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Overview of currently approved veterinary vaccine potency testing methods and methods in development that do not require animal use

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Abstract

Veterinary vaccines must be safe, pure, potent, and effective. Potency tests help ensure that each consistently manufactured batch of vaccine provides a level of protection as determined in the original efficacy study throughout the products shelf life. Currently approved assays range from host animal vaccination and challenge to the quantification of specific protective antigens using *in vitro* technology. The development, maintenance, and update of *in vitro* potency assays continue to be a priority for both the animal health industry and the corresponding regulatory agencies. New assay development emphasis is being placed on assays that currently involve laboratory animal vaccination/challenge such as vaccines containing the *Leptospira* and *Clostridium spp*. antigens. This paper provides an overview of various *in vitro* potency assays available, the factors that can impact the accuracy of these methods, and specific considerations to be taken into account during assay development.

Keywords: *in vitro*; ELISA; potency; titration

1. Introduction

Veterinary vaccines must be safe, pure, potent, and effective. These four attributes serve to build the framework for current regulations on a global basis. Vaccine effectiveness is demonstrated in the host animal immunogenicity studies used to support product registration. These studies establish the minimum antigenic dose known to elicit a statistically significant, clinically relevant protective effect. To be eligible for use, each batch of vaccine released for distribution must meet or exceed this level of efficacy and maintain it through its shelf life. The potency assay is used to establish this link and must therefore be correlated to efficacy.

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Veterinary vaccines can be divided into two large groups: (1) those that contain live presentations of the immunizing antigen and (2) those that contain an inactivated presentation. Antigens may be the whole virus or bacteria and may be prepared with limited purification. Antigens may also be partially purified preparations, either natural or genetically engineered, or purified peptides. Most inactivated vaccines, and increasingly some live vaccines, also contain one or more adjuvants to boost the immune response. In many cases, particularly with bacterial vaccines, the protective epitope is either unknown or is a combination of antigenic proteins. As an additional complication, most veterinary vaccines are sold as large combination vaccines that include multiple antigens. All of these factors contribute to the difficulty of developing and validating *in vitro* potency assays or refining current assays to reduce the use of animals.

2. Types of potency assays

Historically, potency for most animal health vaccines was demonstrated by means of vaccination and subsequent challenge of the vaccinates with the live pathogenic agent. The challenge tests used laboratory animals (including mice, hamsters, and guinea pigs) and often the host animal. Several current vaccines still require these *in vivo* assays, with the most notable including rabies vaccines, Clostridial and Leptospira-containing vaccines, fish vaccines, and certain poultry products. For many vaccines containing live antigens, these tests have been largely replaced by *in vitro* assays that titrate the live antigen content in the vaccines [1]. In other cases, *in vitro* serologic assays have been developed that measure the vaccinated animal's immune response to antigens that have been demonstrated to correlate to efficacy, thereby avoiding the challenge test. Enzyme-linked immunosorbent assay (ELISA) technology has been developed for inactivated products. These ELISAs are based on the *in vitro* quantification of antigens known to be protective in the host. Many of these assays are designed as relative potency assays, in which the potency of the unknown vaccine is compared to a "reference" vaccine that has previously been demonstrated as effective in the host animal. **Figure 1** illustrates an approximation of the percentage of veterinary vaccine potency assays that fall into the various groupings described [2].

