Archives of Pharmaceutical Sciences Ain Shams University 2021; Vol. 5(2): 265-274

OPEN ACCESS

Microbiology and Immunology

Mini review

Mini review on Potency Evaluation of Rabies Vaccine preparations

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Abstract

Rabies is still considered one of the most harmful viral infections of warm-blooded animals. Thousands of people are infected with it each year worldwide. It is a fatal disease unless early treatment is received before the appearance of symptoms. The virus usually enters the human body through a bite wound from rabid animal's saliva; however, it can also be transmitted by other ways such as inhalation of the aerosolized rabies virus with laboratory workers. About, twenty-four thousand die due to rabies in Africa annually and dogs are the main reason for infection transmission. Fortunately, it is easily eliminated by vaccines. The importance of vaccination comes from the fact that it is the only way to limit disease mortality levels; meanwhile, the same vaccine is used for treatment after infection exposure to rabies or as prophylaxis. Therefore, high-quality control must be applied to it to ensure its safety, efficacy, and potency. A potency test is an important tool for experiencing the actual relative potency of manufactured vaccine batches. Because of the high variability of biological products, potency is an effective tool that assures the lot-to-lot consistency of commercial vaccines. In this review, we aimed to discuss the rabies virus and its antigenic structure, different vaccine preparations, quality control of vaccines, different methods used in potency tests for rabies vaccine preparations including *in vivo* and *in vitro* methods. In conclusion, without good quality control, we couldn't ensure consistency in vaccine manufacturing, and without replacement of old methods depending on animals, we couldn't go with global approaches of refinement, reduction, and replacement of animals in quality control tests especially the potency test.

Keywords: *Rabies; Potency; rabies vaccine preparations; quality control; in vivo; in vitro*.

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Citation | Khalil WM, Aboshanab KM, Aboulwafa M M, 2021. Mini review on Potency Evaluation of Rabies Vaccine preparations. Arch Pharm Sci ASU 5(2): 265-274

DOI: 10.21608/aps.2021.101125.1071

Print ISSN: 2356-8380. **Online ISSN**: 2356-8399.

Received 25 November 2021. **Accepted** 31 October 2021.

Published by: Ain Shams University, Faculty of Pharmacy

1. Introduction

Rabies is considered one of the most harmful viral infections of warm-blooded animals and still, thousands of people are infected with it each year worldwide **[1]**. Rabies Disease is a highly dangerous disease that can cause death in case of symptoms to appear before receiving the appropriate treatment. Dog's saliva contains the virus is the main cause of infection **[2]**. Other modes of transmission are documented in literature as inhalation of aerosolized rabies in a laboratory environment **[3]**. About twenty-four thousand die due to rabies in Africa annually and dogs are the main reason for infection transmission **[1]**. Fortunately, it is a vaccine-

preventable disease **[4]** and the most important way to prevent is vaccination of both pets and people **[16]**. The rabies vaccine is a type of inactivated virus vaccine that is prepared either in human diploid cell culture [HDCV] or in purified chick embryo cell culture [PCEC] **[5]**. Rabies vaccination is administrated mostly in two cases: i. pre-exposure prophylaxis in populations with high-risk exposure to rabies due to geographical location and; ii. Occupations and after exposure to animals known or suspected to be rabid. The pre-exposure immunizing course is four doses of rabies vaccine given intramuscularly on the day of infection, the seventh day of the infection, the twenty-one, and the twenty-eight days. The four-dose regimen leads to 100% protection due to antibody levels **[5]**. So, the importance of vaccines comes from the fact that the same vaccine is used for post-exposure prophylaxis or pre-exposure to rabies, therefore, high-quality control must be applied to it to ensure its safety, efficacy, and potency. A potency test is an important tool for testing the actual relative strength of the vaccine. Due to biological products' high variability, potency is an effective tool that ensures different lot consistency **[6]**.

