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Case study of development, validation, and acceptance of a non-animal method for assessing veterinary vaccine potency

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Abstract

This paper describes the development of an *in vitro* assay to replace *in vivo* potency testing for the batch release of inactivated Newcastle Disease virus vaccines. The assay involves the extraction of inactivated antigen from oil emulsion vaccines, the most common adjuvant for poultry vaccines. An enzyme-linked immunosorbent assay (ELISA) is used to quantify the hemagglutinin-neuraminidase (HN) protein of the virus, which was demonstrated to correlate with protection levels.
Validation experiments showed that the method could be used for HN antigen regardless of virus strain or method of inactivation. From the results of these tests it was concluded that this method could replace the *in vivo* methods that were prescribed in European Pharmacopoeia (Ph. Eur.) Monograph 0870 on inactivated Newcastle Disease vaccines. Large quantities of the necessary reagents and reference materials were prepared and tested. The materials were subjected to stability studies to demonstrate their suitability for long-term storage in order to guarantee long-term availability to the international community. A study in three laboratories demonstrated a good correlation of the candidate assay with two existing *in vivo* assays, good transferability of the assay, and excellent reproducibility of the proposed assay. A collaborative study was organized by the European Directorate for the Quality of Medicines & Healthcare (EDQM) to demonstrate the repeatability and the reproducibility of the candidate *in vitro* assay. The suitability of the reference reagent as a Ph. Eur. Biological Reference Preparation was also determined. Fourteen laboratories participated in this study. As a result of these efforts, the assay was included in the relevant Ph. Eur. monograph (01/2007:0870). This paper will address not only the technical aspect of this process but also factors that are considered critical for the success of this project.

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Keywords: veterinary vaccine potency testing; non-animal method; validation; acceptance

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1. Introduction

Vaccines are generally derived from living organisms that are produced in individual batches through biological production processes. These processes are intrinsically variable, and each batch should be considered unique. Therefore, each batch needs to be controlled using validated quality control procedures to ensure safety and potency of these vaccines. The introduction of Good Manufacturing Practice (GMP) regulations and greater attention to consistency of production has not yet replaced the need for final product testing. During the vaccine development phase, *in vivo* animal models involving challenge with live pathogens support the efficacy of the products under study. Very often these *in vivo* models are also the basis for vaccine batch control testing, but there are ample opportunities for the development of *in vitro* alternative assays. Consequently, multiple options exist to reduce, refine, or replace the use of animals in potency and safety testing of vaccines [1].

For some vaccine products, *in vitro* methods will be able to completely replace animal models for the demonstration of potency, especially when protection relies entirely on antibody-mediated response. The combination of *in vivo* (vaccination) and *in vitro* (determination of pathogen-specific antibody response) experimental techniques is usually a first step. In situations where the antibody response is directly related to the amount of antigen in a vaccine dose, and the protective antigen is identified, the *in vivo* step can be eliminated completely if reliable techniques can be developed to quantify the amount of antigen. During the development and evaluation of new test methods, five main stages can be identified: test development, prevalidation, validation (involving a formal interlaboratory study), independent assessment, and progression toward regulatory acceptance [2].

In Europe, the responsibility for evaluation and approval of veterinary vaccine monographs lies with Group 15V (veterinary sera and vaccines) of the European Pharmacopoeia (Ph. Eur.). Before any candidate assay can be included in a monograph, it must be demonstrated that the assay will be suitable for a wide range of similar but not identical products. Many antigen quantification tests have been developed for a range of bacterial and viral antigens, but few have actually been accepted by the authorities as potency assays for release of vaccines.

Ideally, an alternative assay should be applicable for all vaccines on the market, in this case inactivated Newcastle Disease (ND) vaccines. In practice, this means that the assay should work regardless of antigen dose, type of adjuvant, mode of inactivation, and virus strain included in the vaccine. Moreover, if an antigen quantification assay is the method of choice to replace an animal-based assay, a direct relationship between antigen content and protective immune response should be demonstrated. Vaccines produced by different manufacturers should demonstrate similar dose-response effects if the assay is to be widely applicable.

