Validation Study to Evaluate the Reproducibility of a Candidate *In Vitro* Potency Assay of Newcastle Disease Vaccines and to Establish the Suitability of a Candidate Biological Reference Preparation

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SUMMARY

A quantification assay for the Haemagglutinin-Neuraminidase (HN) protein of Newcastle Disease Virus (NDV) has been developed at CIDC-Lelystad as a candidate in vitro potency test for inactivated Newcastle disease (ND) vaccines. In studies performed at CIDC-Lelystad, a high correlation was demonstrated between the results of this candidate in vitro potency assay and the results of the serological potency assay (European Pharmacopoeia monograph 0870; test A) [1-7]. Furthermore, a high correlation between the serological data (Haemagglutination Inhibition-antibody titres) and clinical protection after challenge was demonstrated.

Correlation between in vivo and in vitro potency assays was confirmed in a collaborative pre-validation study [8]. In the pre-validation study three Official Medicines Control Laboratories (OMCLs) determined both the NDV-HN antigen content and the in vivo potency (vaccination-serology and vaccination-challenge) of 6 vaccine batches. The conclusion of the pre-validation study was that a large-scale collaborative study should be organised to validate the in vitro method and the suitability of the reference preparation.

This report describes the outcome of this study. In brief, 14 laboratories (8 OMCLs and 6 vaccine manufacturers) determined the NDV-HN antigen content of 9 different vaccines in 3 independent tests. The vaccine batches were produced by 5 different manufacturers and represent a quantitative range of ND antigen content. One vaccine batch with insufficient potency and one poultry vaccine not containing NDV were included.

Statistical evaluation of the results indicated that the antigen content could be determined with high precision. A good repeatability as well as reproducibility was found. Furthermore all laboratories found a similar ranking of the vaccines, based on the antigen content.

Comparison of the antigen content and the in vivo potency of a series of vaccines with relatively low potencies indicated that a threshold relative antigen level of 7.0 antigen units per dose would discriminate between vaccine batches with sufficient and insufficient potency. An in vitro assay with this threshold level for antigen content did not result in any false positive results and only a limited number of false negative results in the BSP055 study.

We conclude that the in vitro measurement of the antigen content of inactivated ND-vaccines with the proposed method is a reliable alternative potency assay that could be included as a new method in monograph 0870 on ND-vaccines.

KEYWORDS

Newcastle disease vaccine; potency assay; validation study; *in vitro* alternative method; ELISA; European Pharmacopoeia.

1. INTRODUCTION

An NDV antigen quantification assay has been developed at CIDC-Lelystad as a candidate *in vitro* potency test for inactivated ND vaccines. The assay involves the extraction of antigen from oil emulsion vaccines with isopropylmyristate (IPM) followed by an *in vitro* ELISA for quantification of the HN-protein of NDV in the vaccine.

In studies performed at CIDC-Lelystad, (formerly part of ID-Lelystad) for a series of 20 inactivated ND vaccines (the most commonly used ND vaccines in Western Europe) a high correlation (r2 = 0.86) was demonstrated between the results of this candidate *in vitro* potency assay and the results of the serological potency assay (Ph. Eur. monograph 0870; test A). Furthermore, a high correlation between the serological data (HI-antibody titres) and clinical protection after challenge was demonstrated [1]. The results of this study have been reported to the Ph. Eur. Experts Group 15V.

In a subsequent study, in co-operation with EDQM and the IVI (Dr. L. Bruckner), the transferability of the test protocols was demonstrated [2].

With the aim of reducing the use of animals in the batch release of such vaccines it has been proposed that the candidate *in vitro* antigen quantification assay be included as an alternative assay for the batch release of inactivated ND vaccines in addition to the current *in vivo* methods now listed in the Ph. Eur. monograph 0870. As 2 *in vivo* methods are allowed in the Ph. Eur. monograph (method A and B), acceptance of the *in vitro* method would introduce an additional method in the monograph.

Large quantities of coating antibody, conjugate antibody and reference preparations have been prepared by CIDC-Lelystad and can be made available [1].

As the studies performed at CIDC-Lelystad looked promising, the Ph. Eur. Experts Group 15V proposed that further demonstration of the correlation between *in vitro* results as determined by the ELISA and the *in vivo* potency determination by established methods should be performed. In a pre-validation study with 3 OMCLs as participants, the correlation between the results of the *in vitro* method and the established *in vivo* methods, was confirmed. Furthermore, an excellent reproducibility of the proposed method was observed with respect to the ranking of the vaccines included in the study [8].

A large-scale collaborative study could then be organised to further validate the candidate *in vitro* method and to establish a biological reference preparation (BRP).

Successful outcome of this large-scale validation study would hopefully result in the acceptance of an *in vitro* antigen

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quantification assay as an alternative assay for the batch release of inactivated ND vaccines in addition to the current *in vivo* methods.

2. AIM OF THE STUDY

The aim of the validation study was to determine the repeatability and reproducibility of the candidate *in vitro* potency assay. Furthermore the suitability of the reference preparation as a Ph. Eur. BRP was to be determined.

3. PARTICIPANTS

Fourteen European laboratories with experience in performing ELISAs took part in the study. These included 8 OMCLs and 6 vaccine manufacturers. All laboratories reported results using the reporting forms provided. The laboratories have been randomly assigned a code number from 1 to 14 and are referred to as such throughout the report. The participants are listed in alphabetical order at the end of the report in section 10. The assignment of code numbers was random and does not necessarily correspond to the order of the listing.

4. STUDY DESIGN, METHODS, MATERIALS AND STATISTICAL ANALYSIS

4.1 Study design

Laboratories were asked to determine the NDV-HN antigen content of 9 different inactivated vaccines using the candidate *in vitro* method. Samples were coded (numbers 1-9) so as not to reveal their identities. Eight commercial vaccine batches, produced by 5 different manufacturers and representing a range of ND vaccines with different antigen contents were used. Seven vaccines passed all release tests of the manufacturers and therefore were expected to have sufficient potency. The *in vivo* potency of 3 of these vaccines, with relatively low antigen content, was determined by CIDC-Lelystad (see Annex 2). One vaccine batch was shown in previous studies to have insufficient potency (number 2 in this study and number 4 of the first part of BSP055). One commercial poultry vaccine containing a similar oil adjuvant as the other vaccines but without NDV was included as negative control (number 6 in the study).

4.2 Methods

Antigen from oil emulsion inactivated ND vaccines was extracted in the aqueous phase using an extraction technique involving isopropylmyrisate (IPM).

Antigen was quantified using an ELISA. In brief, the ELISA plate was coated with antibodies against the HN antigen of NDV. Plates were then incubated with vaccine extracts and subsequently incubated with a HN-antibody-HRPO conjugate. Finally the OD was measured after incubation with TMB, a substrate of HRPO.

Monoclonal antibodies against the HN-antigen, developed at CIDC-Lelystad, were used for both the coating and the conjugate.

A reference antigen and internal control antigen (supplied by CIDC-Lelystad) as well as a negative control were included on each plate and the activity of the test antigen was determined in terms of a relative potency to the reference standard. Since lyophilised inactivated viruses were used as reference and internal control antigen, no extraction had to be performed.

The following standard operating procedures were provided:

- Extraction of viral antigens from water-in oil emulsion vaccines with IPM;
- ELISA for quantification of the HN-protein of NDV

These complete SOP's were published recently [8].

4.3 Materials provided for the study

EDQM distributed the test samples numbered 1-9 and reagents for the *in vitro* assay. The 9 vaccines to be tested were produced by 5 different manufacturers (Fort Dodge, The Netherlands; Hipra, Spain; Intervet BV, The Netherlands; Lohmann Animal Health, Germany; Mérial, France). Monovalent as well as combination vaccines were included in this study (refer to Table 1). The vaccines contained either NDV strain Ulster, NDV strain LaSota or Clone30 NDV. Seven commercial ND vaccine batches that had been released on the market and were thus presumed to have adequate potency were tested in this study. Although one commercial vaccine (number 2) with low antigen content passed the manufacturers release tests, this batch was proven to be of insufficient potency in a previous study [8]. This batch was included to test the ability of the *in vitro* assay to identify sub-potent batches. One vaccine without NDV was included as negative control (number 6).