The available potency assays for rabies vaccine preparations can be alienated into four different classifications including; i. challenge test; ii. Serum neutralizing test, iii. Enzyme activity assays and iv. Cell-based assays **[7]**. Quality control of rabies vaccine preparations should be conducted at two levels, by the manufacturer, and by a national control authority. The methods for quality control of rabies vaccines were revised by the World Health Organization [WHO] Expert Committee [Technical Report Series 709] **[8]**. In Egypt, the Egyptian Drug Authority is the national control authority that is responsible for testing rabies vaccine efficacy, safety, and potency. Safety and potency are the most important tests that their results must be confirmed so that the product is approved for release to the public. However, the potency test is the most important test at all, as it determines the production capacity to protect against rabies disease **[8]**.

As in whole authorities, the Mouse Challenge NIH test is the main reference text used for potency evaluation **[9]**. This test is dependent on immunization of a large number of mice, followed by challenging them with the rabies virus and one month later, both the live and dead animals are counted followed by calculating the 50% lethal dose of the vaccine and its potency **[7]**. In this test, the determination of an immune response in the animal model would allow the rational development of an effective vaccine. However, proving this correlation is difficult. These animals only represent the interactions of the organism with the immune system **[7]**. In addition, the main limitations of this test are consuming a large number of animals, non-humane endpoint for the test, time-consuming, and high variability **[10, 21]**. In addition, safety-wise it is easier risk management in cell culture lab than in Animal Facility. Even though all tests are done in the animal facility which is fully safety equipped and animals are allowed to live within the test period in individually ventilated cages [IVC cages] but the virulent material injection and technicians who are exposed to contaminated animals increase the risk probabilities **[7]**. Because of all these disadvantages, and because of many approaches for replacement, and/ or reduction, or refinement of laboratory animals in tests, there are many alternative techniques such as serological assays for potency evaluation.

Serological assays measure antibody levels produced by animals which represent vaccine potency **[7]**. So, instead of depending on the number of live animals in the challenge test, we depend on antibody titer. However, this test only reflects the immune response in the first phase

[7]. RFFIT is a serological test for potency determination by measuring the levels of rabies virus neutralizing antibody titers induced after vaccination of mice against Rabies. It is a WHO standard method assay used to evaluate the immunity effect of mice after vaccination **[10]**. PHA is another serological test that was developed to overcome the disadvantages of RFFIT, is cheaper **[1],** and is not affected by the cell type in which the virus was grown **[11]**. In 1959, Russell and Burch had found the 3Rs approach as the guiding principle for using laboratory animals ethically [mainly mice]. This approach encourages the reduction of animal numbers, its replacement, and refinement for pain alleviation.

The NIH test consumes a large number of animals, takes a long time, and has wide variability. Besides, few studies were found in the literature for the correlation of this test with other serological tests.

This review gives an overview of the history of rabies vaccine potency evaluation and global approaches in the enhancement of quality control of vaccines.