Virulent Newcastle Disease virus (NDV) can cause morbidity and mortality in high percentages of chickens in an infected flock. ND is a vaccine-preventable disease, and both live and inactivated vaccines are used to protect chickens against the disease. Live vaccines are generally used to induce protection in young birds. Inactivated vaccines are used to bolster the immune responses in layer flocks and to generate uniform protective antibody titers before the laying period. Inactivated ND vaccines are among the most widely used vaccines in commercial poultry worldwide. In Europe, potency testing of inactivated ND vaccines consisted of either a serological test or a vaccination-challenge test. It has been shown that, for ND vaccines, there is a correlation between vaccine antigen content and the immune response against the hemagglutinin-neuraminidase (HN) [3,4]. This finding was the basis for the development of an NDV-HN antigen quantification assay followed by a long series of studies to demonstrate its suitability.

Inactivated ND vaccines are generally formulated with mineral oil adjuvant to render them more immunogenic. The NDV antigens cannot be determined in this matrix and the development of a non destructive extraction method was undertaken as part of this project.

The *in vitro* assay quantifies the amount of HN antigen in the vaccine after extraction from the oil emulsion adjuvant. The test platform is an antigen-capture ELISA that measures the HN antigen using specific monoclonal antibodies for the detection of this antigen.

This paper does not contain any new experimental data but is an overview of the studies that were conducted and the steps that were taken to promote regulatory acceptance of the NDV-HN ELISA as an alternative to the *in vivo* potency assay for inactivated ND vaccines. As a result of these studies, it is no longer necessary to use animals for the potency testing of inactivated ND vaccines. The current monograph in the Ph. Eur. includes the option to release vaccines on the basis of *in vitro* potency testing only [5].

The development of the NDV-HN ELISA, the initial validation of the assay, and the production of the reagents were conducted by a research team at the Central Veterinary Institute in Lelystad. The international validation studies were organized by a team from the European Directorate for Quality of Medicines & Healthcare (EDQM).

2. Phase 1: development of the NDV-HN antigen quantification assay

A series of vaccination experiments investigated the dose-response effects of oil-emulsion-adjuvanted inactivated ND vaccines [3]. These studies measured the relationship between the vaccine dose and clinical protection after challenge with virulent NDV. Additionally, the serological response was quantified using different serological assays, including virus neutralization, ELISA, and hemagglutination inhibition assays. These experiments were based on immunizations with vaccines diluted in the same adjuvant as was used in their production.

Comparable dose-response effects were found for three inactivated ND vaccines produced by three different manufacturers. These experiments showed that a number of factors influence the calculated potency of inactivated ND vaccines [3]. These factors included the composition of the water-in-oil emulsion of the different companies' vaccines, the time sera were taken after vaccination, and the type of chicken used in the assay (SPF layers versus broiler birds). Three different assays, hemagglutination inhibition (HI), virus neutralization (VN) and an ELISA were used to determine specific antibody responses. Despite differences the correlation with clinical protection was high.

The results indicated that the serological response was directly correlated to the vaccine antigen content, thereby potentially allowing *in vitro* determination of the potency of ND vaccines and thus eliminating the need for an animal-based *in vivo* potency test.

At the time this study was executed, there was not an assay available to determine the antigen content of formulated inactivated ND vaccines. Instead, hemagglutination titers were used to determine the amount of virus before and after inactivation and in extracted virus from oil-adjuvanted vaccines [6]. Because the *in vitro* quantification of a protective antigen, like the HA or another structural protein like the Fusion protein F, was considered to be a more practical approach than vaccination-serology-based assays, quantitative ELISA platforms were developed that could measure HA and F protein in aqueous solutions.

A later study showed that antigen content of the HA and F protein in vaccines correlated with protective antibody response after vaccination [7]. Both proteins induce neutralizing antibodies in chickens. This study demonstrated that a humoral immune response against NDV can fully protect against clinical ND [8].

The primary aim was to develop an ELISA for the quantification of NDV antigen in vaccine extracts. It was decide to primarily focus on the specificity of the ELISA and on the ability to quantify different NDV proteins. The assay needed to be specific and precise. In order to quantify the NDV proteins, the optical density (OD) in the ELISA must depend directly on the amount of virus that is present (linearity). Also, because it was envisioned that the assay would be transferred to many other laboratories, the reproducibility of the assay had to be high.

A standard catching antibody enzyme-linked immunosorbent assay (ELISA) format was used in which plates are coated with antibodies that can bind antigen. The bound antigen is subsequently detected by a 17α-hydroxyprogesterone (HRPO) conjugated antibody. Monoclonal antibodies (MoAbs) were chosen both as catching antibody and for the conjugate for the following reasons: MoAbs allow maximum standardization of the assay; they guarantee availability of good quality standardized reagents for the future; and animals are no longer necessary to produce reagents. Moreover, when MoAbs are used instead of polyclonal antisera, specificity is almost guaranteed. Well-characterized MoAbs against relevant NDV antigens had already been generated for research purposes and were freely available for test development.