Table 1 – *Summary of composition of vaccines used in the study*

Number of samples	Type of sample vaccine	Composition of sample vaccine	NDV Strain
5	Single	NDV	LaSota/Clone30/Ulster
2	Combo	NDV + EDS	Clone30/Ulster
1	Combo	NDV + IB + EDS + TRT	Ulster
1	Negative	Gumboro	-

NDV: Newcastle Disease Virus, EDS: Egg Drop Syndrome, IB: Infectious Bronchitis, TRT: Turkey RhinoTracheitis

Each participant received the following reagents that were distributed by EDQM, for the *in vitro* assay:

Nine inactivated Newcastle disease vaccines:	10 ml of each vaccine
Coating antibody IDNDV134.1 (lyophilised):	4 vials (to be resuspended in 1.5 ml distilled water)
HRPO-Conjugate of IDNDV134.1 (lyophilised):	4 vials (to be resuspended in 1.5 ml ELISA-buffer)
TMB (3,3',5,5'Tetramethyl-benzidine):	2 bottles of 60 ml
Reference antigen (lyophilised):	4 vials (to be resuspended in 1.5 ml ELISA-buffer)
Internal control antigen (lyophilised):	4 vials (to be resuspended in 1.5 ml ELISA-buffer)
IPM:	1 bottle of 500 ml
ELISA-plates:	12 plates
BSA (Bovine Serum Albumin):	5 g

The lyophilised test reagents were produced by CIDC-Lelystad.

4.4 Statistical Analysis and calculation of relative potency

The raw data of the *in vitro* assays were analysed at EDQM using a parallel line model on log-transformed OD-readings. Only optical densities in the dynamic range were included in the analysis. In practice this means that usually not more than 5 consecutive dilutions are used for the calculations.

The relative potencies are expressed as antigen units per dose. The reference antigen (candidate BRP) contains 0.5 arbitrarily assigned antigen units per 125 µl after resuspension of the lyophised content of a vial in 1.5 ml buffer solution (a vial contains thus 6 antigen units). Since most inactivated ND vaccines contain approximately 125 µl water-phase, a vaccine with equal antigen concentration as the reference antigen contains 0.5 antigen units per dose.

In order to obtain antigen units per dose for all vaccines, a correction factor (*f*) had to be applied to the relative potencies in the assays to take into account the different water phase's (W_T) and dose volumes (V_T) according to the formula:

$$f = \frac{V_T}{0.5} \times \frac{W_T}{25}$$

so that a vaccine of 0.5 ml per dose with a water phase of 25% has a correction factor of 1.

An example of the calculation of relative potency against the reference preparation for a series of vaccines with different dose volumes and water-phases is provided in Annex 1.

For each type of assay, the vaccines were ranked within laboratories. An overall ranking of the vaccines per assay type was also determined. This should help to establish the reproducibility of the methods, and the consistency of the assay.

5. RESULTS

First the OD-readings of the blank wells were inspected. In general, the values were well below 0.2 as was considered desirable per protocol. Only in 2 cases, a reading above 0.2 was reported (Lab 7, assay 1, plate 3, row 3 and Lab 11, assay 2, plate 3, row 4). In both cases it was observed that the other blanks on the plate were well below 0.2 and that the quality of the assay did not seem to be affected.

The potencies obtained are listed in Tables 2a to 2c, together with their 95 per cent confidence limits as calculated on the basis of the intra-treatment variation. OD-values of vaccine number 6 were in all laboratories close to the baseline and could therefore not be included in the parallel line analysis. For this vaccine an estimated upper limit of the potency is listed.

It was observed that 55 of the 120 assays showed significant deviations ($p < 0.05$) from linearity, 33 of which were highly significant ($p < 0.01$). It was also observed that 84 of the 120 assays showed significant deviations ($p < 0.05$) from parallelism, 67 of which were highly significant ($p < 0.01$). It is known that the intra-treatment variation is in practice often an underestimation of the true assay-variability because adjacent doses of serial dilutions normally exhibit auto-correlated errors resulting in excessive numbers of significant F-values.

Indeed, the 95 per cent confidence limits are on average only 93 and 107 per cent of the estimated potency, which is a remarkably high precision for an assay with 2-fold dilution steps. Only a small increase of the average intra-treatment variance would already reduce the number of significant non-linear assays to 5 out of 120, which is a normal proportion, whereas the precision would still be remarkably good with confidence limits of 91 and 110 per cent of the estimated potency. This, together with the observation that the nature of the non-linearity does not show any systematic patterns (e.g. quadratic curvature) and that confidence limits which would reflect the inter-assay variability would have to be approximately 70 and 143 per cent of the estimated potency, lead to the conclusion that the degree of non-linearity is of no practical importance.

The large amount of significant non-parallel assays, however, cannot be attributed to an underestimation of the residual variance only. In order to reduce the number of invalid assays to 6 out of 126, the average residual variance would have to be 7 times as large as observed, corresponding to confidence limits of 84 and 120 per cent of the estimated potency. Even though this is still a fairly good precision for a 2-fold dilution step, the systematic patterns of the non-parallelism cannot be ignored. Table 4 shows for each test vaccine and each laboratory the ratio between the slope of the test-vaccine and the slope of the reference preparation (average of the 3 assays). A sign test shows that the slopes of vaccine numbers 1, 4, 7 and 9 are significantly ($p < 0.05$) more shallow than the reference. Vaccine numbers 5 and 8 also tend to be shallower than the reference. Only vaccine numbers 2 and 3 show no difference of the slope.

Despite the structural non-parallelism and the underestimated residual variance, potencies were calculated. The individual results per assay are listed in Tables 2a to 2c. The average results per laboratory and vaccine are listed in Table 3, together with the overall geometric mean. For vaccine number 6, only an upper limit of the potency is listed. A graphical impression is given in Figure 1.

The average repeatability standard deviation of ln-transformed potency estimates is 0.18, which implies that 2 independent assays within the same laboratory result in potency estimates that do not differ by more than a factor 1.7 from each other in 95 per cent of the cases. The average reproducibility standard deviation varies from 0.18 for vaccine 3 to 0.35 for vaccine 2 and is on average 0.24. This implies that 2 laboratories, each having carried out 3 assays, will find mean potency estimates that will not differ by more than a factor 2.0 from each other in 95 per cent of the cases.

As can easily be seen from Figure 1 all laboratories found a similar ranking of the vaccines. All participants rank vaccine number 2 lowest (disregarding vaccine number 6 and the control antigen). Vaccine numbers 4, 5 and 7 being of similar potency it is not surprising that a few inversions in the ranking order appear and similarly so for vaccine numbers 1, 8 and 9. In summary the ranking in all laboratories is identical or very similar: (Vac. 6) < (Control) < (Vac. number 2) < (Vac. numbers 4,5,7) < (Vac. number 3) < (Vac. numbers 1, 8, 9).

