Rabies Vaccines for Human Use: Potency Testing Without Mouse Challenge?

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Summary

The conventional batch potency test for cell culture-based inactivated rabies vaccines involves vaccination and viral challenge of mice. The test is highly variable, very time-consuming, needs huge numbers of mice, and means major suffering to the animals. It was established in the 1950s in the absence of GMP at the National Institutes of Health (NIH) and has been broadly used since then, despite its multiple drawbacks. In line with the modern concept of the consistency approach for vaccine quality, future batch potency control will combine data from a panel of tests throughout manufacturing to provide improved knowledge of product characteristics. Immunogenicity testing is a key element, providing valuable information that complements protein quantification.

Consequently, we propose the development of an immunogenicity assay based on vaccination of mice and determination of neutralizing antibodies. The assessment of the serum response is done by quantification with the WHO standard anti-rabies immunoglobulin based on the Ph. Eur. monograph on potency testing of human rabies immunoglobulin. Points for consideration of a potential impact on the antibody response are the vaccination scheme and the age of the mice. The design for the development of a serological alternative assay should be harmonized worldwide to the greatest possible extent. Global acceptance is a prerequisite for any alternative assay. Therefore, the alternative serological assay for human rabies vaccine potency was presented and discussed in the global context of the World Congress on Alternatives and Animal Use in the Life Sciences.

Keywords: rabies, potency, challenge, alternative, serology

Rabies vaccines for human use currently are tested for potency by a mouse challenge assay. This test was developed by the National Institutes of Health (NIH) and is therefore referred to as the NIH test. Its origins go back to the 1950's, well before good manufacturing practice (GMP) was established. The NIH test soon was recognized to be highly variable, to consume a large number of animals, and to be very distressing to the mice and to the staff performing the test. In the absence of any alternative, it became the mandatory potency test for rabies vaccines worldwide.

GMP now is implemented for production of human rabies vaccines, and further tools and tests have been developed that finally will allow the move away from the NIH test.

From a regulator's point of view, potency testing is important to provide reliable information on vaccine batch performance. Therefore, it is in the interest of Public Health to define and apply the most appropriate concept of potency testing. Historically, potency was considered to be assured by a single test on the final-product level. We now understand that a new concept of controlled production and multi-stage testing without the variable NIH test provides more reliability. Better data quality will enable more reliable performance trending. Alternative tests will cause fewer ethical concerns. Alternative tests also are faster and consequently cheaper, especially when the reduced frequency of retesting is considered.

Manufacturers have the same objectives for a more reliable testing concept: They want to prevent the loss of production by identifying non-conforming material earlier than the final stage. Therefore, they favor the implementation of tools and tests that improve the monitoring to reduce the risk of generating non-conforming batches.

In which direction will batch potency testing go? The testing will evolve to demonstrate consistent batch to batch performance. During licensing, the efficacy of the vaccine is assessed on representative batches. Only efficacious products will get access to the market. All batches produced in the same manner as the original representative batches shall have similar performance. Since biological products show intrinsic variability, the production process needs close monitoring to enable consistent production. Testing of the final product is complemented by relevant in-process controls. This concept is expressed by the consistency approach: Not one test but a panel of tests on critical quality aspects will provide more relevant and reliable information on batch performance and consistency. This will be in line with the refinement, reduction, and replacement of animal tests.

The major variability of animal tests undermines its value in testing functional immune response. For human rabies vaccines, efficacy in the target species, mankind, is of utmost importance. Potency assessment in the murine immune system aims to provide information on an example of a functional immune system. Considering the lethality of the disease, this has been accepted as justified over the decades. However, the need to test in a challenge assay will vanish when a serological assay becomes available. Since protection from rabies is conferred by serum antibodies, as proven by successful post-exposure prophylaxis, the measurement of protective anti-rabies antibodies in serum of vaccinated animals will provide the same certainty as a challenge test. *In vitro* testing of the immunogenic epitopes of the rabies glycoprotein further contributes relevant information without consuming any animals.

Therefore, we propose to improve batch potency control of human rabies vaccines worldwide by the combination of GMP, critical in-process controls, *in vitro* measurement of the neutralizing antibodies generated in response to vaccination, and analysis of the immunogenic epitopes of rabies virus glycoprotein.

