In Vitro Alternative Assay for Newcastle Disease Vaccine

1. Introduction

An Newcastle disease virus (NDV) antigen quantification assay has been developed at CIDC-Lelystad as a candidate in vitro potency test for inactivated Newcastle disease 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). Furthermore, a high correlation between the serological data (Haemagglutination Inhibition-antibody titres) and clinical protection after challenge was demonstrated.

The aim of the feasibility study was to confirm the correlation between the results obtained using the candidate in vitro potency assay and the results from both the in vivo potency assays currently prescribed in Ph Eur monograph 0870, in different laboratories and to determine whether a large-scale validation study of the in vitro method should ensue.

In the feasibility study three Official Medicines Control Laboratories tested the potency of 5 different inactivated Newcastle disease vaccines and one experimental vaccine, using both of the in vivo methods described in the European Pharmacopoeia and the candidate in vitro method. The 6 vaccine batches represented a quantitative range of Newcastle disease virus antigen content and were produced by different manufacturers.

Statistical evaluation of all results indicated that a satisfactory correlation was found in all laboratories between the two types of in vivo tests currently in place, and the candidate in vitro test. An excellent reproducibility of the proposed in vitro method was observed with respect to the ranking of the vaccines included in this study. It is concluded that the results of this feasibility study indicate that a large-scale collaborative study can be organised to validate the in vitro method and the suitability of the reference preparation.

Keywords

Newcastle disease vaccine, Potency assay, Feasibility study, In vitro alternative method, ELISA, European Pharmacopoeia

1. Introduction

An Newcastle disease virus (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 \textit{in vivo} methods now listed in the Ph Eur monograph 0870. As two \textit{in vivo} methods are allowed in the Ph Eur monograph (method A and B), acceptance of the \textit{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].

After evaluation of the studies performed at CIDC-Lelystad by the Ph Eur Experts Group 15V, it was decided that further demonstration of the correlation between \textit{in vitro} results as determined by the ELISA and the \textit{in vivo} potency determination by established methods should be performed before a large-scale validation study of the \textit{in vitro} method could take place.

A validation study to demonstrate both the feasibility and the accuracy of a candidate \textit{in vitro} potency assay for inactivated ND vaccines with a successful outcome would then hopefully result in the acceptance of an \textit{in vitro} antigen quantification assay as an alternative assay for the batch release of inactivated ND vaccines in addition to the current \textit{in vivo} methods.

2. Aim of the study

The aim of the feasibility study was to confirm the correlation between the results obtained using the candidate \textit{in vitro} potency assay and the results from both the \textit{in vivo} potency assays currently prescribed in the Ph Eur monograph for ND vaccine in different laboratories and to thus determine whether a large-scale validation study of the \textit{in vitro} method should ensue.

3. Participants

Three OMCLs with experience in performing the \textit{in vivo} potency assays prescribed in the Ph Eur monograph for ND vaccine took part in the study. All laboratories reported results using the reporting forms provided. The laboratories have been randomly assigned a code number from 1 to 3 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 test the potency of 6 different inactivated ND vaccines using both of the \textit{in vivo} methods described in the Ph Eur monograph 0870 and the candidate \textit{in vitro} method. The participating laboratories had experience in the performance of \textit{in vivo} potency assays for ND vaccines. Samples were coded [1-6] so as not to reveal their identities. Five commercial batches represented a range of ND vaccines with different antigen contents and were produced by different manufacturers. These vaccines passed all release tests of the manufacturers and therefore were expected to have sufficient potency. One experimental vaccine batch with insufficient potency was included in the study (No. 2). This vaccine batch was prepared especially by Intervet BV (Boxmeer, The Netherlands) and contained a very low antigen content.

4.2. Methods

\textit{In vivo}

\textit{In vivo} potency assays were based on method A (serology assay) and method B (vaccination challenge) of the Ph Eur monograph. In order to reduce the use of animals to a minimum the same animals were used for both methods A and B as follows:

For each vaccine to be tested three groups of 20 SPF-chickens (3-4 weeks of age) were vaccinated with different volumes corresponding to 1/25, 1/50 and 1/100 of a dose.

Three weeks after vaccination before viral challenge, blood samples were taken from the animals that were vaccinated with 1/50 dose, and antibody titres were measured in the HI-assay (Ph Eur monograph 0870, test A); the HI titre of each serum was determined three times.