Table 2a – *Results per assay for Plate 1 (units/dose): potency estimates and 95 per cent confidence limits of the estimate per assay*

Lab	Assay	Control antigen Correction factor: 1,00	Vaccine 1 Correction factor: 0,60	Vaccine 2 Correction factor: 1,00	Vaccine 3 Correction factor: 1,60
1	1	6,84 (93% - 107%)	92 (93% - 107%)	2,28 (93% - 107%)	14,4 (93% - 108%)
	2	6,13 (93% - 107%)	75 (93% - 107%)	2,41 (93% - 107%)	12,8 (93% - 107%)
	3	5,90 (90% - 111%)	67 (90% - 111%)	2,25 (90% - 111%)	12,9 (90% - 111%)
2	1	6,40 (96% - 105%)	61 (96% - 105%)	3,87 (95% - 105%)	18,3 (95% - 105%)
	2	6,88 (95% - 105%)	64 (95% - 105%)	5,45 (95% - 105%)	20,1 (95% - 105%)
	3	7,00 (95% - 105%)	58 (95% - 105%)	3,80 (95% - 105%)	18,2 (95% - 105%)
3	1	4,64 (95% - 105%)	67 (95% - 105%)	4,50 (95% - 105%)	25,6 (95% - 105%)
	2	4,17 (97% - 104%)	51 (97% - 103%)	4,00 (97% - 103%)	20,6 (97% - 104%)
	3	3,96 (94% - 107%)	78 (94% - 107%)	3,83 (94% - 107%)	19,7 (94% - 107%)
4	1	3,57 (91% - 110%)	54 (91% - 110%)	4,85 (91% - 110%)	15,1 (91% - 110%)
	2	3,28 (93% - 108%)	47 (93% - 107%)	4,09 (93% - 107%)	20,4 (93% - 108%)
	3	3,86 (94% - 106%)	43 (95% - 106%)	3,80 (94% - 106%)	16,7 (94% - 106%)
5	1	3,50 (95% - 105%)	57 (95% - 105%)	3,36 (95% - 105%)	19,6 (95% - 106%)
	2	2,44 (94% - 107%)	50 (94% - 106%)	3,92 (94% - 106%)	16,2 (94% - 106%)
	3	3,18 (93% - 108%)	54 (93% - 108%)	3,46 (93% - 108%)	18,0 (93% - 108%)
6	1	3,68 (94% - 107%)	61 (94% - 107%)	4,41 (94% - 107%)	20,3 (94% - 106%)
	2	3,38 (95% - 105%)	67 (95% - 105%)	5,33 (95% - 105%)	24,6 (95% - 106%)
	3	4,36 (90% - 111%)	56 (91% - 110%)	5,91 (91% - 110%)	22,3 (91% - 110%)
7	1	3,71 (96% - 104%)	84 (96% - 104%)	3,58 (96% - 104%)	22,1 (96% - 104%)
	2	3,86 (95% - 106%)	73 (94% - 106%)	3,42 (95% - 106%)	19,6 (95% - 106%)
	3	5,53 (92% - 108%)	108 (92% - 108%)	2,56 (92% - 109%)	18,3 (92% - 109%)
8	1	3,01 (92% - 108%)	57 (93% - 108%)	3,23 (92% - 108%)	18,5 (92% - 108%)
	2	3,04 (94% - 107%)	60 (94% - 106%)	3,77 (94% - 106%)	21,1 (94% - 107%)
	3	2,87 (94% - 106%)	80 (95% - 106%)	3,71 (94% - 106%)	16,0 (94% - 106%)
9	1	4,28 (91% - 110%)	58 (91% - 111%)	1,76 (90% - 111%)	15,2 (91% - 110%)
	2	4,33 (93% - 107%)	50 (94% - 107%)	1,74 (93% - 108%)	13,7 (93% - 108%)
	3	5,51 (91% - 110%)	75 (92% - 109%)	2,71 (91% - 110%)	16,4 (91% - 110%)
10	1	4,20 (96% - 104%)	62 (96% - 104%)	3,26 (96% - 104%)	21,7 (96% - 104%)
	2	7,19 (92% - 108%)	82 (92% - 108%)	0,89 (92% - 109%)	19,5 (92% - 109%)
	3	6,92 (91% - 109%)	71 (91% - 109%)	2,25 (91% - 109%)	16,9 (91% - 109%)
11	1	2,57 (93% - 107%)	62 (94% - 107%)	3,94 (94% - 106%)	17,8 (94% - 107%)
	2	2,29 (88% - 114%)	69 (89% - 113%)	4,29 (89% - 113%)	24,9 (88% - 114%)
	3	2,19 (91% - 110%)	56 (92% - 109%)	3,39 (92% - 109%)	21,4 (92% - 109%)
12	1	3,86 (93% - 108%)	65 (93% - 108%)	1,47 (93% - 108%)	11,3 (93% - 107%)
	2	3,59 (94% - 107%)	104 (94% - 107%)	1,44 (94% - 107%)	9,7 (94% - 107%)
	3	3,12 (85% - 117%)	73 (86% - 116%)	1,91 (85% - 117%)	17,1 (87% - 115%)
13	1	n.r.	n.r.	n.r.	n.r.
	2	n.r.	n.r.	n.r.	n.r.
	3	6,03 (92% - 109%)	99 (92% - 109%)	2,95 (92% - 109%)	12,4 (92% - 109%)
14	1	4,67 (88% - 114%)	40 (88% - 114%)	2,93 (88% - 114%)	14,1 (88% - 114%)
	2	5,75 (95% - 105%)	53 (95% - 105%)	4,05 (95% - 105%)	20,1 (95% - 105%)
	3	5,59 (87% - 115%)	37 (87% - 115%)	3,87 (87% - 115%)	16,7 (87% - 115%)

n.r. = not reported

Table 2b – *Results per assay for Plate 2 (units/dose): potency estimates and 95 per cent confidence limits of the estimate per assay*

Lab	Assay	Control antigen Correction factor: 1,00	Vaccine 4 Correction factor: 1,00	Vaccine 5 Correction factor: 1,00	Vaccine 6 Correction factor: 1,60
1	1	6,72 (89% - 113%)	6,8 (89% - 113%)	7,8 (88% - 113%)	< 0,05
	2	6,12 (92% - 109%)	5,6 (91% - 110%)	8,7 (92% - 109%)	< 0,05
	3	4,67 (91% - 110%)	6,1 (91% - 110%)	7,0 (91% - 110%)	< 0,05
2	1	6,60 (93% - 107%)	8,2 (93% - 107%)	11,2 (93% - 107%)	< 0,13
	2	6,29 (91% - 110%)	9,5 (91% - 110%)	12,1 (90% - 111%)	< 0,15
	3	7,48 (92% - 109%)	10,7 (92% - 108%)	15,8 (92% - 109%)	< 0,11
3	1	4,04 (93% - 108%)	9,5 (93% - 108%)	14,7 (93% - 107%)	< 0,37
	2	4,40 (85% - 117%)	10,4 (85% - 117%)	12,6 (85% - 117%)	< 0,35
	3	3,07 (92% - 109%)	9,4 (92% - 108%)	13,0 (92% - 108%)	< 0,30
4	1	3,73 (87% - 115%)	8,5 (87% - 115%)	13,0 (88% - 114%)	< 0,87
	2	3,39 (90% - 111%)	8,2 (91% - 110%)	11,9 (90% - 111%)	< 0,63
	3	2,81 (93% - 107%)	7,7 (94% - 107%)	8,2 (94% - 107%)	< 0,40
5	1	3,14 (92% - 108%)	7,1 (93% - 107%)	10,7 (93% - 107%)	< 0,60
	2	3,04 (90% - 111%)	8,4 (90% - 111%)	11,8 (91% - 110%)	< 0,52
	3	3,54 (93% - 107%)	6,7 (94% - 106%)	9,2 (94% - 106%)	< 0,16
6	1	4,14 (93% - 108%)	9,3 (93% - 107%)	11,1 (93% - 108%)	< 0,09
	2	2,99 (94% - 106%)	10,4 (95% - 106%)	14,8 (95% - 106%)	< 0,10
	3	4,40 (93% - 107%)	13,9 (93% - 107%)	15,4 (93% - 107%)	< 0,13
7	1	3,60 (96% - 104%)	7,7 (97% - 104%)	10,9 (97% - 104%)	< 0,24
	2	4,52 (97% - 103%)	7,5 (97% - 103%)	11,9 (97% - 103%)	< 0,23
	3	3,69 (80% - 124%)	5,6 (81% - 122%)	7,8 (82% - 122%)	< 0,16
8	1	3,70 (94% - 106%)	7,9 (95% - 106%)	8,3 (95% - 106%)	< 0,16
	2	3,01 (95% - 105%)	7,7 (95% - 105%)	8,7 (95% - 105%)	< 0,22
	3	2,64 (93% - 108%)	10,3 (93% - 107%)	8,8 (93% - 107%)	< 0,16
9	1	5,17 (87% - 115%)	5,8 (87% - 116%)	9,3 (87% - 115%)	< 0,27
	2	3,49 (91% - 110%)	6,3 (91% - 109%)	10,2 (91% - 110%)	< 0,37
	3	5,05 (94% - 106%)	5,4 (94% - 106%)	9,6 (94% - 106%)	< 0,39
10	1	4,59 (97% - 103%)	9,5 (97% - 103%)	15,3 (97% - 103%)	< 0,02
	2	6,66 (94% - 106%)	5,9 (94% - 106%)	11,5 (94% - 106%)	< 0,03
	3	6,91 (97% - 104%)	7,8 (97% - 104%)	13,4 (97% - 104%)	< 0,03
11	1	2,71 (94% - 106%)	8,5 (95% - 105%)	8,9 (95% - 105%)	< 0,14
	2	2,60 (95% - 106%)	7,4 (95% - 105%)	9,4 (95% - 105%)	< 0,16
	3	2,06 (88% - 114%)	6,1 (89% - 113%)	7,9 (89% - 113%)	< 0,12
12	1	3,38 (94% - 106%)	4,5 (94% - 106%)	5,3 (94% - 106%)	< 0,01
	2	3,30 (94% - 106%)	4,6 (95% - 106%)	6,0 (94% - 106%)	< 0,03
	3	3,36 (88% - 114%)	6,0 (87% - 115%)	6,8 (88% - 114%)	< 0,01
13	1	n.r.	n.r.	n.r.	n.r.
	2	n.r.	n.r.	n.r.	n.r.
	3	5,98 (93% - 108%)	7,7 (93% - 107%)	7,1 (93% - 107%)	< 0,11
14	1	5,09 (91% - 110%)	6,9 (91% - 110%)	9,5 (91% - 110%)	< 0,14
	2	5,64 (91% - 110%)	6,7 (91% - 110%)	9,7 (91% - 110%)	< 0,16
	3	5,16 (96% - 105%)	6,7 (96% - 105%)	9,2 (96% - 105%)	< 0,26

