029 (*)

Sample 2	
Id.	Vaccine B
Limit tested	1.00000 IU / dose
Probability	0.047 (*)

Sample 3	
Id.	Vaccine C
Limit tested	2.00000 IU / dose
Probability	0.350

Sample 4	
Id.	Vaccine D
Limit tested	3.00000 IU / dose
Probability	0.030 (*)



Executed by: Calculated by: Approved by:

ID: EDQM/Stat/COE

Appendix 3

Proposed criterion for maximum allowed number of non-responders in the group of animals immunised with the standard vaccine (BRP)

Follow-up of BSP105

In the study report of BSP105 a suggestion is made to impose a requirement on the maximum allowed number of non-responders in the group of mice immunised with the standard vaccine (BRP), for example not more than 1 in a group of 6 or not more than 2 in a group of 8.

The overall percentage of non-responders observed in this study ranges from 2.3% for the high potency vaccine B to 7.2% for the sub-potent vaccine C. For the BRP this percentage is 5.9%. Assuming that these percentages represent the normal expected frequency of non-responders, it is possible to calculate the probability that a given number of non-responders occurs in a group of size n.

Table 1 shows the probability of m or more non-responders in a group of n mice, assuming an expected frequency of 6% non-responders (the cumulative binomial distribution with $p=0.06$). For example, in a group of 10 mice we may expect 2 or more non-responders in 11.8% of the assays. An assay should therefore not be declared invalid when 2 non-responders occur because this will occur rather frequently. However, in only 1.9% of the assays we may expect 3 or more non-responders in a group of 10, so that could be a reasonable criterion to declare the assay invalid.

If we set the cut-off for the probability level at 5% we see that the example given in the study report is confirmed by this table: not more than 1 non-responder in a group of 6 mice and not more than 2 non-responders in a group of 7 to 12 mice. Tables for other values of p are given on the next page for information only.

Table 1 – *Expected frequencies of at least m non-responders in a group of n mice (p=0.06)*

p=0.06		Total number of mice tested (n)						
		n=6	n=7	n=8	n=9	n=10	n=11	n=12
Probability of m or more non-responders	$m \geq 0$	100%	100%	100%	100%	100%	100%	100%
	$m \geq 1$	31.0%	35.2%	39.0%	42.7%	46.1%	49.4%	52.4%
	$m \geq 2$	4.6%	6.2%	7.9%	9.8%	11.8%	13.8%	16.0%
	$m \geq 3$	0.4%	0.6%	1.0%	1.4%	1.9%	2.5%	3.2%
	$m \geq 4$	0.0%	0.0%	0.1%	0.1%	0.2%	0.3%	0.4%
	$m \geq 5$	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
	$m \geq 6$	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
	$m \geq 7$	-	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
	$m \geq 8$	-	-	0.0%	0.0%	0.0%	0.0%	0.0%
	$m \geq 9$	-	-	-	0.0%	0.0%	0.0%	0.0%
	$m \geq 10$	-	-	-	-	0.0%	0.0%	0.0%
	$m \geq 11$	-	-	-	-	-	0.0%	0.0%
	$m \geq 12$	-	-	-	-	-	-	0.0%

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Table 2 – *Expected frequencies of at least m non-responders in a group of n mice (p=0.03)*

p=0.03		Total number of mice tested (n)						
		n=6	n=7	n=8	n=9	n=10	n=11	n=12
Probability of m or more non-responders	m≥0	100%	100%	100%	100%	100%	100%	100%
	m≥1	16.7%	19.2%	21.6%	24.0%	26.3%	28.5%	30.6%
	m≥2	1.2%	1.7%	2.2%	2.8%	3.5%	4.1%	4.9%
	m≥3	0.1%	0.1%	0.1%	0.2%	0.3%	0.4%	0.5%
	m≥4	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
	m≥5	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
	m≥6	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
	m≥7	-	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
	m≥8	-	-	0.0%	0.0%	0.0%	0.0%	0.0%
	m≥9	-	-	-	0.0%	0.0%	0.0%	0.0%
	m≥10	-	-	-	-	0.0%	0.0%	0.0%
	m≥11	-	-	-	-	-	0.0%	0.0%
	m≥12	-	-	-	-	-	-	0.0%

Table 3 – *Expected frequencies of at least m non-responders in a group of n mice (p=0.08)*

p=0.08		Total number of mice tested (n)						
		n=6	n=7	n=8	n=9	n=10	n=11	n=12
Probability of m or more non-responders	m≥0	100%	100%	100%	100%	100%	100%	100%
	m≥1	39.4%	44.2%	48.7%	52.8%	56.6%	60.0%	63.2%
	m≥2	7.7%	10.3%	13.0%	15.8%	18.8%	21.8%	24.9%
	m≥3	0.9%	1.4%	2.1%	3.0%	4.0%	5.2%	6.5%
	m≥4	0.1%	0.1%	0.2%	0.4%	0.6%	0.9%	1.2%
	m≥5	0.0%	0.0%	0.0%	0.0%	0.1%	0.1%	0.2%
	m≥6	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
	m≥7	-	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
	m≥8	-	-	0.0%	0.0%	0.0%	0.0%	0.0%
	m≥9	-	-	-	0.0%	0.0%	0.0%	0.0%
	m≥10	-	-	-	-	0.0%	0.0%	0.0%
	m≥11	-	-	-	-	-	0.0%	0.0%
	m≥12	-	-	-	-	-	-	0.0%