REPLACEMENT OF ANIMAL TESTING IN ASSAYING POTENCY OF HUMAN VACCINES AT THE ITALIAN OFFICIAL MEDICINE CONTROL LABORATORY

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Introduction

Several aspects assure human vaccine quality. Briefly, the vaccine production process occurs under Good Manufacturing Practices (GMP), adhesion to which is periodically checked by authorized teams of inspectors. Starting from the raw materials up to the final vaccine product, all production steps are subject to severe quality controls by the manufacturer. Quality testing is an essential element of the process by which medicinal products are released for use. Furthermore, before to enter onto the market, the vaccines for human use have again to be submitted to a control of key parameters. In Europe, the control is performed by a European Official Medicine Control Laboratory (EU-OMCL) according to the Directive 2001/83/EC Article 114 as amended by Directive 2004/27/EC, adopted in the Italian Legislation with the DL.vo 219/2006 (Italia, 2006). The National Centre for Control and Evaluation of Medicines (Centro Nazionale Controllo e Valutazione dei Farmaci, CNCF) of the Istituto Superiore di Sanità (ISS) (Ministero della Salute, 2016) is the Italian OMCL within the European Network as well as a member of the World Health Organization (WHO) National Control Laboratory Network for Biologicals.

For the quality control of medicines, the reference point is the European Pharmacopeia (Ph.Eur.), a compendium of texts on the qualitative and quantitative composition of medicines and tests/methods to be carried out that provide a scientific basis to guarantee the quality during the entire life cycle of a product.

Safety and potency testing are part of the quality control and often involve animal tests. Particularly, for established inactivated vaccines such as diphtheria, tetanus, pertussis, rabies, tick-born encephalitis and hepatitis a large numbers of laboratory animals are used.

Since the elaboration of the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes signed in 1986 (Council Directive 86/609/EEC) (Europe, 1986), an intensive activity has been performed by the European Pharmacopeia Commission to review all animal tests present in the various monographs of the Ph.Eur. to apply the 3Rs principle (Replacement, Reduction and Refinement) proposed by Russell and Burch in 1959 (Russell & Burch, 1959). The 3Rs principle provides a strategy for replacement, reduction, and refinement in animal testing and are an internationally accepted methodological approach for manufacturers and national quality control laboratories.

The establishment of the Biological Standardization Programme of the European Directorate for the Quality of Medicines and Healthcare (EDQM) (https://www.edqm.eu/en/biological-standardisation-programme) in 1991 has provided the means to carry out studies to develop and validate methods promoting the application of 3Rs, which were subsequently incorporated into the monographs and chapters of Ph.Eur.

The Bacterial and Viral Vaccines Sections of the Biological and Biotechnological Products Unit of the CNCF-ISS have participated in many BSP studies, in particular in those which objectives were to refine or reduce *in vivo* potency testing or replacing the *in vivo* potency or safety test with an alternative *in vitro* test.

The quality controls to be performed by EU-OMCLs for releasing a vaccine are indicated in the "Product specific guidelines for immunological products consisting in vaccines" published on the web site of the EDQM. Among the tests to be performed, is included the potency test, i.e. assessment of the biological activity of the vaccine.

Vaccines are very complex and heterogeneous products constituted not only by the specific antigen, but also might contain adjuvant/s, excipients and preservatives. The vaccine composition may interfere with non-animal tests determining the difficulty in performing the potency and safety tests by using physico-chemical test and consequently an *in vivo* test is still required. However, very important efforts have been made to replace, reduce and refine the use of animals for potency and safety testing at the European level.

The present review will provide an insight in the progress and achievements of 3Rs application, in particular in the replacement area, in controlling the potency of different kind of human vaccines at the Italian OMCL/NCL.

Activity performed as OMCL/NCL

At the CNCF, different types of vaccines for human use are controlled before being placed on the market (batch release activity) and/or for post marketing surveillance.

To perform these activities the experts are continuously skilled in ISO 17025 requirements, Ph.Eur., EDQM Guidelines and WHO Technical Report Series.

Below examples of vaccines currently controlled by the CNCF are reported, for which ISS experts have contributed over the years to activities aimed at implementing the 3Rs to *in vivo* assays.