To quantify the antigen in a comparable way over time, the amount of antigen was measured with a relative potency assay. For this purpose, a batch of reference antigen was prepared and lyophilized. Dilution series of vaccine extracts were compared in the ELISA with dilution series of the reference preparation and analyzed with suitable software. The comparison of the resulting ELISA curves adds to the accuracy of the test.

Two different ELISAs were developed simultaneously to detect the HN or the F protein of NDV [9], showing proof of principle. At the same time, a method was developed and validated to extract the NDV antigens from the emulsified vaccines. The development of that extraction method, which was crucial to the success of this project, is described below.

2.1. Antigen recovery from oil-adjuvanted vaccine

Nearly all inactivated poultry vaccines are adjuvanted with mineral oils. Antigen quantification assays generally do not work in this matrix. Therefore it was essential to find a method that could extract antigen in a quantitative way from the water-in-oil emulsions. The extraction method should be mild, with limited or no effect on the antigenic structures of the antigen in order to still allow quantification. Moreover, the relative amount of antigen that was extracted from different vaccines should be roughly the same to allow comparison of the vaccines. Relatively mild extraction procedures were tested that separated water and oil in the emulsions. After the separation the relative amount of the water and the oil phase can be determined. The water-to-oil ratio is not the same for vaccines from different manufacturers, and it needs to be determined to allow calculation of the antigen quantity per vaccine dose. It was not necessary to collect the full aqueous phase for the assay itself. In fact, it was important to be conservative in collecting the water phase because small amounts of residual oil could interact with the antigen quantification assays.

Four different techniques were compared to break the emulsions to obtain the viral antigens that are present in the water phase of the emulsions: freeze thawing, treatment with high concentrations of Tween 80, the "aqueous partition" method [6], and treatment with isopropylmyristate (IPM). Two methods, the aqueous partition method and treatment with IPM, were further investigated in a series of experiments using a large number of vaccines from four different manufacturers. Despite large quantitative differences in antigen content of individual vaccines, a high correlation (R^2 =0.95) was found between the relative antigen content in the vaccines after the different extractions. This showed that, in principle, both methods could be used despite differences in the extraction method. However, with the use of IPM. a pure water phase was obtained, whereas the aqueous partition method extracted only a portion of the inactivated virus.

It was considered an additional advantage that the IPM extraction was already described in the Ph. Eur. as a step in preparation of samples for sterility testing. Results showed that the IPM extraction method is a suitable method for all oil-emulsified inactivated ND vaccines on the European market despite different compositions of the vaccines.

3. Phase 2: in-house validation of the *in vitro* assay, production of standards and reagents

Extensive in-house validation of the developed assays was required before either could be accepted as a general potency assay for ND vaccines. Further validation of two *in vitro* assays would double the amount of work and hence double the cost. It was therefore decided to focus on the most promising assay. The highest correlation between the proposed *in vitro* and the classical potency assay was found between the NDV-HN antigen content and the HI titer after vaccination (R^2=0.87). Thus it appeared logical to continue with the NDV-HN antigen quantification assay.

Titration experiments showed that the sensitivity of the assay was sufficient for the quantification of NDV in all vaccines that were tested. The lowest amount detected was 0.05 hemagglutinating units (HAU). It was demonstrated that all ND vaccines on the EU market contain more antigen than the lowest detectable amount in the NDV-HN ELISA [7].

The assay was shown to be very specific. Ulster, Lasota, and Clone30 NDV strains were tested as both live and inactivated viruses. Comparable responses were observed regardless of inactivation method (formalin versus β-propiolacton). Other poultry viruses such as IBDV, IBV, EDS virus, or reovirus, and three adjuvants (without antigen) supplied by vaccine manufacturers, did not bind in the HN antigen assay, further validating its specificity.

Dilution series of vaccine extracts were analyzed in the ELISA to guarantee optimal accuracy of the test. The ELISA was optimized in such a way that a twofold reduction of the amount of antigen resulted in a twofold reduction of the OD. This linear relationship was demonstrated over a range of five twofold dilutions corresponding with a 32-fold range of antigen content. Linearity was thus proven over a 32-fold range of antigen. The software that is used for the calculation compares 11 different vaccine extract dilutions and allows quantification when linearity is present for at least three dilutions. The 11 twofold dilutions allow accurate estimation of antigen present in vaccines over a 2048-fold range of antigen content. The design guarantees that the antigen content of all ND vaccines can be quantified.

