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3R initiatives for advanced biological safety tests

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Abstract

Pharmacists had a significant hurdle in testing biological safety in humans. Pharmaceutical production is now extensively regulated and supervised. Different regulatory agencies and themodern pharmaceutical industry have collaborated to develop a comprehensive process understanding by implementing appropriate manufacturing process control, validation, and release testing in accordance with international Good Manufacturing Practices (GMP) standards. The incompatibility of particular preparation groups, vaccine components, or additives with the animal species employed is shown by retrospective analysis of Abnormal Toxicity Test (ATT) findings. The ATT, on the other hand, did not identify vaccinations that caused adverse effects on the target species. As a result, the anomalous toxicity test is ineffective for detecting dangerous batches. It is recommended to the DAB that the ATT be omitted because of concerns about drug safety and animal welfare. The evidence for anomalous toxicity testing as a predictor of dangerous batches is dubious at best, and there is no scientific basis for it. Numerous studies of historical ATT data have indicated that this quality control (QC) measure cannot be used to make any meaningful conclusions. Recognizing that strict QC processes can ensure product safety, efficacy, and stability, most regulatory agencies no longer require the ATT for most product classes. The test requirement has been removed from the European Pharmacopoeia, as well as the majority of product classes in the United

States, as part of the replacement, reduction, and refinement (3Rs) strategy. For these reasons, it is advised that the ATT be regularly excluded from pharmacopoeias and other regulatory requirements around the world. Non-animal alternativeshave been developed or are in the process of being validated by international regulatory organisations for the majority of these items. However, due to a lack of efforts on standardisation and application of these *in vitro* tests produced by regulatory authorities,many nations' pharmacopoeias continue to use in vivo techniques for safety testing. *In vivo* potency tests used in the quality control of immunobiological products require too many animals, causing them severe pain and suffering. Much research has been undertaken inrecent decades to evaluate alternative approaches for quality control and batch release of vaccines and other immunological products, particularly for potency tests.

Introduction

Earlier in the 19th century, the testing of biological safety in humans was a great challenge for pharmacists. Interestingly, even after being tested on animals, Koch's tuberculin was found to be unsafe for humans due to the patient's death. The first regulation was introduced by the German government when Behring started to commercialise the diphtheria serum on a large scale in 1894. Thereafter, a State Control Institute (SCI) was set up at Berlin-Steglitz in1896 under the headship of Paul Ehrlich. It was considered that animals' tests had a crucial role in quality control, and government control had proved beneficial to the quality (purity, safety, and potency) of antiserum produced in Germany. Later, the SCI shifted to Frankfurt and became the Royal Prussian Institute for Experimental Therapy in 1899 [1].

The most effective preservatives considered at that time were phenol and cresol. However, their use had to be restricted due to toxicity. Later on, the safety criteria for the serum were established such that if it contained 0.5% phenolorcresol with clear and free form precipitation, it would be safe (Ehrlich, 1896: Berliner Klinische Wochenschrift, 441-443). In large doses, phenol and derivatives may cause harmful effects on the central nervous system and cardiovascular system like dysrhythmia, seizures, and coma. Mice are considered very sensitive to phenol. A mouse injected s.c. with 0.5% phenol in 0.5 ml of serum shows trembling and shaking of the head, while more than 0.5% results in convulsions and death (Throm, 1995). Therefore, the mouse test became a standard test to check the level of phenolor their derivative preservatives in anti-sera, vaccines liked iphtheriaserum, bacterial vaccines (typhoid and cholera), etc [1].

On October 2, 1901, a former milk waggon horse in St. Louis named Jim died due to tetanus. He was used to produce serum containing diphtheria antitoxin. Further, a batch of diphtheria antiserum that had been contaminated in Saint Louis (US) led to the deaths of 12 more children. In the meantime, a similar incident (contamination with tetanus spores) occurred in Italy, resulting in more than 18 cases of tetanus, with 13 fatalities, being reported after treatment of children with diphtheria serum from the Sero Therapeutic Institute of Milan. This casualty, and a similar incident involving contaminated smallpox vaccine, led to the passage of the Biologics Control Act in 1902, followed by the formation of the US Food and Drug Administration in 1906.

In 1901, a guinea-pig test was established as a biological indicator for extraneous clostridial toxins in Germany and thereafter in many other countries [2]. In this test, a large volume of serum, i.e., 10 ml had to be injected by subcutaneous route. Initially, this test was set up especially for the control of diphtheria serum, but later on, it was extended to other sera also. This testing procedure remained unchanged in Germany and was used even up to 1935.

Later on, the German testing standards were amended and the serum sample was considered "safe" if it was free from toxins, in particular tetanus toxin. In the 1940s, a safety test consisting of a mouse test and guinea pig emerged in Germany when governmental regulations for several vaccines were revised. The abnormal toxicity test, as created by the combination of two formerly independent specific safety tests, has become a general safety test. This test was used for control of diphtheria serum and was consecutively applied to others era also [2]. Following World War II, the World Health Organization (WHO) began to develop internationally accepted guidelines; the Abnormal Toxicity Test was first mentioned and eventually became a general testing requirement for immunological and biological medicine used in the human and veterinary fields.

The ATT, as considered a test for freedom from abnormal toxicity, has been mentioned in Appendix 34 of the First Edition of the International Pharmacopoeia, 1956. Both the following tests are applied by injecting 0.5 ml under the skin of a healthy mouse weighing about 20 g and 5.0 ml under the skin or into the peritoneal cavity of a healthy guinea-pig weighing 250-400 g. If neither serious symptoms, nor death, ensue within six days [1].