All animals were thereafter challenged with at least 10\textsuperscript{6} ELD\textsubscript{50} virulent NDV strain Hertz and the mortality and morbidity was monitored (Ph Eur monograph 0870, test B).

A control group with non-vaccinated animals was also included in the experiment (both method A and B).

\textit{In vitro}

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

The 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 the 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.

1) Extraction of viral antigens from water-in oil emulsion vaccines with IPM

Refer to the standard operating procedure provided in Annex 1
2) ELISA for quantification of the HN-protein of NDV

Refer to the standard operating procedure provided in Annex 2

4.3. Materials

CIDC-Lelystad distributed the test samples 1-6 and reagents for the in vitro assay. The 6 vaccines to be tested were produced by 4 different manufacturers (Fort Dodge, The Netherlands; Intervet BV, The Netherlands; Lohmann Animal Health, Germany; Mérieux, France). Monovalent as well as combination vaccines were included in this study. The vaccines contained either NDV strain Ulster, NDV strain LaSota or Clone30 NDV. Five commercial vaccine batches that had been released on the market and were thus presumed to have adequate potency were tested in this study. One experimental vaccine (No 2) with a very low antigen content and insufficient potency (produced by Intervet BV, The Netherlands) was also included to test the ability of the in vitro assay to identify sub-potent batches. During the course of this study it appeared that one of the commercial batches (No. 4), was also of insufficient potency as tested by all participants, although it passed the manufacturers release tests.

Each participant received the following reagents supplied by CIDC-Lelystad for the in vitro assay:

- Coating antibody
- IDNDV134.1 (lyophilised) (to be resuspended in 1.5 ml ELISA-buffer)
- HRPO-Conjugate of IDNDV134.1 (lyophilised) (to be resuspended in 1.5 ml ELISA-buffer)
- TMB (3,3',5,5' Tetramethylbenzidine)
- Reference antigen (lyophilised) (to be resuspended in 1.5 ml ELISA-buffer)
- Internal control antigen (to be resuspended in 1.5 ml ELISA-buffer)
- IPM
- ELISA-plates

For a complete list of materials required for the in vitro assay refer to the standard operating procedures for the extraction of viral antigen and the ELISA quantification of HN-protein of NDV in Annexes 1 and 2.

4.4. Statistical analysis

The raw data of the in vitro assays were analysed at the 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 potencies are expressed in terms of relative potencies per 100 µl of vaccine extract assuming 1 arbitrarily assigned unit per 200 µl of reference material resuspended in 1.5 ml ELISA-buffer. It should be noted that the relative potency of the extract bears no immediate relationship with the potency per dose of the original product, because it depends on the water phase and the volume of a dose. The water phase and dose volume was not the same for all vaccines included in this study. Pre-calculated correction factors, based on the percentage aqueous phase or the dose volume, were applied to vaccines 5 and 6 respectively in order to enable correct ranking of the products and comparisons with the in vivo tests.

The raw data of the in vivo Test A were submitted to an averaging of the log₂ titres per animal per assay. The raw data of the in vivo Test B were submitted to PD₅₀ determination of probit transformed survival rates. It should be noted that most PD₅₀ values are not contained within the dilution range, which may complicate a reliable ranking of the potencies.

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 each assay type.

5. Results

The relative potency estimates based on in vitro assay data are listed in Table 1a. Shown are the results for each assay, the geometric mean across assays within laboratories, and the rank numbers of the vaccines within laboratories. It can be seen that the ranking is identical for each laboratory. However, quantitative comparisons between laboratories show that laboratory 2 finds consistently lower potencies than laboratory 1, and almost consistently lower potencies than laboratory 3 (with vaccine 3 as the only exception). Laboratory 3 finds higher potencies than Laboratory 1 for weaker vaccines, and the opposite for stronger vaccines. This is more clearly illustrated in Figure 1 where the potencies are plotted against each other. It is striking that the potencies of Laboratories 1 and 2 differ by a nearly constant ratio of about 2. This may be the result of a dilution error. However as no laboratory indicated a problem with the dilution scheme it could indicate that the assay is potentially very reproducible, but that the reference behaves differently compared to the test vaccines in both laboratories. For example, the difference between the two laboratories would be much smaller if any of the test vaccines is taken as a reference. Interestingly, reproducibility seems to improve when the Control is used as a reference. The relative potencies are shown in Table 1b (assuming a potency of 0.7 units per 200 µl) and the corresponding plots are shown in Figure 2.