n.r. = not reported

Table 2c – *Results per assay for Plate 3 (units/dose): potency estimates and 95 per cent confidence limits of the estimate per assay*

Lab	Assay	Control antigen Correction factor: 1,00	Vaccine 7 Correction factor: 1,00	Vaccine 8 Correction factor: 0,60	Vaccine 9 Correction factor: 1,00
1	1	6,00 (93% - 108%)	6,2 (93% - 108%)	89 (93% - 108%)	101 (92% - 108%)
	2	6,10 (92% - 109%)	7,1 (92% - 109%)	62 (92% - 109%)	88 (92% - 109%)
	3	5,03 (89% - 112%)	5,9 (89% - 112%)	69 (89% - 112%)	92 (89% - 113%)
2	1	6,27 (96% - 104%)	10,6 (96% - 104%)	75 (96% - 104%)	55 (96% - 105%)
	2	6,46 (96% - 104%)	10,6 (96% - 104%)	82 (96% - 104%)	70 (96% - 104%)
	3	7,40 (93% - 107%)	9,8 (93% - 107%)	86 (93% - 108%)	70 (93% - 107%)
3	1	3,78 (95% - 105%)	12,2 (95% - 105%)	83 (95% - 105%)	88 (96% - 105%)
	2	3,92 (95% - 105%)	11,0 (95% - 105%)	80 (95% - 105%)	87 (95% - 105%)
	3	3,07 (92% - 109%)	8,3 (92% - 109%)	82 (92% - 109%)	67 (92% - 109%)
4	1	3,62 (86% - 116%)	8,5 (87% - 115%)	76 (87% - 115%)	60 (87% - 115%)
	2	3,23 (92% - 108%)	8,0 (92% - 108%)	53 (92% - 108%)	41 (93% - 108%)
	3	2,89 (93% - 108%)	7,6 (93% - 107%)	55 (93% - 107%)	38 (93% - 107%)
5	1	3,64 (93% - 108%)	8,7 (93% - 107%)	60 (93% - 108%)	60 (93% - 107%)
	2	2,46 (91% - 110%)	9,2 (92% - 109%)	77 (92% - 109%)	86 (92% - 109%)
	3	3,27 (95% - 106%)	9,2 (95% - 105%)	46 (95% - 105%)	39 (95% - 105%)
6	1	4,21 (92% - 108%)	10,3 (93% - 108%)	66 (92% - 109%)	41 (92% - 109%)
	2	3,08 (94% - 106%)	13,8 (95% - 106%)	49 (94% - 106%)	63 (95% - 106%)
	3	4,14 (91% - 110%)	17,2 (91% - 110%)	58 (92% - 109%)	81 (92% - 109%)
7	1	3,84 (92% - 109%)	9,4 (92% - 108%)	97 (92% - 108%)	64 (92% - 108%)
	2	4,26 (94% - 106%)	9,3 (95% - 106%)	86 (94% - 106%)	61 (94% - 106%)
	3	4,81 (95% - 105%)	10,6 (95% - 105%)	100 (95% - 105%)	72 (95% - 105%)
8	1	3,32 (94% - 106%)	8,6 (94% - 106%)	77 (95% - 106%)	72 (95% - 106%)
	2	3,00 (96% - 105%)	11,0 (96% - 104%)	79 (96% - 104%)	61 (96% - 105%)
	3	2,81 (95% - 105%)	8,8 (95% - 105%)	91 (96% - 105%)	74 (96% - 105%)
9	1	3,95 (87% - 115%)	5,7 (86% - 116%)	80 (87% - 114%)	68 (87% - 115%)
	2	3,65 (84% - 119%)	7,3 (83% - 120%)	98 (84% - 119%)	98 (84% - 119%)
	3	5,81 (88% - 113%)	6,1 (88% - 114%)	106 (87% - 115%)	69 (88% - 113%)
10	1	4,80 (96% - 104%)	10,4 (96% - 104%)	74 (96% - 104%)	68 (96% - 104%)
	2	6,65 (96% - 104%)	7,9 (96% - 104%)	71 (96% - 104%)	80 (96% - 104%)
	3	7,71 (95% - 105%)	6,7 (95% - 105%)	68 (95% - 105%)	68 (95% - 105%)
11	1	2,50 (93% - 107%)	10,2 (94% - 106%)	78 (94% - 106%)	59 (94% - 106%)
	2	2,08 (93% - 107%)	8,6 (94% - 107%)	83 (94% - 107%)	58 (94% - 107%)
	3	2,15 (86% - 117%)	7,4 (88% - 114%)	66 (87% - 115%)	49 (87% - 115%)
12	1	3,99 (94% - 107%)	5,1 (94% - 106%)	75 (94% - 107%)	71 (94% - 107%)
	2	3,60 (92% - 108%)	4,7 (92% - 108%)	66 (92% - 108%)	50 (93% - 108%)
	3	3,16 (92% - 108%)	4,6 (92% - 108%)	79 (92% - 109%)	57 (92% - 109%)
13	1	n.r.	n.r.	n.r.	n.r.
	2	n.r.	n.r.	n.r.	n.r.
	3	6,86 (94% - 106%)	9,4 (94% - 106%)	166 (94% - 106%)	126 (94% - 106%)
14	1	5,33 (92% - 108%)	11,3 (92% - 108%)	72 (92% - 108%)	64 (92% - 108%)
	2	6,36 (93% - 107%)	8,2 (93% - 107%)	56 (93% - 108%)	23 (93% - 108%)
	3	5,45 (86% - 116%)	7,6 (86% - 116%)	64 (86% - 116%)	54 (86% - 116%)

n.r. = not reported

Table 3 – *Combined results per laboratory (units/dose): unweighted geometric mean of the potency estimates per laboratory and per vaccine and the geometric coefficient of variation (GCV)*