The ATT is a general safety test (using mice and guinea pigs) which is intended to detect non-specific contamin ants causing adverse effects in biological or vaccines intended for parenteral administration and is used as a Quality Control (QC) release test according to pharmacopeial or other regulatory requirements around the world. This animal test has been given various names, including the Innocuity Test (WHO nomenclature), the General Safety Test (US reference) [4], and the Abnormal Toxicity Test (European Pharmacopeia (EP) & Indian Pharmacopeia (IP) nomenclature) [5], but the basic principle is the same: A single injection of a specified volume of a product batch into guinea pigs and/or mice is followed by an observation period. The preparation under the examination passes the test if none of the animals shows signs of ill health, death during the test observation period, or weight not less at the end of the test period than at the time of injection [2]. Repeat the test if more than one animal dies or shows signs of illness. The preparation passes the test if none of the animals in these condgroup dies or shows signs of ill health in the time interval specified.

There is wide variation between pharmacopoeias and international requirements as shown in Table 1 for Europe, the

United States, Russia, China, and India, as well as WHO requirements. Considering the today best practices like GLP or animal welfare, the administration volumes used for Intravenous (i.v.) dosing is not acceptable. For example, a maximum of 5 mL/kg as per most of relevant guideline should be administering resultant a maximum volume of 0.1 mL for mice with a body weight of around 20 g. Following the pharmacopoeias/ requirements (0.5–1.0 mL administration volume), a mouse receives 5–10-fold of the volume considered good practice [6].

Chronology of data analysis

In the early 1900's, the licencing procedures for biological preparations were not established. Even the reliable analytical techniques required to appropriately detect phenol levels in serum products were not in place. Therefore, mice and G. pigs were used as susceptible species for the detection of toxic phenol levels and tetanus toxin in biological preparations, respectively [7,8]. A survey was performed by the Paul-Ehrlich Institute (PEI) with the support of the German Ministry for Education and Research in the period of 1994–1995 to assess the usefulness of this test against human and veterinary sera and vaccines. More than 4367 ATTs for 159 different products were performed using more than 19,000 mice and more than 8,700 guinea pigs. Out of these, only 1.1 percent of ATTs needed a repeat test, and all batches passed the test. However, the test modification was observed for certain vaccines (whole cellpertussis, cholera, and typhoid vaccine are mentioned) due to the inherent toxicity of these vaccines [9]. The results published by the German PEI have even shown that this test does not serve its purpose and does not add any further information to that already obtained from QC release testing under GMP [10]. Kraemer et al.'s historical data, in which 5896 ATTs for 146 preparations were performed using 30193 mice and 12420 G. pigs, revealed that none of the batches were rejected. However, these results show that the ATT is not a useful predictor or control for harmful batches [4,11,12]. As per the US Code of Federal Regulations, Title 21, requires general safety testing be done for biological products (21 CFR, Part 610.1112). However, on the basis of the outcome of these products, the US Food and Drug Administration (FDA) realised that there is a need to evaluate many aspects of a biological product, especially the product's safety, purity, or potency with tests other than those prescribed in part 610". Thus, the FDA amended the regulatory standards for general biological products by adding an administrative procedure for obtaining exemptions from the test requirements. 13: 21 CFR, Part 601.2 states that the licence requirement is exempted for biological products, especially therapeutic deoxyribonucleic acid (DNA) plasmid products, therapeutic synthetic peptide products of 40 or fewer amino acids, monoclonal antibody products for *in vivo* use, or therapeutic recombinant DNA-derived products.

Later on, in August 2014, the FDA also removed the general safety test requirement for biological products. The same proposed to amend the biological regulations in August 2014 by removing the General Safety Test (GST) requirements for biological products. FDA is recommending this action because the existing codified GST regulations are duplicative of requirements that are also specified in biologics licenses or are no longer necessary or appropriate to help ensure the safety, purity, and potency of licenced biological products [13]. In addition, the expert Committee on Biological Harmonisation, constituted by WHO in 2002, also observed that several countries had removed the abnormal toxicity test for most products. This happened because of the implementation and compliance of GMP. Further, it has also been proved that "abnormal toxicity tests did not provide any extra information for assurances of the quality of the product" [14].

European Pharmacopeia has also removed abnormal toxicity testing from the monographs for "parental preparations" [15], "monoclonal antibodies for human use" [16], and "products of recombinant DNA technology" [17]. This was also on the basis of the fact that no additional value could be drawn from abnormal toxicity testing. However, the abnormal toxicity test has been deleted from approximately 80 monographs for biotechnological products, blood products, antibiotics, and vaccines based on the review of historical data [18,19]. In addition, it has strongly recommended exploring the replacement alternatives for safety testing for batch release of existing vaccines [20-22].

Performance of ATT in context of validation of analytical procedures

The International Conference on Harmonisation (ICH Q2 (R1) "Validation of Analytical Procedures") describes the characteristics normally evaluated for impurity tests (limit). The objective of the validation of an analytical procedure is to demonstrate that it is suitable for its intended purpose. The ATT's applicability to these characteristics has been described in detail below [23].

The outcome of ATT results can be affected by many factors other than contaminants, like body weight, species, strain differences, and stress levels of the animals. Moreover, false positive results may be observed due to responses of the active ingredients themselves or formulation components [22]. The tested preparations are optimised for safe use in humans but not in mice or guinea pigs at such high concentrations. In this test, a fixed volume is administered, irrespective of the dose used in humans. Thus, the full human dose may be administered to guinea pigs of 250–400 g body weight or mice of 20 g. In this study, assuming a human body weight of 60 kg, a guinea pig and mice would receive 150-fold and 3000 times the human dose, respectively. In addition, the preservatives and adjuvants used in the vaccine may cause false positive results. There are some examples of drug products that have produced false positive results when injected with high concentrations of benzyl alcohol when used as a formulation component for recombinant protein given by the intra peritoneal route. In addition, a reaction was observed to the high sugar content in an oral paediatric vaccine when administered according to a national pharmacopoeia by i.v. injection [23].