The HI titres of in vivo test A are listed in Table 2. Shown are the arithmetic mean of the titres per repetition, the arithmetic mean of the repetitions per vaccine, and the ranking per laboratory. The three weakest vaccines (vaccine 2, 4 and 6) are ranked identically by each laboratory and this is consistent with the ranking of the in vitro results. The three strongest vaccines are not ranked in the same order, and none of the laboratories finds the same ranking as the in vitro results. However, the inherent variability of the assay can easily cause inversions between
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vaccines that are of similar potency. The ranking based on overall titres (mean between laboratories) is shown to be identical to the in vitro potencies.

The estimated PD_{50} values per dose based on in vivo test B are listed in Table 3, together with the rankings. Again, it can be seen that the three weakest vaccines are ranked identically by each laboratory and consistent with the in vitro results. The three strongest vaccines were not ranked the same by all laboratories. Laboratory 1 found vaccine 3 to be the strongest, whereas the other laboratories found vaccine 5 to be the strongest. The overall ranking is also not identical to the other types of assay. However, the PD_{50} values of these vaccines are based on such a large extrapolation of the probits that these rankings cannot be reliably established. The insignificance of the 'missplacement' of vaccine 5 as the strongest vaccine can more intuitively be judged by direct examination of the survival rates, as is shown in Table 5.

Table 1a – In vitro results

<table>
<thead>
<tr>
<th>Lab</th>
<th>Vac</th>
<th>Assay</th>
<th>Mean</th>
<th>Rank</th>
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</tr>
<tr>
<td>3</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

In the large table are listed the relative potencies per 100 µl (with respect to the reference material) for each individual assay and the geometric mean across the assays. For each laboratory, the first Control refers to the control vaccine included on a plate with vaccines 1 to 3, and the last Control refers to the one included on one plate with vaccines 4 to 6. The highest estimated potency within a laboratory is assigned rank 1 and the lowest estimated potency is assigned rank 6 (Controls excluded).

The small table lists the geometric mean of vaccines 1 to 6 across laboratories and their respective rank numbers.
In the large table are listed the relative potencies per 100 µl (with respect to the Control) for each individual assay and the geometric mean across the assays. For each laboratory, the first reference refers to the reference included on a plate with vaccines 1 to 3, and the last reference refers to the one included on one plate with vaccines 4 to 6. The highest estimated potency within a laboratory is assigned rank 1 and the lowest estimated potency is assigned rank 6 (reference excluded).

The small table lists the geometric mean of vaccines 1 to 6 across laboratories and their respective rank numbers.

<table>
<thead>
<tr>
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<th>Mean</th>
<th>Rank</th>
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<td></td>
<td></td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>1</td>
<td>Reference</td>
<td>0.450</td>
<td>0.606</td>
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<td>1</td>
<td>180</td>
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<td>222</td>
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<td>5.32</td>
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<td>5</td>
<td>72.6</td>
<td>98.4</td>
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<td>6</td>
<td>6.51</td>
<td>7.39</td>
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<td>9.66</td>
<td>7.23</td>
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<td>1.12</td>
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<th>Rank</th>
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<td>4</td>
<td>4.90</td>
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<td>52.1</td>
<td>45.7</td>
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<tr>
<td></td>
<td>Reference</td>
<td>0.435</td>
<td>0.458</td>
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<tr>
<td>2</td>
<td>0.652</td>
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<td>6</td>
<td>7.30</td>
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Table 1b – *In vitro results using the Control as reference*
In the large table are listed the average log₂ titres for each individual assay and the arithmetic mean across assays. The vaccine with the highest average titre within a laboratory is assigned rank 1 and the lowest is assigned rank 6.