		Control antigen			**Test vaccines**								
Lab		Plate 1	Plate 2	Plate 3	1	2	3	4	5	6	7	8	9
1	Mean	6,28	5,77	5,69	78	2,31	13,3	6,2	7,8	< 0,05	6,4	73	94
	(GCV)	(8%)	(21%)	(11%)	(18%)	(4%)	(7%)	(10%)	(12%)	n.a.	(10%)	(20%)	(7%)
2	Mean	6,76	6,77	6,70	61	4,31	18,9	9,4	13,0	< 0,13	10,3	81	65
	(GCV)	(5%)	(9%)	(9%)	(5%)	(23%)	(6%)	(14%)	(20%)	n.a.	(4%)	(7%)	(16%)
3	Mean	4,25	3,79	3,57	64	4,10	21,8	9,8	13,4	< 0,34	10,4	82	80
	(GCV)	(8%)	(21%)	(14%)	(24%)	(9%)	(15%)	(5%)	(8%)	n.a.	(22%)	(2%)	(16%)
4	Mean	3,56	3,29	3,23	48	4,22	17,3	8,1	11,0	< 0,63	8,0	60	45
	(GCV)	(8%)	(15%)	(12%)	(12%)	(13%)	(16%)	(5%)	(28%)	n.a.	(6%)	(22%)	(28%)
5	Mean	3,01	3,23	3,08	53	3,57	17,9	7,4	10,5	< 0,43	9,1	60	59
	(GCV)	(21%)	(8%)	(23%)	(7%)	(9%)	(10%)	(13%)	(13%)	n.a.	(3%)	(30%)	(48%)
6	Mean	3,79	3,79	3,77	61	5,18	22,3	11,2	13,8	< 0,11	13,5	57	59
	(GCV)	(14%)	(23%)	(19%)	(10%)	(16%)	(10%)	(24%)	(19%)	n.a.	(29%)	(16%)	(40%)
7	Mean	4,30	3,92	4,29	87	3,15	20,0	7,0	10,2	< 0,21	9,7	95	66
	(GCV)	(25%)	(13%)	(12%)	(22%)	(20%)	(10%)	(19%)	(25%)	n.a.	(7%)	(8%)	(9%)
8	Mean	2,97	3,09	3,04	65	3,56	18,4	8,6	8,6	< 0,18	9,4	82	69
	(GCV)	(3%)	(19%)	(9%)	(19%)	(9%)	(15%)	(17%)	(3%)	n.a.	(14%)	(10%)	(11%)
9	Mean	4,67	4,50	4,38	60	2,03	15,1	5,8	9,7	< 0,35	6,4	94	77
	(GCV)	(15%)	(25%)	(28%)	(23%)	(29%)	(10%)	(8%)	(5%)	n.a.	(14%)	(16%)	(23%)
10	Mean	5,93	5,96	6,27	71	1,87	19,3	7,7	13,4	< 0,03	8,2	71	72
	(GCV)	(35%)	(25%)	(27%)	(15%)	(95%)	(13%)	(27%)	(16%)	n.a.	(24%)	(4%)	(10%)
11	Mean	2,34	2,44	2,23	62	3,86	21,2	7,3	8,7	< 0,14	8,7	75	55
	(GCV)	(8%)	(16%)	(11%)	(11%)	(13%)	(18%)	(17%)	(9%)	n.a.	(17%)	(13%)	(10%)
12	Mean	3,51	3,35	3,57	79	1,60	12,4	5,0	6,0	< 0,02	4,8	73	59
	(GCV)	(11%)	(1%)	(12%)	(28%)	(17%)	(34%)	(18%)	(14%)	n.a.	(6%)	(9%)	(20%)
13	Mean	6,03	5,98	6,86	99	2,95	12,4	7,7	7,1	< 0,11	9,4	166	126
	(GCV)	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
14	Mean	5,32	5,29	5,69	43	3,58	16,8	6,8	9,5	< 0,19	8,9	64	43
	(GCV)	(12%)	(6%)	(10%)	(22%)	(19%)	(19%)	(2%)	(2%)	n.a.	(23%)	(13%)	(71%)
Overall mean		4,28	4,18	4,22	65	3,13	17,3	7,6	9,9	< 0,14	8,6	78	67
Overall GCV		(38%)	(36%)	(41%)	(25%)	(42%)	(22%)	(24%)	(29%)	n.a.	(29%)	(31%)	(32%)
n.a. = not applicable													

Table 4 – *Relative slopes of the vaccines: average ratios (n = 3) between the slope of the test-vaccine, and the reference vaccine*

Lab	Vac 1	Vac 2	Vac 3	Vac 4	Vac 5	Vac 7	Vac 8	Vac 9
1	1,08	1,01	1,00	0,97	1,01	0,83	1,05	0,95
2	0,92	1,02	1,01	0,98	0,92	0,97	0,94	0,89
3	0,96	1,01	1,02	0,87	0,86	0,88	0,96	0,88
4	0,96	1,00	1,03	0,95	0,93	0,97	0,96	0,95
5	0,95	0,85	0,98	0,85	0,82	0,84	0,95	0,85
6	0,95	0,95	0,94	0,90	0,90	0,90	0,99	0,86
7	0,96	0,97	1,11	1,02	1,08	0,97	1,00	0,95
8	0,92	0,99	1,03	0,95	0,90	0,94	0,94	0,88
9	0,85	1,03	1,21	1,10	1,02	1,02	0,90	0,96
10	0,97	0,66	0,90	0,87	0,94	0,93	0,99	0,93
11	0,93	1,04	1,07	0,94	0,94	1,00	1,00	0,97
12	0,99	1,00	0,89	0,99	0,92	0,88	1,00	0,76
13	0,86	0,90	0,99	0,94	0,92	0,92	0,85	0,79
14	0,92	0,99	0,97	0,91	0,92	0,92	0,93	0,89
Mean	0,94	0,96	1,01	0,95	0,93	0,93	0,96	0,89

6. DISCUSSION

The batch potency of inactivated ND vaccines is routinely measured in vaccination experiments. Many animals are used in these batch release tests. The *in vitro* quantification of the antigen content of inactivated ND vaccines seems a promising alternative to these animal experiments.

At CIDC-Lelystad an *in vitro* assay has been developed to quantify the NDV-HN protein in inactivated ND-vaccines. The results of this assay correlated with the serological response of vaccinated chickens, as well as with clinical protection of vaccinated chickens after challenge with virulent NDV [1,3,7]. After the production of large batches of lyophilised reagents [1] a series of validation studies was performed to evaluate the suitability of this *in vitro* test as candidate potency assay.