Reproducibility: The ATT design differs slightly between pharmacopoeias or international requirements. Kraemer *etal* [11]. Findings revealed that if ATT is conducted with strict adherence to the same study design and protocol (German Pharmacopoeia DAB 10), the biological tests in different laboratories and repeatedly in the same lab have produced significantly different test results.

Reliability: It is a measure of consistency and reproducibility. Considering the lack of reproducibility, the ATT must be classified as unreliable. No adequate positive control (e.g., reference standard) is available as the ATT test seeks for unknown contamination and non-specific toxicity. The quantitative test for impurities or content, the common analytical procedures, is mandatory to demonstrate suitability for the intended purpose. Accordingly, the above-mentioned characteristic used for impurity testing, ATT, is neither reproducible nor specific. Further-

more, ATT would not fulfill international validation criteria for analytical methods. This test lacks explicit acceptance criteria, as no definite endpoint is defined, like signs of ill health [24], significant signs of toxicity [25] and abnormal reactions [26]. In conclusion, this animal test is considered not suitable for its intended purpose. To address validation studies on alternative potency methods proposed to replace in vivo potency testing and the statistical strategy utilized, as well as to propose terminology harmonization and design validation studies for alternative potency methods. A survey of scientific databases was conducted to gather the goods' data on the validation procedures and verify their inclusion in the pharmacopoeias.

The general basis for quality testing in the classic batch release methodology is to demonstrate manufacturing consistency using analytical methodologies. Vaccines, which are complex immunobiological products involving antigens, adjuvants, excipients, and preservatives, were once treated differently, with batches being treated as individual products. As a result, authorities needed to conduct thorough quality control testing of each batch of a licenced vaccination, usually on animals, to ensure lot-to-lot safety and potency [27]. Much research has been conducted in recent decades to evaluate alternate approaches for controlling and batch-releasing biological products, particularly for potency assessments.

Vaccination-challenge tests are still commonly used, despite the possibility of utilizing alternative assays. Several *in vitro* assay options are proposed in the academic and compendia literature to examine the efficacy and safety of immunological preparations that require analytical validation. Test duration, improved reproducibility, low-cost animal tests, and the fact that they are susceptible to methodological validation, which has a beneficial impact on the quality control routine, are only a few of their advantages.

ATT in context of modern quality control

GMP standards, validated manufacturing process and appropriate analytical methods

Now days, pharmaceutical manufacturing is highly regulated and controlled. The different regulatory agencies and the modern pharmaceutical industry have evolved a comprehensive process understanding by establishing appropriate control of the manufacturing process, validation of the manufacturing process, and release testing complying with international GMP standards (Table 1). Currently, many studies are conducted during formulation and process development in the context of different formulation components (including preservatives) to investigate degradation profiles, product compatibility with various materials/surfaces, and leachable, which may be sources for contaminants (Table 1) [28-31].

Today, pharmaceutical compounds are tested extensively by *in vitro* assays, animal models, and clinical trials to check their safety and toxicity profiles in accordance with international (e.g., ICH Technical Requirements for Registration of Pharmaceuticals for Human Use) and national guidelines. A marketing authorization is granted by the relevant health authorities only when a positive benefit-risk assessment can be demonstrated. Now, pharmaceutical manufacturers produce highly developed medicines with well-defined purity and safety characteristics. The risk of contamination is extremely low if a manufacturer complies with GMP rules (e.g., globally recognised regulations [32-34] and if consistency in production is guaranteed [26,35].

Abnormal product contamination is extremely unlikely if the validated manufacturing process is followed. Appropriate analytical methods (e.g., mass spectrometry applications) are capable of detecting contamination and ensure batch-to-batch consistency. Advanced product testing is applied for the extended product characterization and release testing [23].

Table 1: Guidelines and standards for abnormal toxicity test (ATT) of different biologicals

Pharmacopeias / Requirements	Biologics/ Vaccines	SCOPE							
		Blank Control	Animal Quantity	Body Wt. (g)	Dose/ administration volume	Injection route	Observation time	Acceptance criteria	Retest(s) number/ description
European Pharmacopeia	General Test	No	5 mice	17–24 (m)	≤1.0 mL	i.v.	24 h	No animal dies within 24 h or within such time as specified in the individual monograph	One If one animal dies, repeat the test
	Immunosera/ Vaccines	No	5 mice 2 guinea pigs	17–24 (m) 250–400 (gp)	≤1.0 mL (m) ≤5.0 mL (gp)	i.p.	7 days	No animal shows signs of ill health	One If one animal dies or shows signs of ill health, repeat the test
United States	Biological Products (with exemptions)	No	≥5 mice ≥2 guinea pigs	<22 (m) <400 (gp)	≤0.5 mL (m) ≤5.0 mL (gp)	i.p. or following the approved route of product administration	48 h	No animal dies or exhibits any response, which is not specific for or expected from the product and may indicate a difference in its quality. No loss of body weight	Two If the initial test/first repeat test fails, a repeat test may be conducted
WHO [3]	Vaccines	No	5 mice 2 guinea Pigs	17–22 (m) 250–350 (gp)	One human dose ≤ 1.0 mL (m) ≤ 1.0 mL (gp)	i.p.	48 h	No animal dies within at least 7 days or shows significant signs of toxicity	No