The small table lists the arithmetic mean of the vaccines across laboratories and their respective rank numbers.
Table 3 – *In vivo results (Test B)*

<table>
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<th>Vac</th>
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<th>Lab 3</th>
<th>Mean</th>
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<td>1</td>
<td>150</td>
<td>570</td>
<td>482</td>
<td>345</td>
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<td>2</td>
<td>3.90</td>
<td>1.54</td>
<td>5.51</td>
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<td>25.1</td>
<td>9.0</td>
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<td>6</td>
<td>76</td>
<td>171</td>
<td>163</td>
<td>128</td>
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</tbody>
</table>

In the upper table are listed the estimated PD<sub>50</sub>/dose for each individual vaccine and laboratory, together with the geometric mean across laboratories.

In the lower table are listed the corresponding rank numbers for each vaccine within laboratories, and across laboratories.

Table 4 – *Overview of rankings*

<table>
<thead>
<tr>
<th>Vac</th>
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<th>Lab 2</th>
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<th>Overall</th>
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Listed is a summary of the ranking of the six vaccines by the three laboratories using each of the three assay methods, together with the overall ranking (See tables 1 to 3)
Table 5 – Survival rates

<table>
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<th>Dilution</th>
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<td>1/25</td>
<td>16/19</td>
<td>20/20</td>
<td>22/22</td>
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<tr>
<td>1/50</td>
<td>17/20</td>
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<td>24/24</td>
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<tr>
<td>1/100</td>
<td>16/20</td>
<td>18/20</td>
<td>20/22</td>
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</table>

Listed are the survival rates at each dilution of vaccine 1 and 5.

Each plot represents the ln estimates obtained with the in vitro assay compared between two laboratories. Each dot represents a vaccine. The diagonal line is the line of agreement.

Figure 1 – Two-way plots

Figure 2 – Two-way plots using the Control as reference

Each plot represents the ln estimates obtained with the in vitro assay compared between two laboratories. Each dot represents a vaccine. The diagonal line is the line of agreement.
6. Discussion

The batch potency of inactivated ND vaccines is measured in vaccination experiments. Many animals are used in these potency tests. The in vitro quantification of the antigen content of these inactivated ND vaccines seems a promising alternative to these animal experiments. It was shown that quantification of NDV-HN is possible in all vaccines that were tested. Furthermore, the monoclonal antibodies used in the HN-ELISA recognize all known NDV strains. It was demonstrated that the HN-antigen content in inactivated oil-adjuvanted vaccines could be a reliable indicator of vaccine potency [1-7].

In the present study it was found that the in vitro assay, consisting of the extraction of the water phase with the antigens from the oil-emulsions and a quantitative ELISA, could be performed at the different OMCLs without any problem. Furthermore, the correlation between the antigen content of inactivated ND vaccines and the two official in vivo potency tests is confirmed by the participating OMCLs. All participants could identify vaccine batches with insufficient, low and high potency with the use of the antigen quantification assay. Comparison of the test results indicated that a 100% correlation in ranking was found for the 3 vaccine batches with the lowest antigen content. The ranking of the 3 other vaccines with high potency is hampered by the large extrapolations of the probits in the PD050 calculations. These high-potency vaccines seem to induce (almost) maximal protection in the in vivo potency tests.

The relative potencies of the vaccine batches that were tested in the present study could vary up to a factor of two. Although this variation does not prevent the identification of vaccine batches with insufficient potency in the antigen quantification assay, this variation was higher than that found earlier in an international transfer study [2]. The reason for this is not clear. Stability of the lyophilised reference preparation has been demonstrated for a period of two years [6; unpublished data]. Test reproducibility and suitability of the reference preparation should be evaluated in a separate study.

For the determination of a cut-off value for the vaccine antigen content (RP-value) that is indicative of sufficient potency, more data on test reproducibility are required. According to the current Ph Eur monograph for ND vaccine (inactivated) a vaccine passes the potency test if 1/50 the dose has a mean HI titre of 4 log2 (method A) or if one vaccine dose corresponds to at least 50 PD050 (method B), with the results from method B considered the definitive response. Preliminary data from this study indicate that an RP-value of at least 10 would be a safe cut-off value for the identification of vaccine batches with insufficient potency. Determination of a safe cut-off value would automatically imply that some vaccine batches with sufficient but relatively low potency, would not pass the in vitro candidate potency test. However, sufficient potency of these batches can be demonstrated in the in vivo tests.