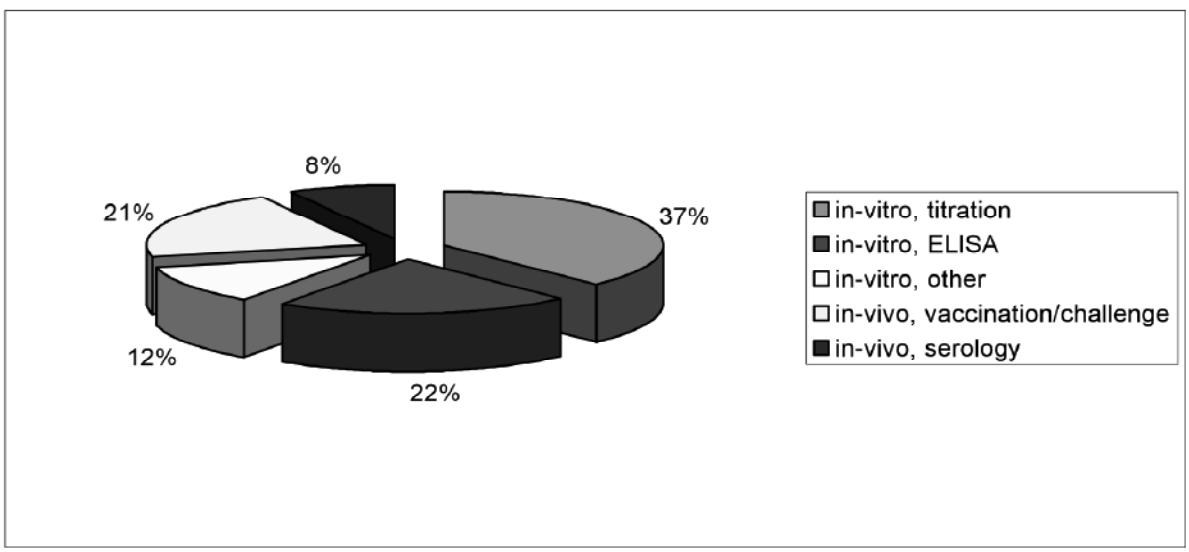

Figure 1. Estimated usage of potency assays for USDA licensed non-poultry non-aquaculture vaccines [1]

For the determination of live viral antigen concentration, a microtiter plate cell culture system is often employed, utilizing tissue culture (50% tissue culture infective dose [$TCID_{50}$]), fluorescent antibody (50% fluorescent antibody infectious dose [$FAID_{50}$]), or plaque-forming units (PFU) as the endpoint. The assay often uses the methods of Reed and Muench or Spearman Karber for quantification [3, 4]. The assay is correlated to the protective dose demonstrated in the host animal immunogenicity study and frequently includes an overage to allow for testing error

and loss during storage (in the U.S., $10^{0.7}$ and approximately $10^{0.5}$ $TCID_{50}$/dose, respectively). The potency of live bacterial vaccines is usually expressed as colony-forming units or CFU. Particle counts can also be used for live bacterial vaccines, but the assay must be designed to differentiate between living and dead microorganisms. Live-vectored recombinant antigens can be quantified using a live antigen titration method similar to those described above; however, in these cases an assay for expression of the inserted gene must be included as part of the potency assay. A significant advantage of live antigen titration methods to determine potency is that the same assay can be used for the determination of antigen content at the time of vaccine assembly and for the final product assay, a somewhat difficult task with inactivated vaccines.

The vaccination of either the target animal or a suitable laboratory animal has historically been viewed as a primary option for potency assays. This is particularly true for inactivated vaccines, in which titration of the live antigen is no longer an option. Animal welfare considerations, cost, assay duration, and assay variability have all been drivers for the animal health industry to find alternative assays. There is still, however, a number of products for which vaccination, followed by challenge, remains the primary assay (rabies vaccines, Leptospira-containing products, many Clostridial vaccines, most fish vaccines, and certain poultry products). A benefit of *in vivo* assays is that the role of the adjuvant in the immune response is directly measured by the assay.

As an alternative to challenge, serology can sometimes be used as an indicator of potency. For serology to be an effective tool, the antibody response to the test vaccine antigen must be the primary protective response in the vaccinated host. As with a potency assay that relies on vaccination challenge, serologic assays also take into account the adjuvant's role in the final formulation. In some cases, a second animal species, often mice, is used as a measure of post-vaccination serologic response. Many of the Clostridial products involve the vaccination of rabbits, followed by the use of mice to titrate the antitoxin response generated by the Clostridial toxoid using a lethal toxin challenge.

The animal health industry has made significant progress over the last 20 years in developing *in vitro* potency assays for inactivated antigens. These *in vitro* assays involve the direct measure of antigen in a vaccine using methods such as hemaglutination, and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). For certain peptide vaccines, high-pressure liquid chromatography (HPLC) or the use of immunologic methods such as immuno diffusion (RID, rocket) or ELISAs may be used. To provide a correlation to efficacy in the host, many of the assays involve the direct comparison of the test vaccine to a reference vaccine that was initially tested for efficacy in the host animal. These assays are referred to as relative potency (RP) assays; they currently form the majority of *in vitro* assays employed by the industry. Because these assays do not measure the role of adjuvant in the immune response, some regulatory agencies require a second assay to measure the adjuvant, e.g., total aluminum content in alum-adjuvanted vaccines.