Russian Pharmacopo-eia [6]	General Test	No	5 mice	19–21 (m)	0.5 mL	i.v.	48 h	No animal dies within the specified follow-up period	One If an animal dies, repeat the experiment with five mice (20 ± 0.5 g)
	Vaccines/ Sera	No	5 mice 2 guinea Pigs	17–20 (m) 250–300 (gp)	One human dose ≤ 1.0 mL (m) ≤ 5.0 mL (gp)	i.p.	7 days	No animal dies within at least 7 days, shows significant signs of toxicity, or a decrease in body weight	One If an animal dies, shows clinical signs of intoxication or a decrease in body weight, repeat experiment under the same conditions
Chinese Pharmacopo-eia [7]	Biologics/ Vaccines	Yes	5 mice 2 guinea Pigs	18–22 (m) 250–350 (gp)	0.5 mL (m) 5.0 mL (gp)	i.p.	7 days	All animals remain healthy and survive the observation period, without any abnormal reaction, and with an increase in body weight by the end of observation period	One If the test fails, it may be repeated once with 10 mice/4 guinea pigs
	Chemicals, Traditional Medicines	No	5 mice	17–20 (m)	0.5 mL (m)	Following the approved route of product administration	2 days	All animals survive the observation period	One If the test fails, it may be repeated once with 10 mice (18–19 g)
Indian Pharmacopeia	Biologics (Blood products)	No	5 mice 2 guinea Pigs	17- 22 (m) 250-350 (gp)	0.5 mL (m) 5.0 mL (gp) Except Factor VIII: 1.5 IU (m) 15 IU (gp)	i.p.	7 days	No of animals shows signs of ill health or died during test observation period	One If one animal dies or shows signs of ill health, repeat the test
	Vaccines	No	5 mice 2 guinea Pigs	17- 22 (m) 250-350 (gp)	One human dose ≤ 1.0 mL (m) ≤ 5.0 mL (gp	i.p.	7 days	No of animals shows signs of ill health or died during test observation period	One If one animal dies or shows signs of ill health, repeat the test
	Enzymes & Hormones	No	5 mice	17- 22 (m)	0.5 ml (m)	i.v.	1 day (Enzymes) 2 days (Hormone)	No of animals shows signs of ill health or died during test observation period	One If one animal dies or shows signs of ill health, repeat the test

[a]The United States Pharmacopoeia (USP 36) refers to US Code of Federal Regulations (21CFR,Part 610) [2].
[b]Exemptions: therapeutic DNA plasmid products, therapeutic synthetic peptide products of 40 or fewer aminoacids, monoclonal antibody products for *invivo* use, or therapeutic recombinant DNA-derived products. IP: Intraperitoneal; IV: Intravenous; M: Mice; GP: Guineapigs.

Validation of alternative methods for quality control of biologicals

Validation is research that determines the reliability and applicability of a method or process for a certain goal. Vaccine potency assays are typically dependent on two factors:

1) The type of vaccine that has been tested and 2) the analytical procedure's specific details, with potency being reported as antigen content or, more commonly, biological activity. Animal-based assays, cell cultures, biochemistry, and, in some circumstances, receptor-lig and binding are among the approaches available [36,37].

Methods that have been validated to reduce refine, or replace animal use (3 Rs) are often those that have been validated through collaborative studies conducted by the company for a specific product or those that have been validated and published by another laboratory. Since empirical information is generated and/or assessed on the reliability and relevance of a test method or approach under standardized and controlled conditions, the validation process is generally accepted to facilitate and/or accelerate international (regulatory) acceptance of alternative test methods or approaches [27].

Various computational methods as *in vitro* testing

Animal testing can also be replaced with computational methods. To replace and reduce the use of animals in safety and efficacy testing, mathematical techniques such as Quantita-

tive Structure-Activity Relationship (QSAR) modelling and Physiologically Based Kinetic and Dynamic (PBK/D) modeling can be used [38].

Quantitative structure-activity relationship (QSAR) models

QSAR models use chemical physicochemical and structural features to predict biological or toxicological traits. The Organisation for Economic Cooperation and Development (OECD) has issued recommendations on how to validate and record QSAR models for regulatory purposes in order to harmonise best practices.

Reading across and grouping

Chemical characteristics canals be anticipated by classifying compounds based on structural and biological similarities, then inferring relevant qualities from related data-rich molecules. In most cases, read-across is done in addition to QSAR to boost understanding in the anticipated attributes. Chemical assessments based on read-across can be aided by employing computational tools like Toxmatch and Toxtree, as well as reviewing toxicological data sources through on-line services like Chem Agora and Che LIST [38].

Kinetic models based physiologically

Physiologically Based Kinetic (PBK) models are mathematical representations of how chemicals spread in humans and other animals. PBK models are used to analyse *in vitro* toxicity data and to simulate internal concentrations following chemical exposure by diet, skin, or inhalation, for example. When they 're-combined with mathematical models of biological response in the target organ or tissue, they're called "Physiologically Based Kinetic and Dynamic" (PBKD) models. This is a mathematical model that models the distribution of chemicals (kinetics) and their biological effects (dynamics) in a variety of *in vitro* settings [38].

As prescribed in WHO guidelines, the quality and safety of vaccine batches are regulated to high standards by national regulatory authorities by using various quality control and safety tests, including the ATT. However, the measures for abnormal results are not well defined in these guidelines, like signs of illness etc. In addition, the quality of animals to be used in ATT, categorised on the basis of microbial colonization, was not mentioned in any of the guidelines. As per Mizukami *et al*., a new and improved method of ATT based on statistical, histopathological, and haematological findings has been used. This method is based on the observation that each vaccine has a specific body weight curve, and this pattern can be used as a reference for evaluating the test vaccine. In addition, histopathological data is useful for determining vaccine quality and safety. Therefore, a new improved ATT method with a combination of his to pathological examination and monitoring will be a novel method used for monitoring the consistency, quality, and safety of different batches of vaccine [39].