Meningococcal serotype B vaccine

A meningococcal vaccine against the serogroup B of *Neisseria meningitides* (MenB) is controlled by the ISS. The Outer Membrane Vesicles (OMV) and three recombinant proteins of MenB in the presence of aluminium hydroxide as adjuvant compose the vaccine. The initial potency test, as well as a pyrogenicity test, were designed as *in vivo* tests.

The initial *in vivo* potency assay, after a complex validation study, has been replaced by the manufacturer by an *in vitro* relative potency test (IVRP) based on the Enzyme-Linked Immunosorbent Assay (ELISA), which is also performed by CNCF to release the MenB vaccine.

The rabbit pyrogen test performed to assay the MenB vaccine pyrogenicity has been substituted by the *in vitro* monocyte activation test (for further details, see contribution of M. Etna *et al.* in this report).

Hepatitis A and B vaccines

There are several types of hepatitis A vaccine.

The *hepatitis A vaccine, inactivated, adsorbed* is a suspension consisting of a suitable strain of hepatitis A virus grown in cell cultures, inactivated by a validated method and adsorbed on a mineral carrier.

The *hepatitis A vaccine – inactivated, virosome* is a suspension of a suitable strain of hepatitis A virus grown in cell cultures and inactivated by a validated method. The virosomes are composed of proteins of an influenza virus strain approved for that particular product and phospholipids are used as adjuvants.

According to the Ph.Eur. (2.7.14 Assay of hepatitis A vaccine) the assay of *hepatitis A vaccine* is carried out either *in vivo*, by comparing, under given conditions, its capacity to induce specific antibodies in mice with the same capacity of a reference preparation, or *in vitro*. The *in vitro* test was approved in June 2014, at the 149th Session of the Ph.Eur. Commission. Until 2014 no standardised *in vitro* test common to all hepatitis A vaccines was available for both manufacturers and NCLs. Indeed, this new method, is based on the determination of the vaccine antigen content by a unique polyvalent enzyme-linked immunosorbent assay (ELISA) developed to appraise all commercially available hepatitis A vaccines. ISS experts participated in the establishment of the hepatitis A reference preparations by using the *in vitro* test and successively replaced the *in vivo* by the *in vitro* test for the control of hepatitis vaccines (Wood *et al.,* 2000; Morgeaux *et al.,* 2015).

Hepatitis B vaccine is a preparation of hepatitis B surface antigen (HBsAg) obtained by recombinant DNA technology.

The assay of hepatitis B vaccine (rDNA) is carried out either *in vivo*, by comparing under given conditions its capacity to induce specific antibodies against the HBsAg in mice or guinea-pigs respect a reference preparation, or *in vitro*, by an immunochemical determination of the antigen content (Ph.Eur., 2.7.15 Assay of hepatitis B vaccine). The Ph.Eur. 2.7.15 does not describe in depth a validated ELISA method applicable to all commercially available HBsAg, therefore manufacturers have developed and validated their own ELISA potency assay and use their own in-house standard preparation.

For Post-Marketing Surveillance, the CNCF tests all hepatitis vaccine by *in vitro* methods.

Polio vaccines

Oral Poliovirus Vaccine (OPV), which is made from alive attenuated Sabin strains of poliovirus, has been the preferred vaccine throughout the WHO Global Poliovirus Eradication Initiative. However, the intrinsic instability of Sabin poliovirus strains in OPV may lead to the generation and accumulation of spontaneous point mutations in the 5′ non-coding regions of the viral RNA of the vaccine, which have been associated with an increased neurovirulence. For this reason, the maintenance of the attenuated phenotype of OPV strains must be tightly monitored during the production to ensure not only consistency of the vaccine but also the safety of the product (WHO, 2014). This requires that every batch of OPV is tested for neurovirulence in monkeys or transgenic mice susceptible to poliomyelitis.

As a potential substitute of animal based neurovirulence test, a highly sensitive quantitative molecular method called MAPREC (Mutant Analysis by Polymerase chain reaction and Restriction Enzyme Cleavage) was developed to quantify the 5′ UTR revertants in monovalent batches of OPV. The MAPREC test demonstrated that the percentage content of specific mutations directly correlates with the results of the Monkey NeuroVirulence Test (MNVT) (Chumakov *et al.*, 1991). CNCF experts have been involved in the studies for development of the assay and the establishment of the WHO Reference Standards for MAPREC assay (Dunn *et al.*, 2009; WHO, 2012). The MAPREC assay was performed at CNCF as part of the OPV batch release tests, but currently it is performed only upon request by other OMCLs.