Consequently, the continued use of mice may be a necessary intermediate to achieve the global abolishment of the NIH challenge test for rabies vaccines. To appreciate the concrete options for a change in the testing of human rabies vaccines, it is important to understand the developments in the veterinary rabies vaccines field.

For veterinary inactivated rabies vaccines, a serological assay already exists (Krämer et al., 2009). It measures the neutralizing antibodies generated in response to vaccination, and it was confirmed as possible, reproducible, and reliable in the international collaborative study BSP105 including Canada, the EU, and the US (Krämer et al., 2010). In Germany, this serological assay has been used for official batch release of veterinary inactivated rabies vaccines for years. Its results are accepted EU-wide as part of mutual recognition of official medicines control laboratories' (OMCLs) results. Apart from Germany, several other EU OMCLs are preparing the implementation of the serological assay. Various Latin American countries are ready to study the assay (personal communication). Moreover, European and non-European companies are starting their product-specific validation. The veterinary serological assay was developed according to the provisions of the Pharmacopoeia Europea (Ph.Eur.). It is intended for routine batch release and consists of a single immunization with a single dilution of each vaccine and reference (in the EU, Biological Reference Preparation 4: BRP4). Virus-neutralizing antibodies are then quantified *in vitro*.

The assay uses far fewer mice, causes less suffering, takes less time, and is less labor-intense, which in turn means lower costs. Since only a single dilution is used, a total of 20 mice suffice to test one batch. This is a major reduction in animal number compared to the veterinary NIH test, which is run with at least 120 mice, depending greatly on the laboratory. At the Paul-Ehrlich-Institut (PEI), the single dilution serological assay comprises groups of 10 mice each that are injected intraperitoneally (i.p.) with 1/5 of the recommended dose volume, equivalent to 0.2 ml. The reference BRP4 is adjusted with PBS to the minimum requirement of 1 IU/dose. The vaccine batch is only adjusted if the manufacturer's minimum requirement is higher than 1 IU/dose.

The report of the international collaborative study BSP105 recommends that each laboratory find its own optimal mouse group size in a range between 8 and 12 mice. Furthermore, if the manufacturer's minimum requirement is higher than 1 IU/dose, the reference may be adjusted to this specific requirement so that no pre-dilution of the vaccine itself is needed.

After 14 days, blood samples are taken and the sera are tested for rabies virus-neutralizing antibodies by a serum neutrali-