3.1. Production of biological standards and reagents

Large stocks of lyophilized biological reagents for the NDV-HN ELISA were prepared. These included a reference antigen (3HAU Ulster strain formalin-inactivated NDV), a control antigen (6HAU inactivated Lasota strain NDV), coating monoclonal antibody, and conjugate of monoclonal antibody to HRPO. Ulster and Lasota/Clone30 are commonly used in inactivated vaccines. Also, formalin and β-propiolacton are the preferred methods for inactivation of NDV by manufacturers, so these inactivation methods were used to ensure that the reference and control antigens would show similar characteristics when compared with antigens extracted from the vaccine samples. For each of the reagents, 5000 vials were prepared. Each vial contained enough material for at least three 96-well ELISA plates, thereby allowing the testing of at least nine vaccines per set of vials.

The reference antigen became the European Biological Reference Preparation (BRP) after its suitability was established in a large validation study [10].

The stability of the reagents was demonstrated by real-time and accelerated stability studies. There was no systematic decrease in the activity of the reference antigen and the control antigen stored either at -20˚C for 3.5 years or at room temperature after 30 weeks of storage. Continuous monitoring of the reagents is necessary to guarantee optimal performance over time [11].

4. Intermediate independent assessment of the development and validation phase: submission of dossier to Group 15V of the European Pharmacopoeia

A dossier containing the information described above was submitted to Group 15V of the Ph. Eur. for evaluation. The response was positive, and Group 15V recognized the potential for the antigen quantification assay to replace the *in vivo* potency test. Group 15V suggested a two-step approach for final validation and *in vitro* test approval. Although submitted data on 21 vaccine batches showed a high correlation between the proposed *in vitro* assay and the *in vivo* assay, additional prevalidation studies by independent laboratories were requested. Subsequently, a larger study involving both Official Medicines Control Laboratories (OMCLs) and manufacturers' quality control laboratories was conducted to validate the *in vitro* method and determine the suitability of the reference preparations.

4.1. International validation study to evaluate the correlation between in vivo and in vitro assays

Studies performed at CIDC-Lelystad demonstrated a high correlation between the results of the candidate *in vitro* potency assay and the serological potency assay (Ph. Eur. Monograph 0870, Test A). Furthermore, a high correlation between the serological data (hemagglutination inhibition-antibody titers) and clinical protection after challenge was also demonstrated. The aim of the study, which was proposed by Group 15V was to confirm, in different laboratories, the correlation between the results obtained using the candidate *in vitro* potency assay and the results from both of the *in vivo* potency assays currently prescribed in Ph. Eur. Monograph 0870. The proposed study also sought to determine whether a large-scale validation study of the *in vitro* method should be conducted.

In the feasibility study, three OMCLs tested the potency of five inactivated ND vaccines and one experimental vaccine, using both of the *in vivo* methods described in the Ph. Eur. and the candidate *in vitro* method. The six vaccine batches represented a quantitative range of NDV antigen content and were produced by different manufacturers. Statistical evaluation of all results indicated a satisfactory correlation 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 [12]. Based on the results of this study, a large-scale collaborative study was organized to validate the *in vitro* method and to determine the suitability of the reference preparation.

5. Phase 3: international validation study and establishment of a suitable biological reference preparation

In collaboration with EDQM, the next step, a large validation study was initiated to assess the performance of the NDV-HN ELISA and establish the suitability and strength of the proposed BRP. This study, known as BSP055, was part of the European Biological Standardization Programme.

In brief, 14 laboratories (eight OMCLs and six vaccine manufacturers) determined the NDV-HN antigen content of nine different vaccines in three independent tests. The vaccine batches were produced by five different manufacturers and represented a quantitative range of NDV antigen content, and combination vaccines. Previous research had shown that the HN content in inactivated ND vaccine doses may differ by a factor of 100 [13]. The tests included one vaccine batch with insufficient potency and one poultry vaccine not containing NDV. Statistical evaluation of the results indicated that the antigen content could be determined with high precision. Good repeatability and reproducibility were demonstrated. 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. This study concluded 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 [5].