2. Rabies Virus Antigenic structure

The length of Rhabdo-viruses is roughly a hundred and eighty nm, and their width is five nm. The genome encodes five proteins: nucleoprotein [N], phosphoprotein [P], matrix protein [M], glycoprotein [G], and polymerase [L]. All the species have 2 main structural components; a spiral ribonucleoprotein core [RNP] and an encompassing envelope. Within the RNP, the genomic RNA is firmly sheathed by the nucleoprotein. Two different microorganism proteins, the phosphoprotein, and the massive protein [L-protein or polymerase] are related to the RNP. The glycoprotein shapes around four hundred trimeric spikes that are firmly organized on the virus surface. The M protein is linked each with the envelope and therefore the RNP and perhaps the central protein of the animal virus. The fundamental composition and structure of the hydrophobic virus are delineated within the longitudinal antigenic part of the rabies virus which is known as the glycoprotein that has the main function in antibodies induction **[11]**. These antibodies are crucial in the neutralizing virus. So, these antibodies are called virus-neutralizing antibodies **[12]**. Besides, it is responsible for T cells stimulation that by default expresses cytotoxic activities. Because of these extraordinary roles, it is currently used in vaccination **[13, 14]**. There is a new vaccine, known as the DNA vaccine. Rabies virus glycoprotein has many antigenic sites. Till now, there is no serious study keen on evaluating these sites **[15]**, although these sites have great importance in antibodies induction and therefore may help in vaccine development. There is a new study in February 2021, which can successfully isolate 264 monoclonal antibodies **[15]**. A percentage of 97% of monoclonal antibodies recognize antigenic sites **[15]**. Rabies virus antigenic sites play an important role in the disease pathogenies **[16, 17, 18]**. In addition, it is discovered that to sustain viral pathogenicity, the expression of viral proteins and conservancy of many viral elements must be maintained [18], especially G protein must be controlled. In Lab, on dealing with glycoprotein “G Protein” as antigen, complement fixation assay could be easily performed as it is used as virus neutralization of antibodies assays **[19]**. We could increase its protective activity by purification. Displaying high potency, it is suggested to be the ideal vaccine against rabies disease **[19]**.

3. Rabies Vaccine Preparations

Louis Pasteur was the first who administrate a cure for rabies disease over 100 years ago. After that, rabies vaccines, and many other

vaccines as well have been developed a lot **[20]**. In 1955, the manufacturing of vaccines has been modified from animal nerve to embryonated eggs. Then in 1960, the embryonated eggs are replaced with human diploid cells cultures **[21]**. In 1974, in France, comparative methods of test vaccine against reference vaccine are first established **[22]**. In the 1980s, there is a great development in cellular substrates used for vaccine preparations such as primary explant cells of baby kidney hamster, chicken embryo, or diploid cells from rhesus, and finally cells from continuous lines [Vero cells] **[23]**. At the end of 1989, the health authorities stopped the administration of these vaccines and replaced them with human diploid cells vaccines **[9]**. Inactivated rabies vaccines could be Purified Vero cellular vaccine for human use; rabies vaccine produced human diploid cells **[8]**. Purified Vero cellular vaccine gives high protection levels and excessive immunogenic effect. But it, also, gives a low virus titer which results in problems in large-scale manufacturing of rabies vaccine with economic price having the same quality. The Microcarrier technique developed by van Wezel was performed after the manufacture of inactivated poliomyelitis vaccine in Vero cells, researchers applied it to expand a human rabies vaccine. The produced vaccine needed further purification so that the residual cellular DNA could be eliminated. It is called the purified Vero cellular rabies vaccine [PVRV]. Purified chick embryo cell vaccine for humans is produced in primary chick embryo cells derived from specific pathogen-free [SPF] eggs. It is a freeze-dried preparation including purified and concentrated rabies virus antigen inactivated with B-propiolactone. So to sum up, there are three types of rabies vaccines: a. nerve tissue-based vaccines which is developed by Louis Pasteur based on the attenuated virus in nerve tissues **[24]**, b. The new generation of rabies vaccines is made from inactivated virus followed by propagation in embryonated egg cells, c. cell culture-based rabies vaccines **[25]**.

4. Quality control of rabies vaccine preparations

Quality control of rabies vaccine is one of the most crucial practices that is done for ensuring lot to lot consistency. It is performed on a finished product **[7]**. There are some techniques in research and control of biological products used nowadays without any development since they are firstly performed **[9]**. Potency evaluation is one of these techniques. There are two main methods of potency testing; *in vivo* and *in vitro* methods **[26]**. Vaccines differ from pharmaceutical products in their size; vaccines are large complex molecules **[27]**. This means that it is variable from batch to batch, each batch is considered a matchless batch. Other reasons for the variability are virus strains variability, contamination of starting materials, manufacturing facility, and workers. On controlling these factors, quality control represented in quality, safety, and efficacy of each batch is achieved **[7, 8]**. Quality control tests are conducted at specific different steps during manufacturing to ensure good manufacturing practices **[8]**. All these concepts depend on the exclusivity of each batch of vaccines. Vaccine production uniformity means that each vaccine batch has quality, safety, and potency. However, any change in shift reliance on final product testing will need specific control scheme requirements. Consequently, the improvement and validation of 3Rs based alternative methods for vaccines potency testing for consistency establishment in different batches are critical and of major importance before moving the product to international markets **[28, 29]**.