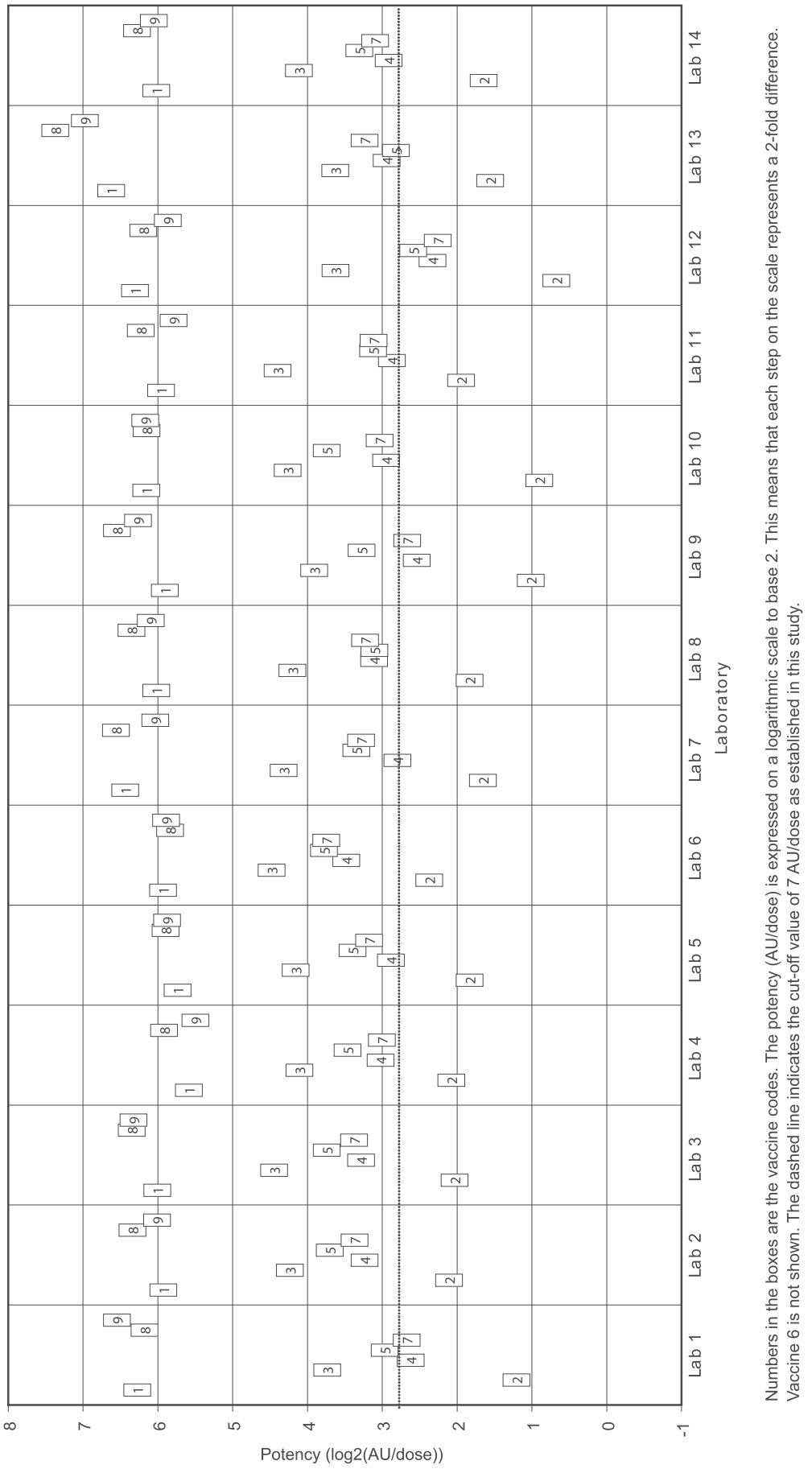
First in house validation tests were performed. This was followed by a transferability study where 2 independent laboratories studied the suitability of the protocols [2, 6]. Satisfactory results of these studies resulted in the organisation of a collaborative feasibility study by EDQM within the Biological Standardisation Program (BSP055 study, part 1). In this pre-validation study 3 OMCL's confirmed the good correlation between the antigen content of inactivated ND-vaccines and both the serological response after vaccination and clinical protection after vaccination and challenge with virulent NDV-Herts [8]. The positive results of the pre-validation study were followed by this large-scale collaborative validation study to determine the reproducibility of the test results and the suitability of the reference preparation. Furthermore positive results of this study should also lead to a proposal for a threshold antigen level that would be indicative of sufficient potency.

Although in a large number of analyses significant deviations from linearity and parallelism was observed, a high assay precision was found in this study. Two independent assays within the same laboratory are estimated not to differ by more than a factor 1.7 from each other in 95% of the cases. Two laboratories, each having carried out 3 assays, would be expected to find mean potency estimates that differed not more than a factor of 2.0 from each other in 95% of the cases. These results indicate acceptable repeatability as well as reproducibility.

The observation that many assays show significant deviations from parallelism and/or linearity is a potential problem. The residual error estimated from the observed assay variation is very low compared to the slope, resulting in high F-ratios and too optimistic confidence limits. Since the reproducibility of the assay is very satisfactory and a good correlation with *in vivo* results has been shown, it may be questioned whether the degree of non-parallelism and non-linearity justifies the rejection of so many assays or even the entire method. If the F-ratios and confidence limits are calculated on the basis of a slightly higher but still acceptable residual variance (e.g. 80 to 125 per cent of the estimated potency) most assays would be statistically valid. In this respect the method can be regarded as superior to the older, more variable methods, which are not able to detect this small deviation from parallelism or linearity, would there be any.

During development and validation of the NDV antigen quantification assay a lyophilised antigen preparation was used as reference. Advantage of the use of such a reference is the long-term stability [6]. In this way validation of new reference vaccine batches in animal tests every two years because of the limited shelf life of these batches, can be avoided. The high precision of the antigen quantification assay in combination with the long-term stability indicates the suitability of this lyophilised reference preparation as BRP.

Figure 1 – *Overview of potency estimates per laboratory and per vaccine*



Numbers in the boxes are the vaccine codes. The potency (AU/dose) is expressed on a logarithmic scale to base 2. This means that each step on the scale represents a 2-fold difference. Vaccine 6 is not shown. The dashed line indicates the cut-off value of 7 AU/dose as established in this study.

Most important is to avoid false positive results in the antigen quantification assay, i.e. vaccine batches with insufficient potency (less that 50 PD50 per dose) should not pass the *in vitro* test. We therefore propose a threshold antigen level of 7.0 antigen units per vaccine dose, related to the candidate BRP used in BSP055 (6 AU/vial), as indicator of sufficient potency (Annex 2). This would lead to insufficient results in all 49 *in vitro* tests performed in 14 different laboratories for the vaccine batch with insufficient potency *in vivo* (number 4 in BSP055-1 and no 2 in BSP055-2). However, some false negative results cannot be avoided as can easily be seen in Figure 1 of BSP055-2. In this study a threshold antigen level of 7.0 antigen units per dose would lead to false negative results for vaccine number 4 in 4 of 14 laboratories and for vaccine number 5 for 1 of 14 laboratories. One participating laboratory (no. 12) had clearly lower results for a series of vaccines in comparison with all other laboratories and scored false negative results for both vaccine number 4 and 5. Also in BSP055-1 a threshold antigen level of 7.0 antigen units per dose would lead to false negative results for vaccine number 6 in 5 out of 10 *in vitro* tests and mean results in 2 of 3 laboratories. Since vaccine numbers 4 and 5 of BSP055-2 both have relatively low antigen levels and sufficient but moderate *in vivo* potency, the number of false negatives is limited and seems acceptable. Furthermore, introduction of an *in vitro* test in the monograph would leave the possibility open to retest *in vivo* vaccine batches that scored insufficient in the *in vitro* antigen test.

7. STABILITY OF THE REFERENCE MATERIALS

Stability studies performed at CIDC-Lelystad on the Reference Antigen and the Control Antigen stored at −20°C over a period of 3.5 years show no systematic decrease in the activity of either material. In accelerated degradation studies samples of the reference antigen stored at room temperature for up to 33 weeks showed no systematic decrease in activity however samples stored at 37 °C showed a small decrease after 8 weeks.

This data suggests the material will be stable for extended periods at −20°C and below. The stability of the material will continue to be monitored by EDQM and its collaborators throughout the duration of use of the BRPs.

8. CONCLUSION

In conclusion, the satisfactory results of this large-scale collaborative validation study and the preceding validation studies strongly argue for the introduction of this *in vitro* test as additional method for potency measurement of inactivated ND-vaccines in the Ph. Eur. This introduction would hopefully reduce the use of animals for routine potency testing of these vaccines significantly.