6. Phases 4 and 5: independent assessment and regulatory assessment

By now, a convincing data package had been collected and was again submitted to Group 15V. They considered the data sufficient, and the decision was made to include antigen quantification as an optional method to determine potency of ND vaccines. The monograph text is very general and allows companies to develop and use their own antigen quantification assay, but it specifically mentions the suitability of the reagents that were prepared in the course of this project.

> 2-4-2-1. *Antigen content.* The relative antigen content is determined by comparing the content of haemagglutinin-neuraminidase antigen per dose of vaccine with a haemagglutinin-neuraminidase antigen reference preparation, by enzyme linked immunosorbent assay (2.7.1). For this comparison, *Newcastle disease virus reference antigen BRP, Newcastle disease virus control antigen BRP, Newcastle disease virus coating antibody BRP and Newcastle disease virus conjugated detection antibody BRP are suitable*. Before estimation, the antigen may be extracted from the emulsion using *Isopropylmyristate* or another suitable method. The vaccine passes the test if the estimated content is not significantly lower than that of a batch that has been found to be satisfactory with respect to immunogenicity (section 2-3-1) [5].

The inclusion of the HN antigen quantification assay in Ph. Eur. Monograph 0870 and the transfer of the reagents to EDQM was the final action of the project. Practical work on this project was concluded two years before.

7. Conclusion and discussion

This paper describes the development and validation of an antigen quantification assay that has replaced the use of animals for vaccine potency testing of ND vaccines. Moreover, it describes the process that has led responsible European authorities to include this method as an official method in the Monograph 0870 in the Ph. Eur. This result was achieved in a relatively short time span from the initial development of the assay through approval by the authorities. The course of the project closely followed the five phases that are mentioned in the introduction, from development of the assay to regulatory acceptance. The results demonstrate that a well-managed project, a focused approach, and a solid data package can successfully result in replacing animal-based assays even in a highly critical regulatory environment.

Development of the assay started in 1999. All the studies, including the designation of a European BRP, were concluded and published in 2004. The European authorities granted preliminary approval in 2005, and the assay has been included in the monograph for inactivated ND vaccines since 2007. The NDV-HN ELISA described in this paper was the first antigen quantification assay that was approved for potency testing of veterinary vaccines as an alternative to already existing *in vivo* assays.

A number of factors highlighted below have contributed to the success of this project. Some factors are specifically linked to this assay, but others are more general in nature. Other projects that aim to get alternative assays accepted for wider application may benefit from the experience gained in this project.

After development of the assay and initial validation in our laboratory, it was believed that the antigen quantification assay held great potential for poultry vaccine manufacturers. Not only did the assay perform well, but it was also based on sound research that was published in peer-reviewed journals.

A strategy that would allow wider application was developed and, under strict project management, goals were established. The development of a robust and reliable extraction method and the production of large batches of high-quality reagents were regarded as two of the key elements to success. Because a lengthy validation process was envisioned, including the performance of international collaborative assays, variability was minimized through the use of the same reagents and references for all studies described in this paper. Additionally, stability assays following Veterinary International Conference on Harmonization (VICH) guidelines were carried out to demonstrate suitability of the reagents and reference materials. Sufficient reagents were produced to last beyond the scope of the validation project, and the dossier that was submitted to the regulatory authorities showed that reagents of similar quality could be made available in the future.

Extensive in-house validation, as described above, was executed. At the time, there were no guidelines on the validation of *in vitro* alternatives for animal testing. Guidance for validation of quality control assays was found in the Ph. Eur and in the VICH guidelines that had been approved in 1999 on validation of analytical methods [14,15]. These guidelines focus primarily on chemical analytical assays. However, largely the same principles apply for immunochemical assays, and with minor adaptations they were considered suitable for this immunological assay. The VICH guideline on stability testing, approved in 2001, also proved to be very useful [16]. However, clear guidance on what is needed to develop an acceptable validation data package for regulatory acceptance of alternative assays is still needed.

Special focus was placed on robustness of the assay. Assay methodology was transferred to other laboratories that had not previously performed the assay, helping to optimize the written protocols. In later studies, this would prove to be very helpful because feedback indicated that the written procedures were unclear. In the international validation study, in which 14 laboratories participated, transferability and robustness were a concern. Therefore, efforts were made to minimize the assay variables as much as possible. To achieve this, the assay reagents were provided to the participants in a kit that included all the necessary reagents that might otherwise have introduced variability, such as the ELISA 96-well plates, IPM for the extraction of the antigens, bovine serum albumin, and the substrate for the detection of the bound HRPO-conjugated antibody.