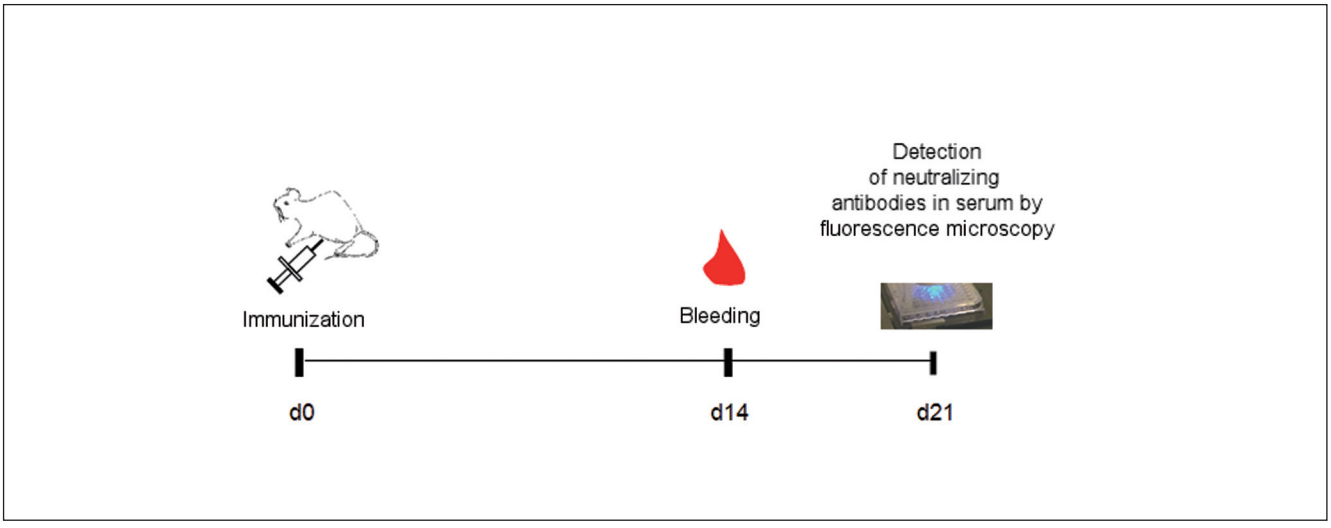

Fig. 1: Schematic procedure of the veterinary serological assay

zation test (SNT). The SNT is run on BHK21 cells and uses virulent rabies virus (CVS). This is an important feature of the assay in terms of regulatory safety to check for neutralization of virulent rabies virus. Non-neutralized virus multiplies on the cell layer and is detected by fluorescently labeled specific antibody. This procedure ensures that only the relevant neutralizing antibodies are measured by the assay. The SNT is a feasible modification of the Ph.Eur. method "rapid fluorescent focus inhibition test" (RFFIT) for human rabies immunoglobulin for use on 96-well plates. Serum samples are analyzed in replicates of four and diluted in a log2 series. A log5 step is included in the last row to enable the read out of highly concentrated sera. In parallel to the test sera, a quantification standard, i.e., WHO Standard human Rabies Anti-Immunoglobulin (RAI), is titrated in a given starting concentration of 2 IU/ml.

After addition of virus, the plates are incubated for 48 hours, stained for rabies virus with fluorescent antibody, and read by microscope. The ED_{50} of each sample is calculated statistically. In the next step, the ED_{50} is compared to the ED_{50} of RAI. Since RAI is used in a given concentration, the antibody concentration – the so-called "serum activity" of all test sera – is quantified in IU/ml. At PEI, all calculations are performed by CombiStats, the official statistical computation program for OMCLs.

The serum activities of the batch test group are then compared to the serum activities of the BRP group by a one-sided statistical test. The test tells whether the batch induces significantly higher neutralizing antibody levels than the reference. The acceptance criterion is $p \leq 0.05$. Any result of the p value below or equal to 0.05 means that the batch is significantly better than the potency limit tested for. Concretely stated, the serological assay implies an additional safety margin: To pass the test, the batch needs to prove significantly better than the reference. In contrast, the highly variable quantitative result in the NIH test may be formally acceptable even if it is just above the minimum requirement. Due to its generally poor precision, the NIH test reliability is lower, despite the use of more animals.

For validation, the veterinary serological assay was tested in direct correlation to the NIH test (Krämer et al., 2009). Due to the huge NIH test variability, it was only possible to link high, medium, and low concentrations of a vaccine dilution series instead of concrete values. Nevertheless, accuracy of high, medium, and low concentrations was demonstrated. Moreover, titration profiles of different virus strains covering Virus fixe Pasteur, Flury LEP, Pasteur RIV, Pasteur VP12, and SAD Vnukovo in PBS dilution confirmed the dose response relationship of the SNT.

Most importantly, the veterinary serological assay was tested for its ability to discriminate between batches that conformed to the requirement limit and those that did not. For this, various conforming and non-conforming batches were studied. Since it is difficult in the EU to get non-conforming material, it must be highlighted that the five non-conforming batches were natural and not artificially prepared. These batches failed the NIH test and also failed the serological assay. Equivalence of pass and fail decisions was demonstrated by PEI and in the BSP105 collaborative study involving 13 laboratories. This can be considered a truly good correlation when we keep in mind the huge variability of the NIH test, which even exceeds the variability of other animal challenge tests, such as the diphtheria potency assay.

