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Replacement of *in vivo* human rabies vaccine potency testing by *in vitro* glycoprotein quantification using ELISA – Results of an international collaborative study

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ABSTRACT

Three different ELISAs quantifying rabies glycoprotein were evaluated as *in vitro* alternatives to the National Institutes of Health (NIH) *in vivo* potency test for batch release of human rabies vaccines. The evaluation was carried out as an international collaborative study supported by the European Partnership for Alternatives to Animal Testing (EPAA). This pre-validation study, the results of which are presented in this paper, compared three different ELISA designs, assessing their within- and between-laboratory precision. One of the ELISA designs was proposed to the European Directorate for the Quality of Medicines & HealthCare (EDQM) and accepted for an international collaborative study under the umbrella of the Biological Standardisation Programme.

1. Introduction

The current immunization/virus challenge mouse test (NIH[1] test [1]) used to evaluate vaccine potency before batch release of human rabies vaccines was established more than 50 years ago. However, this test is associated with a number of issues: the need for live rabies virus involving costly biosafety measures; high variability of the test; and, as the main driver of the present study, the ethical concerns about the large numbers of animals used and the severity of the procedure [2]. Furthermore, replacement of the NIH test with an *in vitro* alternative could reduce batch testing costs appreciably, shorten lead times for release [3], and thereby help prevent rabies vaccine shortages. Therefore there are sound scientific, ethical and economic benefits to replacement of the NIH test.

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[1] *Abbreviations:* ANSM, Agence Nationale de Sécurité du Médicament et des Produits de Santé; BSP, Biological Standardisation Programme; CVS, Challenge Virus Standard; EBL, European bat lyssavirus; EDQM, European Directorate for the Quality of Medicines & HealthCare; EPAA, European Partnership for Alternatives to Animal Testing; EURL ECVAM, European Union Reference Laboratory for Alternative Approaches to Animal Testing; Flury LEP, Flury Low Egg Passage; GSK, GlaxoSmithKline; ICCVAM, Interagency Coordination Committee on the Validation of Alternative Methods; INCQS, Instituto Nacional de Controle de Qualidade em Saúde; IS, International Standard; mAb, monoclonal antibody; NIBSC, National Institute for Biological Standardisation and Control; NIH, National Institutes of Health; PEI, Paul-Ehrlich-Institut; PM, Pitman-Moore; PV, Pasteur Virus; SP, Sanofi-Pasteur; SII, Serum Institute of India.

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Provided the content of glycoprotein has been determined before blending of the vaccine, a serological test may be used for batch release [4,5]. For this test, groups of mice are vaccinated with the batch of vaccine to be tested and, after two weeks, the mice are bled and the sera are tested for rabies antibodies in an *in vitro* neutralization test. Such a test has been validated for veterinary rabies vaccines [4,5] and is currently under development for human rabies vaccines by the WHO (Ute Rosskopf, personal communication), even though the first investigation on its use for human rabies vaccine was performed at the US FDA nearly 40 years ago [6]. However, the complete avoidance of animal use is an achievable objective because it is accepted that protection offered by rabies vaccine is due to the presence of virus-neutralising antibodies to the native virus-associated (trimeric) form of the transmembrane glycoprotein G [7–12]. The European Pharmacopoeia now recommends the use of validated serological or immunochemical assays as alternatives to the NIH test [13]. Consequently, the possibility of validating *in vitro* assays, such as an ELISA, for batch release testing of non-adjuvanted vaccines is entirely feasible, especially as such tests are already used by some manufacturers and national control laboratories for monitoring consistency of manufacture and/or blending of human rabies vaccines.