Growing international harmonization

In line with international regulation, the ATT, in most instances, is not required as a part of QC release analysis for the majority of product classes. EP, for example, does not require ATT for parenteral preparation, [15] monoclonal antibodies for human use [16], and products based on recombinant DNA [17] technology, because this test yielded no additional results. As a consequence, the European Convention on the Protection of Vertebrate Animals used for Experimentation and Scientific Purposes has deleted the ATT has been deleted from approximately 80 monographs for biotechnological products, blood products, antibiotics, and vaccines based on the review of historical data [18,19]. In addition, it has been strongly recommended to explore replacement alternatives for testing of batches of existing vaccines [20,21]. Title 21 of the US Code of Federal Regulations requires general safety testing be done for biological products (21 CFR, Part 610.112). Similarly, the FDA realised nearly a decade ago that the safety, purity, or potency of these products could be evaluated using tests other than those prescribed in part 610. Four [4] Thus, the FDA also modified the biologics regulations regarding general biological products standards by adding an administrative procedure for obtaining exemptions from the general safety test requirements. 21CFR, Part 601.2 [36] specifies that the test is exempted as a requirement for licence applications for therapeutic DNA plasmid products, therapeutic synthetic peptide products of 40 or fewer amino acids, monoclonal antibody products for *in vivo* use, or therapeutic recombinant DNA derived products. Therefore, the a forementioned agency does not require ATT for most of the products, but a batch already released for the EU and or the United States would have to be tested for abnormal toxicity in other countries, for example, the Russian Federation [40] and China [26] to be released for the local market. However, most of the studies revealed that no batch that met European Medicine Agency (EMA) or FDA approved specifications found apositive result in either of these countries (apart from false positive test results). Further, in 2002, the WHO Expert Committee on Biological Harmonization also noted that the abnormal toxicity tests had been wiped out from one region of the world for most of the products. This was connected to the effective enactment of, and compliance with, good manufacturing practices [14].

For safety tests, the panel strongly recommended encouraging the deletion of GSTs and Target Animal Batch Safety Test (TABST) from all national/jurisdictional legal requirements and international guidance (e.g., *Ph.Eur*. monographs/WHO recommendations, OIE guidelines). The panel also endorsed GSTs for human vaccines and other biological products and submitted proposals to relevant European Directorate for the Quality of Medicines (EDQM) expert groups and the WHO External Committee on Biological Standardization (ECBS) group. Moreover, they also suggested that GST and TABST be used for veterinary vaccines and other biological products and even initiated discussion at the World Organization for Animal Health (OIE) level. The European Partnership for Alternative Approaches (EPAA *via* member organisations) and the EU commission recommended exploring means to contact key countries at legislator level, potentially *via* local meetings or targeted group workshops. There was general consensus among the members that this is the right time to strive for the global deletion of GSTs, ATTs, and TABSTs and the international regulatory acceptance of appropriately validated non-animal approaches for vaccine batch potency testing. They also observed that in many cases, the *in vivo* tests had been established before vaccine licencing procedures had been implemented. Furthermore, they noticed that many *in vivo* methods only allow "pass-fail" assessments where as in vitro assays that use advanced analytical methods potentially enable more reproducible qualitative and quantitative product assessments. Therefore, the panel emphasised the need for selecting the best test method for a given purpose so that the best science should prevail and the need for the application of appropriately validated non-animal methods to ensure the supply of vaccines of acceptable quality [41]. In addition, they observed

that, in principle, regulators and industries are willing to accept appropriately validated non-animal methods and need to build up the regulatory authorities' confidence in the new methods. Moreover, to achieve international acceptance of a specific assay or an overarching testing strategy, it is imperative to ensure that all relevant regulators are involved. The WHO and the OIE are key players in pursuing this task since national authorities are required to make communications to these organisations. From the European perspective, all initiatives should further consider ongoing 3Rs-relevant work at the EDQM and the EMA *via* its *Joint Committee for Medicinal Products for Veterinary Use/Committee for Medicinal Products for Human Use Ad-hoc Expert Group on the Application of the 3Rs in Regulatory Testing of Medicinal Products* (JEG3Rs) [41].

In 2013, the European Partnership for Alternative Approaches to Animal Testing (EPAA) came up with the project Harmonisation of 3Rs in Biologicals, including a wide variety of products such as hormones, immunoglobulins, blood products, and vaccines. They even noticed that these biologicals are generally more complex in nature and have to require strict Quality Control (QC) for marketed human and veterinary vaccines in terms of consistency of each batch. These QC strategies include evalidated production processes and analytical techniques that may include animal tests or non-animal *in vitro* methods and approaches. They also observed that in some jurisdictions, specific animal tests for the QC of vaccines and other biologicals have been wiped out or replaced by *in vitro* approaches, whereas in some jurisdictions, the same animal tests may still be required. These regional regulatory differences, however, may lead to the unnecessary continuance of scientifically unsupported animal testing if a product is intended for several international markets [41].

Against this background, an international workshop on "Modern science for better quality control of medicinal products: Towards global harmonization of 3Rs in biology "was conducted by EPAA and took place on September 15th and 16th, 2015 in Egmond a Zee, The Netherlands. However, this workshop focused mainly on vaccines, while also addressing other biologicals too. The workshop came up with conclusions after a brain storming session following different case study discussions. They stressed the need to speed up the uptake and harmonization of the 3Rs principle in regulatory testing requirements for biologicals [40]. The panel also observed that even after uptake of a new method, e.g. in the respective pharmacopoeia monographs, further initiatives may be necessary to provide product-specific evidence to individual authorities that the new method is indeed able to detect inconsistent batches. However, the industry may play a key role in broadening the use of the new methods by collaborating with those responsible for the pharmacopoeias or with national control laboratories, such as, in Europe, the Official Medicines Control Laboratories (OMCLs) [40].