The veterinary serological assay, intended for routine use only, applies a single dilution and therefore does not provide a quantitative potency estimate of the vaccine batch but rather a qualitative final result. However, since the serum activities of all mice are quantified against the WHO International Standard RAI, the response pattern of the whole group of mice can be monitored. We have observed that passing batches show a different pattern from failing batches. Product-specific patterns

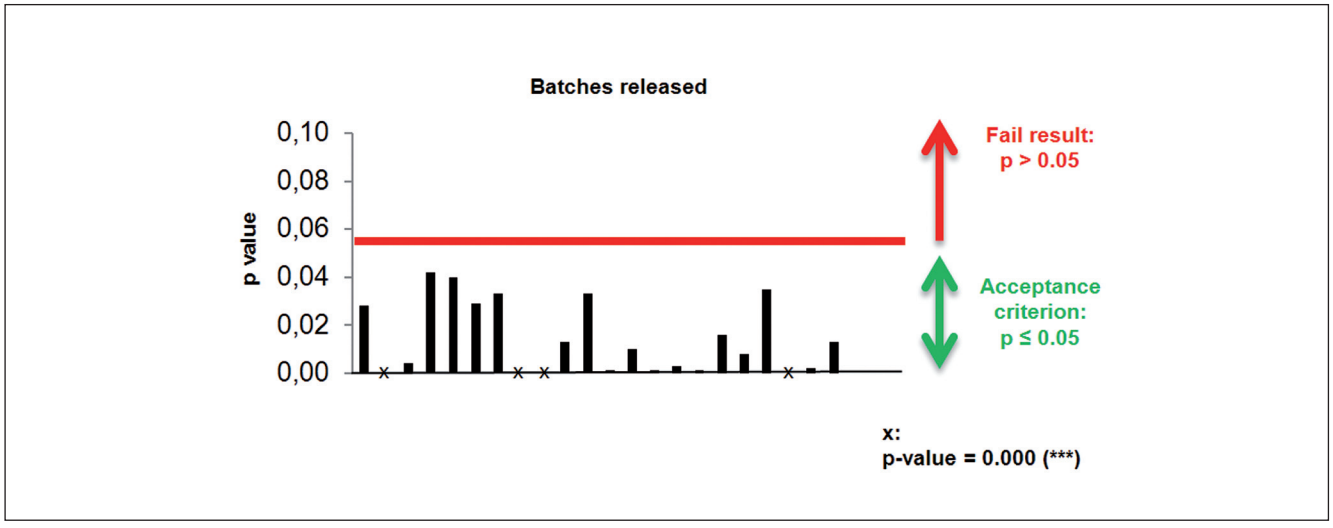

Fig. 2: Batch release results obtained by the veterinary serological assay
A batch passes the serological assay if it induces significantly higher serum activity in mice than the reference does. The level of statistical significance is expressed by the p value. Passing batches show $p \leq 0.05$.

may also be recognized (unpublished results). Therefore, the single dilution assay already provides important semi-quantitative trending information. If validation shows that the dilution tested *in vivo* lies in the linear response range, the single dilution assay will contribute to consistency. In the future, performance limits may be set.

In contrast, the NIH test provides one unreliable potency estimate that is obtained by consuming many more mice. Even depicting the NIH test raw data per dilution cannot contribute as much as the serological assay, since the NIH test only reads out qualitatively (protected/non-protected).

Still, for special purposes such as generation of stability data, impact assessment of manufacturing changes, and calibration of reference material, quantitative results are needed. The single dilution serological assay already provides semi-quantitative information. The variable NIH test could honestly be classified as semi-quantitative as well. A multi-dilution serological assay format could provide sufficiently reliable quantitative results. The availability of such an assay is a prerequisite for the complete abolishment of the NIH test. Therefore, PEI has developed a multi-dilution assay format. First results are promising (unpublished results).

The development of the veterinary serological assay at PEI as an alternative to the NIH challenge test was guided by the Ph.Eur. monograph 0451 – the legally relevant document – for inactivated veterinary rabies vaccines. It already described fundamental elements of a serological assay with respect to vaccination scheme and read out test. Therefore, the concrete assay development adapted and optimized this description.

For human rabies vaccines, the situation is different: only the NIH challenge test is described in worldwide regulations. Consequently, there is the potential to modify and improve the serological batch potency test design.

In the meantime, the development of an alternative serological assay for human rabies vaccines has started at PEI. Since a change in the potency testing of human rabies vaccines would have an immediate global impact, communication with regulators and industry on a worldwide level is highly important and has been initiated. The WHO has already communicated great interest in this project.

It is important that the activities work in parallel:

- communication with regulators on a global scale regarding how to move forward and to cover the requirements;
- laboratory development to prepare now for global acceptance;
- input, feedback, and material provision from industry.