Tick-born encephalitis virus vaccine

Tick-Borne Encephalitis Vaccine (TBEV) (inactivated) is a liquid preparation of a suitable strain of TBEV grown in cultures of chick-embryo cells or other suitable cell cultures and inactivated by an appropriate validated method.

The potency is determined by comparing the dose necessary to protect a given proportion of mice against the effects of a lethal dose of TBEV, administered intraperitoneally, with the quantity of a reference vaccine preparation of TBEV necessary to provide the same protection. Today, two alternative *in vitro* methods are under evaluation in the VAC2VAC project (see below). One is based on an ELISA method and another one aims at identifying novel biomarkers of innate immune response, predictive of vaccine immunogenicity. In particular, a platform based on human peripheral blood mononuclear cells (PBMC) was shown to be able to discriminate among the conforming and not-conforming antigen present in the vaccine final formulation by studying type I interferon (IFN) gene signature analysis by quantitative real time PCR (Signorazzi *et al.*, 2021). This cell-based assay, together with other immunochemical analyses, could be used for batch-to-batch assessment of the TBE vaccine, reducing and eventually replacing *in vivo* assay for potency testing.

Diphtheria, tetanus and combined vaccines.

The replacement of the *in vivo* potency test for tetanus (T) and/or diphtheria (D) component of monovalent vaccines or combined with other antigens such as pertussis (acellular, aP; whole cell, wP), hepatitis B, inactivated polio viruses and *Haemophilus influenzae* type b glycoconjugate has so far not taken place. These vaccines are quite complex in composition and interference from the different drug substances, adjuvant and excipients increase the difficulty in setting up of *in vitro* tests.

Thus, the potency testing of the diphtheria and tetanus toxoids, active drug substances of anti DT vaccines, is still conducted *in vivo* (Ph.Eur. 2.7. 6 Assay of diphtheria; 2.7. 8 Assay of tetanus). Equal groups of animals are immunized with scalar doses of a reference preparation and with the test vaccine; successively, they are challenge with a lethal dose of the respective toxin. The number of survivals in the different groups is used to calculate the vaccine potency. However, thanks to EDQM collaborative studies, to which also CNCF has participated, the *in vivo* lethal challenge assays have been refined over time with the introduction of serology assay for diphtheria and tetanus, and the use of humane end-points (Hendriksen *et al.*, 2001; Winsnes et.al, 2004). For the serology assay, the immunized animals are not challenged with the toxins, but instead they are bled and the sera tested by an immunoassay for the presence of antibodies against diphtheria or tetanus toxins. Furthermore, in order to reduce the animal use, an *in vivo* single dilution assay, after proper validation, can substitute the *in vivo* multi-dilution assay. WHO also strongly support these alternative tests, but their implementation is not simply since a product specific validation is always necessary.

The CNCF receives at control few batches per year of T or DT vaccine (tetanus max 10; diphtheria <10). A reduction in animal use has been applied by substituting the multiple dilution assay with the single dilution assay, which requires 50% less animals. Furthermore, the end-point for tetanus toxin challenged mice is not anymore, the death, but the paralysis (refinement).

It is a policy of the OMCL network to test for potency only the first final fill lot/batch in case more than one is produced from a final vaccine bulk. Of course, this has brought to a large reduction in animal use respect to more than 20 years ago, when every new fill lot/batch was tested for batch release.

Even if the potency assay for tetanus, diphtheria and acellular pertussis vaccines is still an *in vivo* test, some animal test for these kinds of vaccines has been replaced by non-animal-based test and included in the Ph.Eur. For example, the demonstration of absence of toxin in the toxoid bulks, has been replaced by *in vitro* test: the absence of diphtheria toxin, as well as pertussis toxin, is performed currently using VERO cells or CHO cell, respectively. For tetanus toxin a new *in vitro* assay (Behrensdorf-Nicol *et al.*, 2014) as a potential alternative to the mandatory guinea-pigs test for the absence of toxin in tetanus toxoids (Ph.Eur. 0452 and 0697) is the objective of a forthcoming EDQM collaborative study.