Crucial to success was the excellent and fruitful collaboration with the EDQM in Strasbourg. EDQM plays an essential role in the establishment of new BRPs, development of new monographs, coordination of the OMCL network, and organization of proficiency testing and international collaborative assays. The decision to involve EDQM, as the most relevant regulatory authority, early in the project was part of a deliberate strategy to achieve rapid results. EDQM had just launched the first version of the CombiStats statistical program that can be used for parallel line analysis (http://combistats.edqm.eu/). CombiStats is a computer program for the statistical analysis of data from biological dilution assays or potency assays. It can perform calculations according to Chapter 5.3 of the Ph. Eur. (5th edition 01/2005, 6th edition 01/2008, and 7th edition 01/2011). This and the analytical support of EDQM staff proved to be invaluable to the project. By working closely with EDQM and employing their network of OMCLs, the project became more manageable. The validation studies were carried out as part of the Biological Standardisation Programme (10,12) that runs under the auspices of EDQM. EDQM still holds and distributes the stocks of the references, standards, and reagents for the antigen quantification assay.

Sufficient funding was also essential for the success of this project. Initially the project was funded largely by the Dutch Ministry of Agriculture. The international prevalidation and validation studies were supported by EDQM under their Biological Standardisation Programme. More funding is needed to generate the necessary data that can support regulatory acceptance of alternative assays. Too many tests that have shown promising results will never be used outside the laboratory where they were developed. Lack of funding is certainly one of the reasons, but it should also be considered that validation of assays, production of reagents, and the struggle with the regulatory authorities are not attractive for most researchers. Experience with other potential alternatives to animal testing has shown that the processes can be lengthy and frustrating. Vaccine manufacturers often do use *in vitro* assays for the release of their own products. These assays have been developed for in-house use only, and often their existence is unknown to people interested in alternatives to vaccine testing. There is no benefit for the vaccine manufacturers to develop these assays and make them more widely available because it is costly and it will mostly benefit the competitor who would then save money on assay development.

OMCLs and manufacturers have supported the development and validation of the NDV-HN assay by participating in collaborative studies. At CVI other assays were developed that quantify antigens in infectious bursal disease virus and infectious bronchitis virus. These assays have a good correlation with *in vivo* potency [9,17], but lack of funding has hampered further validation.

The vaccine manufacturers were very interested in the antigen quantification assay, and discussions with industry were always constructive. Recently, research that was performed by a vaccine manufacturer showed that the method of inactivation did have an effect on the reactivity of the virus in the NDV-HN ELISA [18]. BPL inactivated NDV antigens have a higher reactivity in the assay than antigens that are inactivated with formalin. It can be concluded from these results that the assay should not be used to compare vaccines directly because different inactivation methods are used by the manufacturers. They do, however, recognize the ELISA as an attractive alternative to the existing *in vivo* potency tests because it can be used by vaccine manufacturers for the release of their vaccines.

It should be mentioned that the project focused very much on the European vaccines and on approval of the assay by European regulatory authorities. Inactivated ND vaccines are produced and used worldwide, and they generally have the same composition. In principle, this assay has the potential to be used worldwide, but regulatory hurdles need to be overcome before this is a reality. European manufacturers that produce vaccines for global marketing will in some cases still have to perform *in vivo* potency testing to satisfy local regulatory requirements. The worldwide adoption of this and other assays would not only reduce the number of animals used for potency testing of poultry vaccines but would also minimize the need to handle virulent NDV in veterinary biosafety level 3 laboratories.

This paper shows that alternatives to vaccine potency testing in animals can be developed, validated, and accepted by regulatory authorities within a reasonable amount of time. Strict project management, a well-designed strategy, good consultation with manufacturers and regulatory authorities, and targeted validation were crucial for the success of this project. To date, regulatory authorities have accepted only a few antigen quantification tests for human and veterinary vaccines. Examples in the human field are hepatitits A, hepatitis B, and inactivated polio virus (Ph. Eur, World Health Organization [WHO]), and human papillomavirus (WHO). For veterinary vaccines, the only approved assays are canine leptospiral vaccines (Ph. Eur and U.S. Department of Agriculture [USDA]), inactivated swine erysipelas (USDA), and inactivated ND vaccines (Ph. Eur). This clearly shows the reluctance to harmonize already accepted and authorized assays. Focused strategies and funding are needed to make already developed antigen quantification tests available for wider application and acceptance by regulatory authorities.

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