5. Potency test of rabies vaccine preparations

Potency is well-defined as “the specific ability or

capacity of the product, as indicated by appropriate laboratory tests or by adequately controlled clinical data" **[30]**. Potency evaluation is a crucial control method for determining the actual relative strength of vaccines **[31]**. Due to the high variability of biological products, potency test is important in ensuring lot-to-lot consistency. Controlling the failure of potency tests could estimate that a batch of vaccines could give insufficient immunogenicity. Because of the variability of vaccine effectiveness and immune response of humans, potency test is not always 100% accurate. This means that a vaccine with a potency not less than 2.5 IU/mL -in the case of rabies vaccine- not always gives 100% protection in 100% cases **[6]**. In the improvement of vaccine formulations as well as control tests; from the factors to be considered; is the protective immune response's nature. These factors could suggest better immunity substitutes, in addition, they may more precisely predict batches efficacy and assure batch-to-batch consistency. Correspondingly, the exposed antigen dose and duration effects, as well as the antigen presentation nature and extrinsic cytokines generation, could be identified and be in correspondence with the potency of vaccine as an extra indicator of vaccine potency **[6]**. So, the Potency evaluation for rabies vaccines could be categorized as shown in **Fig. 1**.

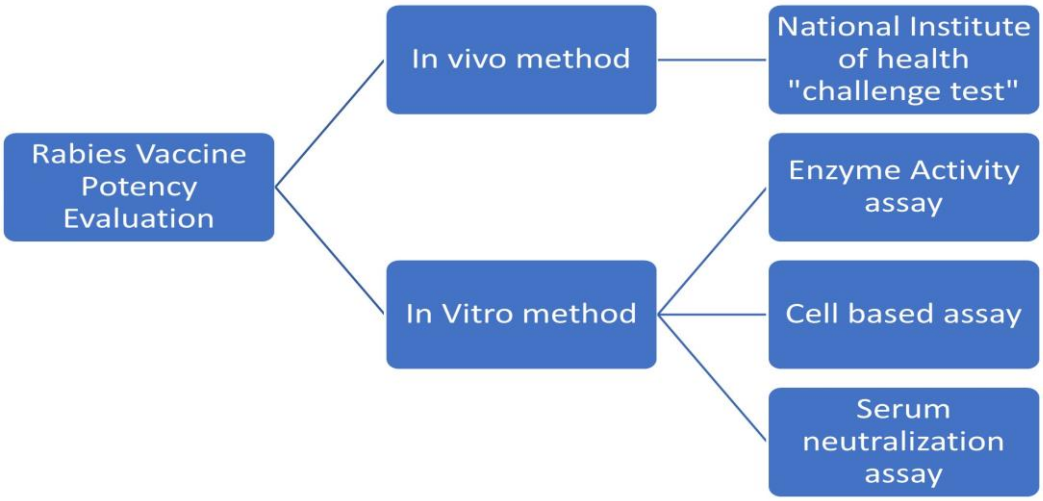

Fig. 1. Potency evaluation for rabies vaccines

5.1. *In vivo* methods of potency test

National Institute of health "Challenge Test"