For the non-EU market, DT vaccines are usually combined with wP instead of with aP. The WHO has qualified many DTwP-HepB vaccines that are used in the vaccination campaign of several developing countries. The potency assay for wP foresees an intracranial challenge (WHO, 2013) with a lethal dose of virulent *Bordetella pertussis*. Therefore, in 2008, a proposal for an alternative serology potency test was submitted to the European Centre for the Validation of Alternative Methods (ECVAM), resulting in approval and funding (von Hunolstein *et al.*, 2008). Currently, this serological potency testing for wP is under evaluation by the manufacturers belonging to the Developing Country Vaccine Manufacturer Network (https://dcvmn.org/), which produce vaccines with wP. The evaluation is performed within the remit of a project funded by the National Institute for Innovation in Manufacturing Biopharmaceuticals, USA (https://niimbl.force.com/s/current-projects). A CNCF expert participate to the project as chair of the Steering Committee.

Discussion and conclusion

A huge effort has been made by the Ph.Eur. to replace, reduce and refine the animal tests for potency and safety testing of human vaccines (Lang *et al.*, 2018). In recent years, good results have been achieved to this aim with the contribution of industries, OMCLs and regulatory agencies.

In the field of batch release, an important contribution to a drastically reduction in animal use has been provided by the requirement of the Directive 2001/83/EC relating to medicinal products for human use. The Official Control Authority Batch Release (OCABR) performed by any given member state shall be mutually recognized by all other member states requiring OCABR for that product. That means that no other OMCLs of the Network controls a specific vaccine batch when already controlled and released by another OMCL.

Substitution of *in vivo* tests is not an easy task, as vaccines are complex and demonstration of equivalence of an alternative method is so far very difficult. To help transition from *in vivo* to *in vitro* methods, the Ph.Eur. Commission developed a new general chapter on the "Substitution of *in vivo* method(s) by *in vitro* method(s) for the quality control of vaccines" (5.2.14, in force since January 2018). In particular, the Ph.Eur. Commission gives advice on how to validate alternative methods in case a direct comparison with the *in vivo* test is not possible, in particular for the vaccines established long time ago (diphtheria, tetanus, rabies, TBEV).

New tests for established vaccines such as diphtheria, tetanus, acellular pertussis, TBEV, rabies, etc., are under evaluation in the "Vaccine batch to vaccine batch comparison by consistency testing" (VAC2VAC) which is a wide-ranging collaborative research project funded by IMI2 which aims to develop and validate quality testing approaches for both human and veterinary vaccines using non-animal methods (www.vac2vac.eu).

The consistency approach has been introduced some years ago and is based upon in depth characterization of the vaccine during development. In addition, the strict application of GMP assures that the quality of batches produced over time are similar for quality, safety and efficacy

to clinically evaluated batches (consistency in production) (De Mattia *et al.*, 2011). The concept is easily applicable to the recently new-generation vaccines that are well characterized as human papilloma virus like-particle or polysaccharide conjugate vaccine for *Haemophilus influenzae* type b, meningococcal serogroup ACWY and pneumococci.

The *in vitro* potency tests, that have been developed based on ELISA assay, are a surrogate of the *in vivo* method, as they do not measure the biological activity of the vaccine, but the content per dose of the antigen/s on the basis of relevant immune epitopes. For example, the IVRP for *N. meningitidis* serotype B, based on ELISA, quantifies the content of the OMV and recombinant proteins (NHBA, NadA, fHbp) present in the vaccine using specific monoclonal antibodies developed to recognize the epitopes relevant in the protection. Monoclonal antibodies are critical reagents, not available commercially as generally developed and produced by the vaccine manufacturer. As all biologicals to be used as reagents in *in vitro* assays, also the monoclonal antibodies need to be well characterized, have assigned a shelf life and be available over the years. Thus, sustainability (and cost) of the reagents for *in vitro* assay are aspects that deserve special attention also from the National Regulatory Authority when approving an *in vitro* assay in replacement of an *in vivo* one.

A great effort to replace *in vivo* potency test is currently ongoing during the development of new vaccines. An example is given by the vaccines against Covid-19, where non-animal methods were identified and proved suitable, during the clinical trials, to indicate immunogenicity and safety of the product.

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