To certify acceptable control, the new methodologies have to meet the important quality parameters that are crucial to maintaining the safety and efficacy of the product established at the time of licensure. There was consensus that the consistency approach is the key approach to promoting the regulatory acceptance of new assays. Significantly, it was also observed that adequate information was available, so in principle, a transition to *in vitro* methods could beachievedwithoutadditionalclinicaltrialsorarequirementforaso-calledone-to-onecomparison of the *in vivo* and *in vitro* methods. Discussions that are on-going at different expert groups, such as the EDQM Group, [14] to provide a conceptual framework in support of the replacement of *in vivo* methods without a requirement for clinical trials or one-to-one method comparisons when scientifically justified, were described. Finally, it was also observed that financial incentives were required to promote the validation and use of new, non-animal test methods on the manufacturer's side. For instance, in Germany and in the United Kingdom, fees for the variation of the marketing authorisation may be reduced or waived in the event that 3Rs methods are introduced for the QC of the product [40].

Table 2: Current status of Abnormal Toxicity Test (ATT).

S.No.	Agencies/ Countries	Recommendations
1.	EDQM, 2015c (Status 2015)	36 European Pharmacopoeia monographs for human vaccines still stipulate under *General Provisions* in the Production section that *"The production method is validated to demonstrate that the product, if tested, would comply with the test for abnormal toxicity for immunes era and vaccines for Human use (2.6.9.)"*
2.	*In Europe*	Despite the deletion of the ATT as a lot release test in Europe, manufacturers producing for the global market may still perform the ATT, since it is stipulated by international requirements (e.g.World Health Organization [WHO]) and national requirements (e.g.Russia, China, Japan, Argentina, Mexico).
3.	*WHO 2013 a: Adiphtheria vaccine*	Test for innocuity*"…on the final lot may be omitted from routine lot release once the consistency of production has been demonstrated"* subject to approval of the national regulatory authority.
4.	*WHO2014a: Tetanus vaccine*	
5.	*WHO2014B*	
6.	India, personal communication	Allow waivers of the ATT.
7.	Brazil, personal communication	Moving towards deletion of the ATT.
8.	USFDA,2015	Revocated the General Safety Test (GST), since *"GST Requirements are no longer appropriate to help ensure the safety, purity, and potency of licensed biological products."*
9	EPAA workshop(Section7.2ofreport)	Deletion of the ATT/GST/test for innocuity from regulatory requirements was one of the topics discussed at a recent EPA Aworkshop.
10	EP work programme	The deletion of the ATT from all remaining products specific EP monographs was initiated.

3Rs in safety tests for immunoglobulins, sera and vaccines

A large number of animals are used in routine worldwide for batch releasing of biologicals, especially for immunoglobulins, sera, and vaccines, as a statutory requirement specified inmonographs of national (e.g. FDA, USDA) or international pharmacopoeias (e.g. European Pharmacopoeia). Safety of biological includes specific toxicity (related to the vaccine components such as antigen or adjuvant) and a-specific toxicity. In general, animal-based testing is found to be very expensive and time-consuming, and the outcome of these tests is highly variable. Therefore, the 3R development approach might be driven, which is more consistent and reliable for certain biologicals like immunoglobulins, sera, and vaccines. Test Box 1 and Table 3 provide an overview of 3R methods that have been incorporated into monographs of EP and other pharmacopeia.

Text box 1: Summary of *in vivo* and *in vitro* Safety Tests for Immunoglobulins, sera and vaccines.

Biologicals

A. Tetanus vaccine for Human use (absence of toxin and irreversibility of toxoid)

***In vivo* test:** In G. Pigs- develop signs of tetanus if presence of tetanus toxin

***In vitro* test:** Behrendorf-Nicol *et.al*. (2013) developed BINACL Eassay [42].

Remarks: There producibility, validation of the Binding & Cleavage (BINACLE) assay is on-going under the umbrella of the EDQM Biological Standardisation Programme (BSP).

B. Acellularpertussis vaccines (test for residual pertussis toxin and irreversibility of pertussis toxoid

***In vivo* test:** Chinese Hamster Ovary (CHO) cell assay (Gillenius *et al*., 1985) [43]. This assay cannot be used for the testing of adjuvanted a cellular pertussis vaccines because of inherent cytotoxicity of adjuvants.

***In vivo* test:** Histamine Sensitisation (HIST) assay carried out in mice (EDQM, 2015e; WHO, 2013b) [44] in which sensitised mice die when challenged with a normally non-lethal histamine dose (Corbel and Xing, 2004). [45]. This assay is considered to have high intra- and inter-laboratory variability and in order to meet the statistical requirements for a valid assay, several repetitions are often necessary (Bache *et al*., 2012; Isbrucker, 2012) [46].

***In vitro* test:** Indirect CHO-cell based assay *alternatives to the murine Histamine Sensitization Test (HIST)* hosted by the UK National Centre for the National Centre for the Replacement, Refinement and Reduction of Animals in Research (NC3Rs) is a suitable alternative for replacement of HIST and ready for product-specific validation at Manufacturer level.

Remarks: The results of the collaborative study will be published in *Pharmeuropa Bio & Scientific Notes*. Incorporation of the method into the Ph.Eur. monograph and other national and international regulatory requirements was recommended. The recently revised WHO recommendations for a cellular pertussis vaccine (WHO, 2013b) already fore see the possible use of an alternative method to the HIST and state that if an alternative assay isused, it should be atleast assensitive and specific as a validated HIST assay and should be
Approved by the national regulatory authority.