Mouse Challenge NIH test is the main reference text used for potency evaluation **[29]**. This test depends on immunization of a large number of mice, followed by challenging them with the rabies virus, and one month later, both the live and dead animals are counted followed by calculating the 50% of the lethal dose of the vaccine and its potency **[26]**. In this test, the determination of an immune response in the animal model would allow the rational development of an effective vaccine. However, proving this correlation is difficult. These animals only represent the interactions of the organism with the immune system **[32]**. In addition, the main limitations of this test are consuming a large number of animals, non-humane endpoint for the test, being time-consuming, and its high variability **[9]**. Additionally, In-vivo testing is difficult, highly

costing and time-consuming. As previously mentioned, the shelf life of the vaccines is less than two years, and performing the potency test needs roughly eight weeks, which is time-consuming. These days, since the restricted ethical regulations, particular animal facility requirement such as individual cages and ventilation equipment raises the animal's husbandry cost. In many western countries, most control laboratories are directed towards in-vitro testing, especially which is alternatives to the methods that cause severe pain and suffering **[33]**. These testing methods mainly depend on large animal numbers that can exceed 100 animals per test and mostly, the animals show severe clinical signs. As a result, decision-makers are always encouraged to follow 3Rs depending on new techniques. Not only ethically and economically, but also, scientifically, potency tests depending on animals should be substituted. In the rabies vaccine, the results show low reproducibility in both intra and inter-laboratory, leading to an increased chance of repeating the test by the manufacturers **[28]**. In the challenge test, determination of the immune response in the animal model correlated with protection against a certain disease would simplify the rational development of an effective vaccine. However, finding such a correlation is quite difficult. These animal-based testing only evaluates the interactions of the animal with the innate, humoral, and cellular immune systems. While, it is problematic to improve animal-based testing strictly for human pathogens such as rabies virus, due to the presence of very specific surface proteins interacting with the receptors in host cells **[17]**. One of the important steps that are required for improving animal-based testing is molecules' characterization that has a very important function in the link between viruses and the host cells in clinical studies **[22]**. In Addition, there is animal-based testing that is not similar to human interactions. In regulatory requirements, the testing animals are to be challenged intra-cerebral, not through broken skin, which is the normal way of rabies virus for entrance. Additionally, the potency testing does not present data about either booster immunization's effect or the time interval for acquiring immunity **[14]**. NIH test is the most appropriate potency test for Rabies vaccine preparations. It is used since 1950 for the evaluation of inactivated rabies vaccines potency via measuring the degree of protection in the vaccinated mice challenged with rabies challenge virus standard [CVS]. The NIH Potency Test has several limitations and drawbacks. The routes of vaccination and challenge are different from the accepted immunization and natural infection routes in domestic animals. Accordingly, differences between the clinical response in the NIH test and the response in naturally exposed vaccines can occur. It has been recognized that the virus enters the bloodstream after IC injection allowing the neutralization of the challenge virus in the peripheral circulation. After IC administration by five minutes, only 2-8% of the original virus is recoverable in the brain and meninges. Moreover, the IC injection results in trauma results in immunoglobulin M [IgM] production, which does not occur in naturally exposed domestic animals **[34]**. Most inactivated rabies vaccines are targeted to produce an immunologic response to the rabies virus glycoprotein [G protein] **[35]**, a protective antigen that is found in all rabies virus isolates. Although protection verified by the assay is associated with G protein serum virus-neutralizing antibody [VNA] titers, differences in CVS challenge virus and wild-type viruses' infectivity and pathogenic characteristics have been made known to significantly affect measured vaccine potency **[18]**. NIH test has been the sole test for potency evaluation for rabies vaccines since 1973 **[36]**. Because of all these disadvantages, and because of many

approaches for replacement, and/or reduction, or refinement of laboratory animals in tests, there are many alternative techniques such as serological assays for potency evaluation.