The reproducibility of the assay and the suitability of the reference preparation should be the subject of a large collaborative validation study. Preferably, as many OMCLs and vaccine manufacturers as possible should participate in this study. Satisfactory results of such a large collaborative study should provide a solid base for the acceptance of this assay as an in vitro alternative for the present potency tests for inactivated ND vaccines.

7. Conclusion

In this study a satisfactory correlation has been shown between the two types of in vivo tests currently in place, and the proposed in vitro test. An excellent reproducibility of the proposed method was observed with respect to the ranking of the vaccines included in this study. Although a cut-off value for sufficient vaccine antigen content still has to be defined, all laboratories were able to discriminate between vaccine batches with sufficient and insufficient potency with the use of the candidate in vitro potency test.

Since the monoclonal antibody used in the ELISA recognizes all NDV-strains that could be used to make inactivated vaccines, it is concluded that the in vitro test is a candidate reference test.

The results of this study support the idea to organise a large collaborative study to further validate the in vitro assay. The reproducibility of the antigen quantification assay and the suitability of the proposed reference should be the subject of this collaborative study.

8. Literature


In Vitro Alternative Assay for Newcastle Disease Vaccine


9. List of participants (presented in alphabetical order by surname of principle investigator)

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Annex 1

Extraction of Viral Antigens from Water-in-Oil Emulsion Vaccines with Isopropyl myristate

Standard Operating Procedure

1. Aim and application

After extraction of the viral antigens from the oil emulsions, the inactivated antigens can be quantified to measure for vaccine potency.

2. Definition/abbreviations

IPM: Isopropyl myristate

3. Utensils

3.1. Disposables
— Isopropylmyristate: IPM (at least 98%) Sigma

3.2. Materials
— Centrifuge with buckets for 15 ml tubes (e.g. Minifuge GL/Minifuge RF) Heraeus
— 15 ml centrifuge-tubes (2096 blue max™ Jr.) 15 ml polypropylene conical tubes;  
  Note: do not use polystyrene tubes Becton Dickinson
— Minishaker: e.g. MS1 Minishaker IKA-Works.Inc

4. Methods

4.1. Precautions

IPM is stored at 4-8 °C. IPM can become solid at lower temperatures.

4.2. Preparations

The centrifuge is cooled at 8 °C.

4.3. Method

— The vaccines and the IPM are taken out of the refrigerator directly before use.
— The vaccines are shaken vigorously for 10 sec. to obtain a homogenous emulsion.
— Two ml of vaccine is added to 8 ml IPM in a 15 ml tube (note: polypropylene tubes should be used).
— The mixture is shaken vigorously for 1 min (Minishaker: maximal).
— The tubes are centrifuged for 10 min (1000 g, 8 °C)
— The upper part (IPM + oil) is removed (approximately 9 to 9.5 ml)
— The lower phase is the water-phase with the antigen. The colour and clarity of this phase can vary between different vaccines (extracts can be turbid).

5. Storage

Preferably, the extracts are used directly in an antigen quantification assay.
Annex 2

ELISA for Quantification of the HN-Protein of Newcastle Disease Virus

Standard Operating Procedure

1. Aim and application

Newcastle disease virus (NDV) is a single stranded RNA virus and belongs to the family of Paramyxoviridae. Since virulent NDV-strains cause mortality and disease in poultry, vaccines have been developed in order to prevent the disease. Inactivated ND vaccines are used to induce high levels of antibodies in hens before the laying period. The potency of each new vaccine batch must be tested in a vaccination experiment. The antigen ELISA has been developed to quantify NDV-HN in vaccine extracts as alternative for the serological potency assay. The test can also be used to identify NDV for other purposes.

1.1. Detection limit

The lowest quantity of virus that gives a positive reaction in the ELISA is 0.05 haem-agglutinating units (HAU), i.e. 5,000 CCID₅₀.

2. Definition/abbreviations

The ELISA is a specific antigen measurement of the HN-protein of Newcastle disease virus.

CCID₅₀ The statistically determined quantity of virus that may be expected to infect 50% of cell cultures to which it is added

ELISA Enzyme-Linked ImmunoSorbent Assay

DAS-ELISA Double Antibody Sandwich ELISA

HAU Haem-Agglutinating Units

HN Haemagglutinin Neuraminidase

HRPO Horse Radish Peroxidase

Mab Monoclonal antibody

NDV Newcastle Disease Virus

OD Optical Density

PBS Phosphate Buffered Saline

TMB 3,3',5,5' Tetramethylbenzidine

3. Principle

The test for the detection of the HN-protein of NDV is a Double Antibody Sandwich (DAS) ELISA.