C. **Whole-cellpertussis vaccines**

***In vivo* test:** Mouse Weight Gain Test (MWGT) has been criticised for its lack of specificity, sincenotonly active pertussis toxin but also other toxins (e.g.endotoxin) typically present in whole-cellpertussis vaccines could decrease the weight gain of mice.

***In vitro* test:** Van Straaten-van de Kapelle *et al*. (1997) [47] compared in a collaborative study the performance of several *in vitro* and *in vivo* assays designed to detect endotoxins (*in vitro* Limulus Amoebocyte Lysateassay) orpertussis toxin (*in vivo* MWGT, leukocytosis promotion test, HIST; CHO assay). None of the tests performed well with regard to inter-laboratory reproducibility, most likely due to the variety of protocols used by the participating laboratories. The accuracy of the MWGT was lower than that of the other assays. Van Straaten *etal*. (2002)[48] proposed to combine the mouse toxicity and immunogenicity test in one animal model. Specific toxicity is determined by measuring endotoxin levels (weight reductio16 h post vaccination) and pertussis toxin levels (increase in leukocytes after 7 days), whereas serum antibody levels after 28 days are used as a measure of immunogenicity.

Remarks: Since the introduction of a cellular pertussis vaccines in1990s, whole-cellpertussis vaccines have lost their importance in Europe and other regions. However, due to the low production costs and since the relative protective efficacy of the best whole-cellpertussis and a cellular pertussis vaccines are comparable, whole-cell pertussis vaccines remain the vaccine of choice in many developing countries. In the interest of the 3Rs, it might be worth to explore whether any of the methods mentioned above or those described is applicable for specific toxicity testing of pertussis vaccines.

D. Livepoliovaccine (Neurovirulence test)

***In vivo* test:** Historically, non-human primates are used for neurovirulence testing (see review by Leven book, 2011) [49].

***In vitro* test:** WHO-collaborative study (Dragunsky *et al*., 2003) *in vivo* tests based on transgenic mice carrying the human poliovirus receptor (TgPVR21mice) can be used instead of non-human primates for neuro virulence testing of poliovirus serotypes 1, 2 and 3 (WHO, 2002; 2014c). *In vitro* methods (mutation analysis by PCR and restriction enzyme cleavage; MAPREC) are available for monitoring individual mutations in each of the three poliovirus serotypes, which are associated with reversion to neurovirulence (see review Leven book, 2011) [49]. Since the MAPREC for poliovirus serotype 3 correlates well with *in vivo* neuro virulence, it is used as a screening method and only bulks passing should be tested *in vivo* (EDQM, 2015h; WHO, 2014c) [50,51]. Neverov and Chumakov (2010) propose massively parallel sequencing (MPS) for identifying and quantifying the mutation.

profiles of oral polio vaccines. As reported by Rubin (2011), [52] MPS based methods may facilitate the monitoring of the genetic consistency of live viral vaccines, and in the case of oral polio vaccine have the potential to replace the in vivo neurovirulence test.

Remarks: The WHO announced in 2013 an international collaborative study that will assess the utility of massively parallel sequencing for monitoring molecular consistency of oral polio vaccine. The study involves national control authorities and vaccine manufacturers and will also develop common approaches, standards, and acceptance criteria needed for introduction of the new method to regulatory decision-making (WHO, 2013c) [53]. Preparation of the study is on-going and testing will start in early2016.

Tetanus toxoid models

Two more *in vitro* models for assessing the potency of human IgG against tetanus have been validated. An international joint investigation found that an Enzymatic Immunoassay (EIA) and a Toxin Inhibition Assay (TIA) had good reproducibility, precision, and repeatability. The EIA and TIA were both submitted to a collaborative investigation in order to be verified as high potency products. The tests were able to differentiate between samples with low, medium, and high potency by utilising the precision concept of reliability, which measures intra-(repeatability) and inter-(reproducibility) laboratory variances [54].

Pertussis models

A serological ELISA was designed to assess the humoral response elicited by the whole-cell vaccine as an alternative to the intracerebral challenge model, confirming that antibody titer son challenge day could predict mouse survival. The vaccination potency values were similar, but ELISA had superior reproducibility. Animal discomfort was reduced, while the number of animals employed was reduced by 25%. The Chi-square test was used to ensure that the results were homogeneous, and the variance analysis and regression correlation coefficient were used to assess the repeatability. The estimated potency analysis was used to determine reproducibility and dependability (geometric mean, mean variance, and Chi-square p-values) [35,37].

Diphtheria models

A serological assay was utilised in a collaborative study to test the efficacy of diphtheria and tetanus toxoid vaccinations for human use. In *vitro* toxin neutralisation experiments on Verocells were compared to the in vivo VC (Vero cells) assay in guinea pigs or intradermal challenge. The results revealed that antitoxin potency measured by the Vero cell assay and diphtheria ELISA was highly linked with neutralisation test potency. The results of the trials compared potency estimates, and the derived correlation coefficients revealed that the SA and VC tests are very similar [27,56].

Integrated Techniques for Testing and Evaluation (IATA) are adaptable approaches to chemical safety assessment that combine and translated at a from diverse methods and sources. IATA can combine novel approach methodologies like high through put screening and high content imaging methods, as well as computational approaches that are employed not only for data collection, but also for analysis and integration, in addition to traditional *in vitro* and in vivo tests [57].

Table 3: Safety testing and 3R progress in human and veterinary vaccine quality control (European Pharmacopoeia) [56].