5.2 *In vitro* methods of potency test

In vitro methods are divided into three categories as follows

5.2.1. Enzyme Activity Assays "enzyme-linked immunosorbent assay [ELISA]"

ELISA is depending on recognizing specifically the native form of Rabies virus glycoprotein" the most important antigenic part in rabies virus" **[37]**. ELISA has the capability of distinguishing between a potent and non-potent lot of different rabies vaccines. ELISA approves its ability to substitute reference test -NIH test- in potency evaluation **[38]**. The drawbacks of ELISA assay are low specificity, and not suitable for adsorbed vaccine or brain tissues, besides the need for well-trained specialists and highly expensive equipment **[39]**. Its mode of action needs a biopharmaceutical receptor or protein binding that could be determined via immunoassay methods such as ELISA. A case in which the binding assay would be suitable would be therapeutic monoclonal antibodies **[40] [41]**. An enzyme activity assay typically tests the therapeutic product's single functional domain activity, reliant on the mode of action. This assay can be used to show biological potency in conjunction with the binding assay.

5.2.2. Binding Assay

The Antibody Binding Test, a new method for the potency evaluation of inactivated Rabies vaccines, was first invented by the three scientists Arko, Wiktor, and Sikes. Its principle is that Rabies virus antigen binds to serum antibody during an incubation period of 60 min. It is one of the best methods for the evaluation of the amount of antigen in a Rabies vaccine. In Binding Assay, the vaccine potency can be determined biochemically using biochemical assay **[41, 42]**. Spectrophotometric, fluorometric, colorimetric, chemiluminescent, and light scattering tests can all commonly be used for vaccine activity detection **[17]**.

5.2.3. Cell-Based Assay

It is favored when the biopharmaceutical contains different functional domains which will all have to be intact for the product to be effective **[43]**. Many tests are categorized as cell-based tests, for example, the Rapid focus fluorescent inhibition test [RFFIT]. It is The World Health Organization [WHO] standard assay for the determination of antibody levels and immunity degree against rabies vaccination **[10]**. It is highly recommended to substitute NIH in potency evaluation of rabies vaccines **[43]**.

5.2.4. Serum neutralizing assays

It could be carried out by assay in mice, and it is called challenge test or by the plaque-reduction technique **[44]**. Tests in mice, as mentioned before, have many disadvantages, such as time-consuming as it needs many days before titrations are completed, a well-equipped animal facility so that it could keep a large number of mice, tissue culture plates which are quite expensive **[45]**. It is complicated by the presence of nonspecific reactions. So, the serum neutralization test is performed *in vitro* as a passive haemagglutination method [PHA] which is developed to overcome other tests' drawbacks. There is a high correlation between titers obtained with mice sera by the PHA method and those obtained by SN tests **[46]**. This method is quite cheaper and easier than any other methods such as ELISA and complement fixation methods **[19]**.

Conclusion

Conclusively, there is a rigorous requirement

for the quality control of finished rabies vaccine preparations. There are great developments in testing methods that help in the reduction of animals used in testing procedures such as serological methods, cell culture, ELISA, etc. However, many limitations face the implementation of these new methods- mainly in low-income countries- because of the difficulty and high cost of these methods. Therefore, great funds and support are needed to help these countries for applying these methods. In addition, countries that do not have problems with funds, face challenges in implementation due to lack of cooperation between health and regulatory authorities, many steps are needed for a complete change and replacement of old methods by the new ones. Methods validation is a crucial step before implementation and results compared with the standard method – challenge test- in our case" rabies vaccine potency testing". After all, WHO guidelines must be followed in all methods used. There is an urgent need for global implementation within a specific time with the help and support of regulatory authorities, to be able to reduce the number of animals and level of variability results from these tests and also reduce the time needed for undertaking tests.

Acknowledgments

The authors would like to thank and express their gratitude to all members of the Viral Control Unit and Animal house at Central Administration of control of Biologicals and innovative products and clinical studies, Egyptian Drug Authority, Egypt for providing facilities that allowed this work to be achieved.

Declarations

Ethics approval and consent to participate

Not applicable

Consent to publish

All authors have read and agreed to the published version of the manuscript.

Availability of data and materials

Data analyzed during this study are all included in the main manuscript.

Competing interests

No competing interests were declared by the authors.

Funding statement

No funding source was received

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