3. Considerations for developing in vitro potency assays

Of primary concern in developing an *in vitro* assay is the identification of appropriate target antigens(s) for the assay. The ideal assays measure a protective immunogen or immunogens through the use of the following:

- Purified proteins that elicit protection in vaccinated animals
- Antibodies to proteins that neutralize the infectivity of the agent in an *in vitro* assay
- Monoclonal antibodies to the protein that passively protect animals against challenge

Additional considerations in developing appropriate *in vitro* assays include the correlation of the assay to host animal efficacy; assay interference by the adjuvant(s), other antigens, and/or vaccine components; accuracy, precision, and the sensitivity of the assay; and the demonstration of reference stability over time.

The majority of animal health inactivated vaccines are adjuvanted, raising two additional concerns: assay interference and the immunological impact of the adjuvant in the product specifications. Assay interference attributable to adjuvants can include the direct interference of the adjuvant on the assay (e.g., high background, nonparallelism) and the impact caused by the direct binding of the antigen to the adjuvant. As industry moves toward the increased use of *in vitro* assays, regulators in certain regions have been concerned with assessing the impact of the adjuvant on serial potency. This can be accomplished through a variety of physical and chemical assays, including particle sizing, aluminum concentration, and HPLC, and through the use of tightly controlled (Good Manufacturing Practice [GMP]) production systems and component specifications. However, this will likely require development and validation of additional assays by industry.

In vitro relative potency assays have additional requirements that need to be factored into assay development. In these assays, the reference vaccine serves as "the stake in the ground" linking the assay to host animal efficacy. In order for this to be valid, the relationship between the dose-response curves of the reference with that of representative vaccine serials must be evaluated; and parallelism must be demonstrated through the linear region that encompasses the critical assay range. Because the reference is the key indicator of efficacy, stability of the reference needs to be understood and demonstrated, and plans must be established to qualify a new reference preparation as needed.

The recently developed Leptospira vaccine assays serve an as example of the progress made by the U.S. Department of Agriculture (USDA) and industry in moving toward ELISA test methods, but they also illustrate some of the potential concerns. The Leptospira vaccines are commonly used across multiple species, including canine, bovine, and porcine, and are complex combination vaccines that include multiple Leptospira serovars and often other antigens. The current *in vivo* Leptospira potency assay involves a hamster vaccination / challenge assay. This is a lethal endpoint challenge assay and includes testing for each of the multiple serovars of Leptospira contained in the product. The test is time consuming, involving a period of 5+ weeks. Because the Leptospira are zoonotic, the assay also involves a significant exposure risk to laboratory and animal care personnel. To address these issues, USDA personnel have developed a sandwich ELISA potency test that uses a polyclonal rabbit capture antibody and specific mouse monoclonal detecting antibodies. Supplemental Assay Methods (SAM Nos. 624-627 [5]) have been published, and U.S. veterinary biological manufacturers are in the process of adopting these assays. Hurdles that remain to be overcome include addressing potential assay interference due to the various adjuvant formulations used by the industry and interference caused by other antigens and components of the various vaccines.

4. Other new potential in vitro assay technologies

The use of the quantitative polymerase chain reaction (qPCR) assay has been proposed as an alternative potency assay due to its ability to determine the number of organisms based on the nucleic assay copy number. However since the qPCR assay does not measure either the expression or conformation of the target antigen, its use is limited to a potential supportive assay in combination with another assay. HPLC has shown promise as an additional potential assay method for use in the quantification of the new purified peptide or subunit vaccines. The use of cell culture toxicity as a replacement for the mouse in clostridial antitoxin determination has also been proposed [6].

5. Conclusion

Significant progress has been made in the development, maintenance, and update of *in vitro* potency assays, and this continues to be a priority for the animal health industry and the regulatory agencies. New assay development emphasizes assays that currently involve laboratory animal vaccination/challenge, such as vaccines containing the rabies, *Leptospira*, and C*lostridium spp*. antigens.

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