Principle of the assay is that ELISA-plates are coated with antibodies binding the HN-protein of NDV. Thereafter an antibody-enzyme conjugate binds to NDV. After addition of enzyme substrate, a staining develops when the conjugate is present.

For coating of the ELISA plates monoclonal antibody IDNDV134.1 is used. The hybridoma producing these antibodies was generated at ID-Lelystad by immunising mice against the velogenic Dutch isolate PMV1/92/152608. The monoclonal antibody recognises a linear epitope of NDV-HN (amino-acids 335-355). The antibodies are produced by purifying culture supernatant of the hybridoma cells using protein-G columns. The same monoclonal antibody is conjugated with HRPO to visualise the viral antigen. After binding of the conjugate, TMB is added as substrate of HRPO. When conjugate is present a coloured reaction product is produced that can be quantified using an ELISA-reader that measures absorption at a specific wave length. The OD is a measure for the concentration of NDV-HN that is present.

4. Equipment and reagents

4.1. Disposables

– High-binding ELISA-plate

– Coating-buffer

– 0.05 % Tween 80

– ELISA-buffer

– TMB (3,3',5,5'Tetramethylbenzidine)

– 0.5 M H₂SO₄

– Coating antibody IDNDV134.1

– HRPO-Conjugate of antibody IDNDV134.1

4.2. Materials

– Variable volume pipette (e.g. Pipetman P10, P200 and P1000).

– Incubator: 37 °C ± 2 °C

– Refrigerator: 4 °C

– Titertek Multiscan ELISA reader

– Centrifuge

4.3. Reference

NDV ELISA Reference antigen (lyophilised NDV-Ulster, formalin-inactivated)

5. Methods

5.1. Precautions

– Bring all reagents to room temperature before use.

– Treat all biological materials as potentially infectious.

– Wear gloves when using TMB ELISA substrate.

5.2. Preparations

NDV-antigen is extracted from oil emulsion vaccines with the use of isopropylmyristate (IPM).
5.3. **Instrument adjustment**

The temperature of the incubator is 37 °C

5.4. **Scheme of the test**

— All samples are diluted from the left to the right, using twofold dilution steps.

— On each ELISA plate 6 wells are used as blanks (no antigen present).

— Six dilutions of both the reference and the control antigen are tested in duplicate.

— Eleven dilutions of the vaccine extracts are tested in duplicate.

5.5. **Method**

5.5.1. **Coating of the ELISA-plates**

— Dissolve the content of a vial with lyophilised coating-antibody in 1.5 ml aqua dest.

— Dilute 20 × in coating-buffer, e.g. 0.5 ml coating-antibody + 9.5 ml coatings buffer (enough for one ELISA-plate).

— Add 100 µl of the diluted solution of coating-antibody to each well.

— Plates are covered with plate sealers.

— Incubate 2 hr at 37 °C.

— Wash the plates 2 × 3 times with 0.05 % Tween80, using an ELISA-washer. Turn the plate 180° after the first 3 washes.

— When no ELISA-washer is available, the plates may be washed by hand. In this case 3 washes with 200 µl PBS/0.05 % Tween80 should be performed.

— Tap the plates dry on absorbing paper.

— These plates, covered with plate sealers, can be stored at 4 °C for maximally one week. The coated plates are not washed again before use.

5.5.2. **Antigen incubation**

— Dissolve the content of 1 vial with lyophilised internal control in 1.5 ml ELISA-buffer.

— Dilute the vaccine-extracts 4x in ELISA-buffer: e.g. 100 µl vaccine-extract + 300 µl ELISA-buffer.

— Add 100 µl ELISA-buffer to all wells, but not to A1, B1, A7, and B7.

— Add 200 µl of the reference solution to the wells A1 and B1.

— Add 200 µl of the internal control solution to the wells A7 and B7.

— Add 100 µl of the diluted vaccine A extract to wells C1 and D1.

— Add 100 µl of the diluted vaccine B extract to wells E1 and F1.

— Add 100 µl of the diluted vaccine C extract to wells G1 and H1 (See scheme of the 96-wells plate).