Based on the results of this study it is therefore proposed to introduce this *in vitro* method and to establish the reference preparation used in the study as a Ph. Eur. BRP with an assignment of 6 antigen units per vial.

Determination of suitability of a test vaccine, in relation to the reference preparation would then be based on an assigned cut of value. A detailed evaluation of the appropriate cut-off level is provided in annex 2, which includes reference to *in vivo* potency estimates of selected test vaccines used in this and the previous study, in comparison to the results of the proposed *in vitro* assay.

A review of the data suggests that a cut-off value of 7 antigen units per dose would be suitable (refer to Annex 2).

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11. ABBREVIATIONS

Abbreviations: AU: Antigen Units; BRP: Biological Reference Preparation; BSP: Biological Standardisation Programme; CIDC: Central Institute for Animal Disease Control; EDQM: European Directorate for the Quality of Medicines; EDS: Egg Drop Syndrome; ELISA: Enzyme-linked immunosorbent assay; GCV: Geometric Coefficient of Variation; HI: Haemagglutination Inhibition; HN: Haemagglutinin-Neuraminidase; HRPO: Horse Radish Peroxidase; IB: Infectious Bronchitis; IPM: IsoPropylMyristate; IVI: Institute for Virology and Immunoprophylaxis; IVRP: *in vitro* Relative Potency; n.a: not applicable; ND: Newcastle Disease; NDV: Newcastle Disease Virus; n.r: not reported; OD: Optical Density; OMCL: Official Medicines Control Laboratory; PD50: Dose inducing protection in 50% of the animals; Ph Eur: Pharmacopée Européenne/European Pharmacopoeia; SOP: Standard Operating Procedure; TMB: 3,3′,5,5′Tetramethylbenzidine; TRT: Turkey Rhino Tracheitis; Vac: Vaccine; µl: microliter

12. REFERENCES

[1] Maas RA, Oei HL, Claassen IJTM. Dossier on the development and validation of an *in vitro* antigen quantification test as candidate potency assay

for inactivated Newcastle disease vaccines. The Netherlands: ID-Lelystad; Dec 2000.

[2] Maas RA, Oei HL, Claassen IJTM. Transferability study candidate *in vitro* potency assay for inactivated Newcastle disease vaccines. The Netherlands: ID-Lelystad; Dec 2000.

[3] Maas RA, de Winter M, Koch G, *et al.* Development of an *in vitro* potency test for inactivated Newcastle disease vaccines. In: Progress in the reduction, refinement and replacement of animal experimentation. Utrecht, NL: Elsevier; 2000:959-967(vol. 31 Dev. in Animal and Veterinary Sciences).

[4] Maas RA, de Winter M, Venema-Kemper S, *et al.* Antigen quantification as *in vitro* alternative for potency testing of inactivated veterinary viral poultry vaccines. *Vet. Quart.* 2000;**22**:223-227.

[5] Maas R, van Diepen M, Komen M, *et al.* Antigen content of inactivated Newcastle disease oil emulsion vaccines as *in vitro* indicator of potency. *Developments in Biologicals* 2002;**111**:167-173.

[6] Maas R, Komen M, Oei H, *et al.* Validation of a candidate *in vitro* potency assay for inactivated Newcastle disease vaccines. *Developments in Biologicals* 2002;**111**:317-323.

[7] Maas RA, Komen M, van Diepen M, *et al.* Correlation of haemagglutinin-neuraminidase and fusion protein content with protective antibody response after immunisation with inactivated Newcastle disease vaccines. *Vaccine* 2003;**21**:3137-3142.

[8] Claassen I, Maas R, Daas A, *et al.* Feasibility study to evaluate the correlation between results of a candidate *in vitro* assay and established *in vivo* assays for potency determination of Newcastle disease vaccines. *Pharmeuropa-Bio* 2003;**1**:53-66.

Annex 1 - Example Calculation of Potency

CombiStats. Version 2.01

Project	BSP055
Assay ID	Lab 8, Assay 2, Plate 1

Standard		
Designation	cB RP Batch 1	
Ass. Pot.	6 AU/vial	
Resuspension	1 vial/1500 µl	
Incubation	100 µl/well	
Doses	(1)	(2)
1/1	1.791	1.738
1/2	0.985	0.989
1/4	0.549	0.554
1/8	0.315	0.306
1/16	0.209	0.195
1/32	~~0.120~~	~~0.120~~

Sample 1		
Designation	Control antigen	
Ass. pot.	? AU/vial	
Resuspension	1 vial/1500 µl	
Incubation	100 µl/well	
Doses	(1)	(2)
1/1	1.062	1.021
1/2	0.557	0.555
1/4	0.330	0.329
1/8	0.197	0.191
1/16	~~0.128~~	~~0.135~~
1/32	~~0.087~~	~~0.090~~

Sample 2		
Designation	Vaccine 1	
Ass. pot.	? AU/0.3 ml	
Extraction	0.3 ml/75 µl	
Incubation	100 µl/well	
Doses	(1)	(2)
1/8	~~3.228~~	~~2.983~~
1/16	~~3.162~~	~~3.422~~
1/32	~~3.309~~	~~3.479~~
1/64	~~2.860~~	~~2.945~~
1/128	~~1.994~~	~~2.003~~
1/256	1.278	1.303
1/512	0.762	0.836
1/1024	0.451	0.505
1/2048	0.270	0.280
1/4096	0.168	0.176
1/8192	~~0.112~~	~~0.112~~

Sample 3		
Designation	Vaccine 2	
Ass. pot.	? AU/0.5 ml	
Extraction	0.5 ml/125 µl	
Incubation	100 µl/well	
Doses	(1)	(2)
1/8	1.779	1.763
1/16	0.934	0.911
1/32	0.479	0.520
1/64	0.316	0.293
1/128	0.196	0.188
1/256	~~0.134~~	~~0.134~~
1/512	~~0.095~~	~~0.099~~
1/1024	~~0.078~~	~~0.091~~
1/2048	~~0.085~~	~~0.078~~
1/4096	~~0.081~~	~~0.072~~
1/8192	~~0.061~~	~~0.061~~

Sample 4		
Designation	Vaccine 3	
Ass. pot.	? AU/0.5 ml	
Extraction	0.5 ml/200 µl	
Incubation	100 µl/well	
Doses	(1)	(2)
1/8	~~2.989~~	~~3.036~~
1/16	~~2.569~~	~~2.674~~
1/32	1.498	1.838
1/64	0.779	0.925
1/128	0.430	0.497
1/256	0.263	0.292
1/512	~~0.184~~	~~0.184~~
1/1024	~~0.118~~	~~0.136~~
1/2048	~~0.099~~	~~0.104~~
1/4096	~~0.075~~	~~0.079~~
1/8192	~~0.068~~	~~0.074~~

Explanations:
This template in CombiStats shows all relevant information for the correct calculation of the antigen content per dose.
The reference antigen is assigned 6 AU/vial. Resuspended in 1500 µl this gives 1 AU/250 µl and 0.4 AU in the first well.
A dose of vaccine 1 is 0.3 ml, so the assumed potency is specified in AU/0.3 ml. The water phase is 25%, so the extraction is 75 µl.
A dose of vaccine 2 is 0.5 ml, so the assumed potency is specified in AU/0.5 ml. The water phase is 25%, so the extraction is 125 µl.
A dose of vaccine 2 is 0.5 ml, so the assumed potency is specified in AU/0.5 ml. The water phase is 40%, so the extraction is 200 µl.
Each well contains 100 µl of the indicated dilution of these extracts.
The ODs which are not included in the calculations are barred out.

Model: Parallel lines.
Design: Completely randomised.
Transformation: y' = ln(y)
Variance: Observed residuals.

Common slope = 0.787221 (0.784487 to 0.789955)
C = 1.00046
g = 4.64 E-04

EDQM/Stat/COE

Example Calculation of Potency

CombiStats. Version 2.01.