An ICCVAM workshop held in 2011 [2,3] concluded that the NIH test should be replaced urgently but that direct correlation of any alternative (e.g. an ELISA that quantifies rabies glycoprotein) with the NIH test was neither necessary nor feasible in view of the latter's high variability. In contrast, the alternative test should show agreement with the immune response in humans and should therefore discriminate between potent and sub-potent batches [14]. The topic was subsequently selected as a priority in the vaccines consistency approach project of the EPAA [15] because of the large numbers of animals used and the animal welfare issues associated with the current NIH test. The EPAA is a voluntary collaboration between the European Commission, companies and trade associations from seven industry sectors, which promotes the application of the 3Rs (replacement, reduction and refinement of the use of animals in research). In an EPAA workshop held in 2012 [15], the participants agreed that a standardised sandwich ELISA calibrated against the current international rabies reference standard would be an ideal solution for human rabies vaccines potency testing. The participants agreed that an international collaborative (pre-validation) study should be carried out to select the most appropriate ELISA reagents and assay design. Based on the success of this approach, the selected assay would undergo formal validation under the umbrella of the Biological Standardisation Programme (BSP) of the EDQM/Council of Europe and EU Commission. To pursue this proposal, an international working group was established with partners from both manufacturers and regulatory bodies. The working group was supported by the EPAA and co-chaired by representatives from the Institut Pasteur (Noël Tordo) and Sanofi-Pasteur (Jean-Michel Chapsal, now independent). The pre-validation study was carried out with the goal of evaluating various ELISA designs in the hands of manufacturers and their National Control Laboratories for batch release, in parallel with the NIH test, for their ability to discriminate sub-potent from potent batches and to test vaccines from different manufacturing processes. To achieve this goal, the study included a set of test vaccines of different manufacturing processes, strains and potencies with independent evaluation of the findings.

The results of the study were presented at an EPAA workshop held in May 2015 and attended by the majority of the working group. This paper describes the results of this study and the conclusions and recommendations of those members of the international working group who are the authors or whose contributions are acknowledged below.

2. Materials and methods

2.1. Vaccine samples

A panel of sub-potent and/or potent batches of freeze-dried rabies vaccines was provided to the participants in the collaborative study by three manufacturers: the Instituto Nacional de Controle de Qualidade em Saúde (INCQS), GlaxoSmithKline (GSK) and Sanofi-Pasteur (SP). These vaccines were derived from different virus strains: Pasteur Virus (PV) from INCQS, Flury Low Egg Passage (Flury LEP) virus from GSK and Pitman-Moore (PM) virus from SP. The potent and sub-potent batches are referred to in Table 1 as "normal" (potent), "degraded" (sub-potent) and "50% spiked normal" (i.e. a 50/50 mixture of "normal" and "degraded" batches). The degraded batches were obtained by heat inactivation of the rabies vaccine batches. INCQS and GSK provided only their "normal" samples. The "degraded" samples of the GSK vaccine were prepared by each laboratory just before testing according to a heat treatment protocol submitted by the manufacturer, and these were then used to prepare the "50% spiked normal" samples. SP provided their own "normal" and "degraded" lyophilised samples and the mixtures of "50% spiked normal" were prepared by each testing laboratory just before use. All the samples ("normal", "degraded" and "50% spiked normal") were tested by the manufacturer for their potency by the NIH test and, in some cases as detailed below, for their rabies virus glycoprotein G content using their in house methods.

2.2. Reference

The WHO 6th International Standard (IS) for Rabies Vaccine (NIBSC code: 07/162 [16]) was used as follows: for NIH mouse protection tests, the potency assigned to the IS is 8 IU per ampoule i.e. 8 IU/mL when reconstituted in 1 ml of distilled water; for *in vitro* assays such as ELISA, the assigned rabies virus glycoprotein G content is 3.3 IU per ampoule or 6.6 IU/mL when reconstituted in 0.5 mL of distilled water. The instructions for use were provided to the participants in the shipment of materials and the IS was used to calculate the titre of each vaccine sample tested.

2.3. Participating laboratories

Five laboratories took part in the study: three were public sector National Control Laboratories and two were private sector manufacturers. They were, in alphabetical order, the Agence Nationale de Sécurité du Médicament et des Produits de Santé (ANSM, France), the US Food and Drug Administration (FDA, US), GlaxoSmithKline (GSK, Belgium), the Paul Ehrlich Institut (PEI, Germany) and Sanofi Pasteur (SP, France). The laboratories are not further identified in this paper, and the order above does not correspond to laboratories numbered #1–5 in the text.

2.4. ELISA methods

The collaborating partners used their own in-house ELISA protocols (or assays) (see Table 2). Thus, Lab #1 used the Institut Pasteur murine D1-25 mAb [17] (also referred to in the literature as D1) for both ELISA plate coating and virus detection [11,14,18]. Labs #2 and #4 both used a polyclonal rabbit antibody for plate coating, and the murine TW17 mAb for virus detection. Labs #3 and #5 used the same ELISA involving the murine WI 1112 mAb from the Wistar Institute [19,20] (also referred to in the literature as 1112 or TJU 1112) for plate coating and the D1-25 mAb for virus detection. In effect therefore, the five laboratories tested three different ELISA formats (designated as A, B and C in Table 2).

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