When more than 3 vaccines are tested, the reference and internal control are tested on each ELISA-plate.

— Make dilution series of the reference antigen, using twofold dilution steps:
  - transfer 100 µl from wells A1 and B1 to wells C1 and D1.
  - transfer 100 µl from wells A2 and B2 to wells E1 and F1.
  - transfer 100 µl from wells A3 and B3 to wells G1 and H1.

The last 100 µl from wells A6 and B6 are discarded.

— Make dilution series of the internal control antigen, using twofold dilution steps:
  - transfer 100 µl from wells A7 and B7 to wells A8 and B8.
  - transfer 100 µl from wells A9 and B9.

The last 100 µl from wells A12 and B12 are discarded.
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— Make dilution series of the vaccine-extracts in wells C1 - H1 to C11 - H11 (do not use C12 – H12), using twofold dilution steps:
  - transfer 100 µl from wells C1 - H1 to wells C2 - H2 and mix,
  - transfer 100 µl from wells C2 - H2 to wells C3 - H3 and mix, etc.
The last 100 µl from wells C11 - H11 are discarded.
— Wells C12 to H12 are blank wells to measure the background OD: no antigen is added to these wells.
— Plates are covered with plate sealers.
— Incubate 4 hr at 37 °C.
— Wash the plates 2 × 3 times with 0.05 % Tween80, using an ELISA-washer. Turn the plate 180° after the first 3 washes.
  When no ELISA-washer is available, the plates may be washed by hand. In this case 3 washes with 200 µl PBS/0.05 % Tween80 should be performed.
— Tap the plates dry on absorbing paper.

5.5.3. Conjugate Reaction
— Dissolve the content of 1 vial with lyophilised conjugate in 1.5 ml ELISA-buffer.
— Dilute the conjugate 20 × in ELISA-buffer, e.g. 0.5 ml conjugate + 9.5 ml ELISA buffer (enough for one ELISA-plate).
— Add 100 µl conjugate to all wells.
— Plates are covered with plate sealers.
— Incubate 1 hr at 37 °C.
— Wash the plates 2 × 3 times with 0.05 % Tween80, using an ELISA-washer. Turn the plate 180° after the first 3 washes.
  When no ELISA-washer is available, the plates may be washed by hand. In this case 3 washes with 200 µl PBS/0.05 % Tween80 should be performed.
— Tap the plates dry on absorbing paper.

5.5.4. Staining
— Add 100 µl TMB-substrate to all wells.
— Incubate 15 min at room temperature.
— Stop the enzyme-reaction by adding 100µl 0.5 M H₂SO₄ to all wells.
— Measure the Optical Density (OD) at 450 nm, using an ELISA-reader. Store the data on a floppy disk.

6. Results
6.1. Calculation
The raw data from the ELISA-reader are used to calculate the relative potency of the samples. The CombiStats Software (EDQM, Strasbourg, France) may be used.

6.2. Judgement of the test results
Preferably the OD-values in the blank wells should be below 0.20.
With use of the CombiStats software the linearity and parallelism of the dilution curves is checked.

6.3. Representation of the test results
The Relative Potency (RP) of the samples is calculated.

7. Storage
All lyophilised materials and the TMB substrate are stored at 4 °C. Coated ELISA-plates can be stored at 4 °C for maximally 1 week.
ELISA-buffer can be stored at 4 °C for maximally 1 week.

8. Literature

9. Buffers
Coating buffer
Dissolve 2.65 g Na₂CO₃ in 500 ml demineralised-water (A).
Dissolve 4.2 g NaHCO₃ in 1000 ml demineralised-water (B).
Add A to B until the pH is 9.6.
Coating buffer can be stored at 4 °C for one month.

ELISA buffer
1 liter of PBS (pH 7.4) is made by dissolving in demineralised-water:
— 8.0 g NaCl
— 0.2 g KCl
— 0.2 g KH₂PO₄
— 1.15 g Na₂HPO₄
To obtain 100 ml of ELISA buffer:
— 0.5 ml 10 % Tween80 is added to 99.5 ml PBS,
— 5.1 g NaCl is added
— 1.0 g Bovine Serum Albumen (BSA) is added.
ELISA buffer can be stored at 4 °C for one week.