Clearly, combined effort is needed to advance and to move away from the NIH test. The worldwide acceptability of alternative potency testing is a prerequisite for any real change.

The obvious success of the veterinary serological assay in the international collaborative study and in routine official release testing in Germany paves the way for a change to human rabies vaccine testing. The aim is to abolish the human NIH test worldwide and to replace it with a multi-test consistency approach. This will lead to the availability of more relevant and reliable data. OMCLs, industry, and universities in the EU are in line with this. We have indications that Asian countries and other major players also would welcome a quicker and less variable potency assay. Global networking and exchange of ideas will contribute to this aim. We truly appreciate the commitment of other parties.

In the EU, we recently brought about a regulatory achievement: The Ph.Eur. monograph for inactivated rabies vaccines for human use 0216 will be modified. Until now, it described the NIH test as the only potency assay. In the future, it also will include the option for a validated serological assay. This was

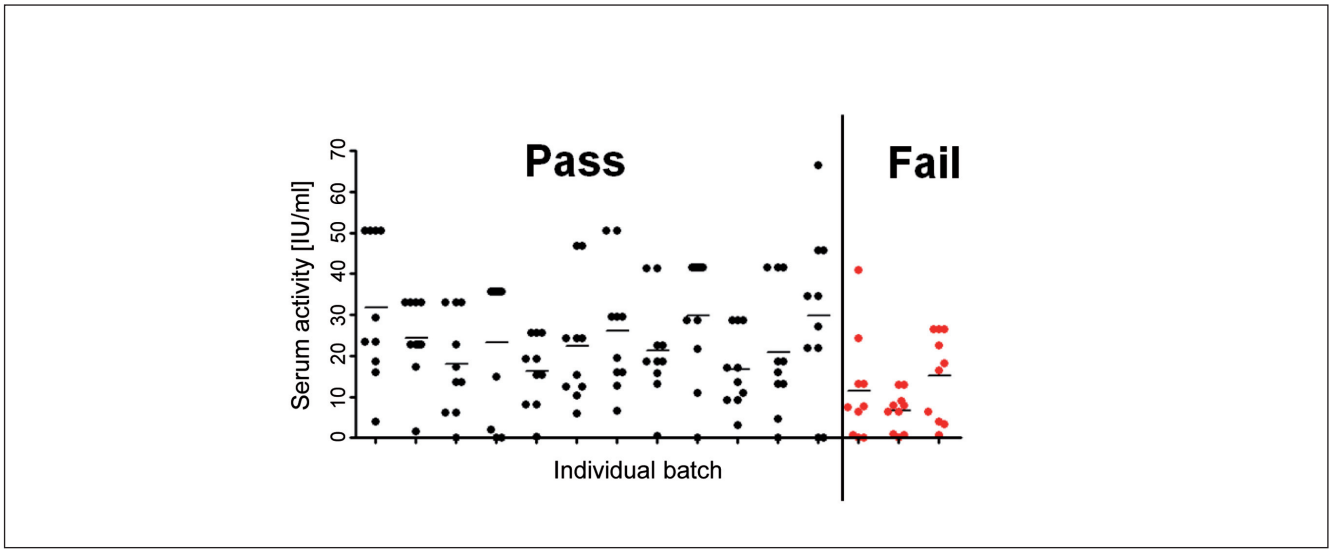

Fig. 3: Semi-quantitative trending information obtained by the veterinary serological assay

The monitoring of individual serum activities of all mice of the batch test group points at different patterns of acceptable and non-acceptable batch performance. This valuable information can contribute to the batch release decision certainty.

motivated by lessons learned from the veterinary assay: it is important to involve regulators early. The concrete allowance of a serological assay in the monograph essentially facilitated several important issues:

– the assay development;
– the EU-wide acceptance of serological results;
– the commitment of other OMCLs to switch to the serological assay.

In line with the aim to promote the consistency approach, the importance of glycoprotein measurement also was strengthened by requiring the test on the in-process and final-lot level.

A joint effort led to the success: the revision of Ph.Eur. monograph 0216 is in preparation! This is a strong signal to other regions of the world as well.

Continued communication between experts on a global scale is necessary. We are finally on the way to conceiving potency testing of human rabies vaccines without the mouse challenge.

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