Project	BSP055
Assay ID	Lab 8, Assay 2, Plate 1

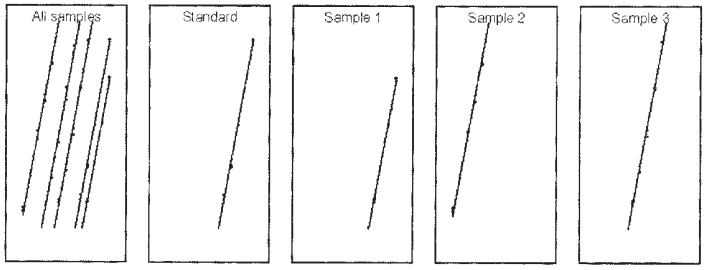
Source of variation	Degrees of freedom	Sum of squares	Mean square	F-ratio	Probability
Preparations	4	0.780115	0.195029	75.570	0.000 (***)
Regression	1	23.8196	23.8196	>1000	0.000 (***)
Non-parallelism	4	0.041258	0.010315	3.997	0.013 (*)
Non-linearity	13	0.070225	0.005402	2.093	0.059
Standard	3	0.018275	0.006092	2.360	0.098
Sample 1	2	0.005995	0.002997	1.161	0.331
Sample 2	3	0.003145	0.001048	0.406	0.750
Sample 3	3	0.036889	0.012297	4.765	0.010 (*)
Sample 4	2	0.005922	0.002961	1.147	0.335
Treatments	22	24.7112	1.12324	435.230	0.000 (***)
Residual error	23	0.059358	0.002581		
Total	45	24.7705	0.550456		

Sample 1			
(AU / vial)	Lower limit	Estimate	Upper limit
Potency	2.85280	3.04104	3.24069
Rel. to Ass.	?	?	?
Rel. to Est.	93.8%	100.0%	106.6%

Sample 2			
(AU / 0.3 ml)	Lower limit	Estimate	Upper limit
Potency	56.7904	60.3053	64.0235
Rel. to Ass.	?	?	?
Rel. to Est.	94.2%	100.0%	106.2%

Sample 3			
(AU / 0.5 ml)	Lower limit	Estimate	Upper limit
Potency	3.55027	3.76889	4.00075
Rel. to Ass.	?	?	?
Rel. to Est.	94.2%	100.0%	106.2%

Sample 4			
(AU / 0.5 ml)	Lower limit	Estimate	Upper limit
Potency	19.7776	21.0711	22.4523
Rel. to Ass.	?	?	?
Rel. to Est.	93.9%	100.0%	106.6%



Annex 2 - Establishment of a candidate Biological Reference Preparation and proposal of a cut-off value indicative of sufficient potency

SUMMARY

3 vaccine batches from the BSP055-2 study that had relatively low antigen content were measured for *in vivo* potency. Results showed that all were above release specification level. Combination of these results with data previously generated in the first part of the BSP055 study leads to a proposal of a threshold antigen level of 7.0 antigen units per dose. This threshold level would not lead to any false positive results for the data in the BSP055 studies and would result in only a limited number of false negatives, even though several vaccine batches were tested that showed borderline potency.

1. AIM

Generation of data to determine a threshold for the relative antigen content that is indicative of sufficient *in vivo* potency of inactivated ND vaccines.

2. EXPERIMENTAL DESIGN

In the BSP055-2 validation study 14 laboratories performed an antigen quantification assay for inactivated ND-vaccines as a candidate *in vitro* potency test. Nine vaccine batches with a range of antigen content were used in this study, including one vaccine batch with insufficient potency.

The potency of 3 vaccine batches, from 3 different manufacturers, was determined *in vivo* at CIDC-Lelystad using the two potency tests that are described in the European Pharmacopoeia (EP2002:0870). These 3 batches were selected because of their relatively low antigen content. Since the vaccine batch with the lowest antigen content had already been tested a number of times *in vivo*, the three batches with the next lowest antigen content that were derived from different manufacturers were selected. The results of these *in vivo* tests will be compared with previously generated *in vivo* results of the first part of the BSP055 study.

3. RESULTS

The results of both the vaccination-serology test and the vaccination-challenge test are presented in Table 5. A good dose response effect was obtained for the serological results for all 3 vaccine batches. As reported previously the HI-antibody titres after vaccination with 1/50 dose were all clearly below the threshold for sufficient potency, i.e. a HI-titre of 4. However, all 3 batches induced sufficient clinical protection after challenge with virulent NDV strain Herts. For one batch no significant regression was found for the protection after vaccination with different doses. However, since the total number of chickens that were protected (31 of 60) was more than 50% and since 12 of 20 chickens were protected after vaccination with 1/50 dose, we conclude that this vaccine batch contained more than 50 PD50 per dose.

These results were compared with previous data from the first part of BSP055, plus additional tests on the vaccine batch with insufficient potency performed at CIDC-Lelystad. All data are summarised in Table 6.

Since PD50 values of more than 100 have very large confidence intervals, these values were all scored as > 100.

4. DISCUSSION AND CONCLUSION

Two of the three vaccine batches from the BSP055-2 study that were tested had clearly sufficient potency in the vaccination-challenge assay. Although the potency assay for the third vaccine batch was not valid (no significant regression), this batch also seemed to have sufficient potency based on the survival rates observed.

After evaluation of all data on the vaccines that were tested in the candidate *in vitro* potency assay as well as in the Pharmacopoeial *in vivo* assays, we conclude that a very good correlation exits between the antigen content of these inactivated ND-vaccines and the potency *in vivo*.

For determination of a threshold antigen level that is indicative of sufficient potency, a comparison with the results of the vaccination-challenge assay should be made, since the HI-antibody titres were often below the threshold level of 4.0 and varied considerably between laboratories (see BSP055-1: reference 8).

We therefore propose a threshold antigen level of 7.0 antigen units per dose.

In the BSP055 studies such a threshold did not lead to any false positive results and only a limited number of false negative results were observed.

Table 5 – *Potency assays vaccines BSP055-2*

Vaccine	Dose	Mean HI antibody titre (log2)	Protected	PD50/dose	Mean IVRP AU/dose
3	1/25	4.5	20/20	> 100	17.3
3	1/50	3.4	20/20		
3	1/100	2.2	12/20		
4	1/25	1.9	11/20	> 50*	7.6
4	1/50	1.3	12/20		
4	1/100	0.6	8/20	81	9.9
5	1/25	3.0	20/20		
5	1/50	2.0	16/20		
5	1/100	0.8	7/20		
Control		0.1	0/20		

*No significant regression

IVRP: *in vitro* relative potency, AU: antigen units IVRP: *in vitro* relative potency, AU: antigen units

Table 6 - *Antigen content of inactivated ND-vaccines and in vivo potency*

Vaccine	BSP055 Study	Mean IVRP* AU/dose	PD50/dose	Mean HI antibody-titre after vaccination with 1/50-dose (log2)[c]
2	1	0.5	1.5[a] - 3.9[c] - 5.5[b]	0.1
4 - 2	1 - 2	3.8 - 3.1	9.0[a] - 24[c] - 25[c] - 31[a] - 49[c]	0.9
6	1	5.6	76[c] - >100[a] - >100[b]	1.2
4	2	7.6	>50[c]	1.3
5	2	9.9	81[c]	2.0
3	2	17.3	>100[c]	3.4
5	1	57	>100[a] - >100[c] - >100[b]	3.7
3	1	119	>100[a] - >100[c] - >100[b]	3.6
1	1	155	>100[a] - >100[c] - >100[b]	4.2

* Mean value in BSP055 study. IVRP: *in vitro* relative potency, AU: antigen units

[a] Measured at OMCL 2 (BSP055-1)

[b] Measured at OMCL 3 (BSP055-1)

[c] Measured at CIDC-Lelystad (BSP055-1 and BSP055-2)