Vaccine	Name of safety test	Animal used	3R alternative	Type of R	Status European pharmacopeia
Human vaccines	Abnormal toxicity	Mouse & guinea Pig	Deletion oftest	Replacement	Accepted on condition Of demonstrated consistency
Diphtheria	Residual toxicity	(Guine a pig (intradermal)	VEROcelltest	Replacement	Accepted
Whole cell Pertussis vaccine	Weight-gaintest	Mouse	Numbers ofanimals	Reduction	Accepted
Oralpolio	Neuro-virulence	Monkey(intracerebral)	a)Trangenicmouse or b) PCR method	a) Refinement b)Replacement	a) Under validation b) Under validation
Veterinary vaccines	Target animal safety test	Target animal	Deletion oftest	Replacement	Accepted on condition Of demonstrated consistency
Avian vaccines	Extraneous agents	Chicken	Cell culture	Replacement	Accepted

Impediments in the implementation of 3R methods in immunoglobulins, sera and vaccines

Although a large number of 3R methods are being developed by many institutes and regulatory agencies, the success of 3R development in regulatory testing is expressed in terms of the acceptance of these models by regulatory authorities. The biggest challenge is to get a validation process in a multi-laboratory study. Generally, it has been observed that the validation of 3R models is highly complex and time-consuming, much like the serological alternatives in tetanus vaccine potency testing, which took almost 17 years between method development and acceptance of the serological approach by the European Pharmacopoeia. However, test acceptance by the regulatory authorities does not necessarily imply that these 3R methods are being implemented as a routine test in every laboratory. Although this is in conflict with existing regulations on the use of animals in biomedical research (e.g.art.7.3.of EU Council Directive 86/609/EEC states that "In a choice between experiments, those which use the minimum number of animals, involve animals with the lowest degree of neuro physiological sensitivity, cause the least pain, suffering, distress or lasting harm and which are most likely to provide satisfactory results shall be selected"). There are several reasons why laboratories are not willing o rare notable to meet these requirements [59]. This area few obstacles which are generally encountered during implementation of 3R:

Lack of uniformity

This is supposed to be the biggest challenge in 3R implementation in the area of biological quality control. It is not necessary that the acceptance of a 3R method by one regulatory authority be acknowledged by the other authorities. The International Conference on Harmonization (ICH) might play a leading role in harmonization, but unfortunately, the focus is not up to snuff in biological quality control and animal testing.

Cost factor: The change in test procedure by the manufacturer requires approval from the registration authorities of each country where the product is licensed. In addition, the manufacturer required more manpower to compile the data and write the variation. Moreover, laboratories are also charged by the competent authorities to cover the administrative costs.

Lack of interest: Generally, *laboratories* might also lack the incentive to improve animal welfare. Even many international and national guidelines on animal experimentation and testing do not recognise humane endpoints, and they still use traditional lethal or severe clinical endpoints.

Practical aspects: The execution of some 3R alternatives requires laboratories to show consistency in production and testing. For example, to waive the safety test, the application has to be based on data from at least ten consecutive batches. In the event that only a few batches of a specific vaccine are produced per year, this might take a considerable number of years.

Lack of training: The paradigm shift from *in vivo* methods to *in vitro* like animal tests to HPLC would require special training of staff and the need to appoint well-trained technicians, but many institutes are reluctant to do so. Although these few challenges, as described above, have been observed in the implementation of 3R, substantial progress has been made with regard to 3R development and acceptance, especially in vaccines, resulting in a huge reduction in the numbers of animals and a saving of time. Moreover, there is a need for extensive and rigorous implementation of the 3R approach by harmonizing guidelines or mutual acceptance of data to offer training courses in new 3R test methods and encourage laboratories to work in line with existing regulations that favor 3R use. Recently, the bottom line of the consistency approach is that each vaccine batch produced at the vaccine production facility is one of a series of batches produced from the same seed lot. Consequently, the new batch shares many of the characteristics of the previous batches produced from the same seed lot [56]. This permits a new strategy of quality control. However, it is strongly believed that the implementation of the consistency approach will contribute significantly towards the elimination of the use of animals in regulatory required vaccine quality control.

Conclusion

As per the different rules and regulations, all biological are governed by different pharmacopoeias which control safety measures. In view of its unproven and questionable suitability to detect contaminants and increase product safety, the *in vivo* ATT is no longer a suitable test for quality control of various biological products. Moreover, as evident from retrospective analysis of historical data, great variability in test performance from various laboratories, including the number of animals, dosage administration, test duration, and performance of ATT in the context of validation of analytical procedures, is fully justified to completely eliminate abnormal toxicity testing from pharmacopoeias and other regulatory requirements. On the other hand, modern quality controls such as GMP-GLP principles, comprehensive validated manufacturing processes, and elaborative appropriate analytical methods have resulted in highly regulated and controlled pharmaceutical manufacturing; abnormal product contamination is extremely unlikely if these validated manufacturing processes are strictly followed. Currently, many studies are conducted during formulation and process development in the context of different formulation components (including preservatives) to investigate degradation profiles, product compatibility with various materials/surfaces, and leachable, which may be sources for contaminants (Table 1) [28-31]. A local/regional organisation, a worldwide organization such as the WHO, and a third external entity, where the process is centralized with expertise in biological product standards, should be included in the validation study arrangement. This setup could be used globally to ensure that the new approaches are harmonised and accepted internationally.

Validation studies that are widely approved by regulatory bodies are difficult to conduct, especially where validation facilities have not yet been created. The terminology employed is crucial, because it necessitates both global harmonisation and the use of proper statistical methodologies. Animal use in immunobiological potency tests may be reduced, refined, or even replaced as a result of studies, development, validation, and harmonisation of alternative control methodologies.

Different international bodies have strongly recommended completely eliminating abnormal toxicity testing from pharmacopoeias and other regulatory requirements. Based on the rationales provided in this paper on the basis of scientific inputs and regulatory agencies 'recommendations, the use of animals for ATT has no relevance. However, still, lab animals are extensively used in other safety tests according to a number of pharmacopoeias and other regulatory requirements for certain product classes due to a lack of harmonisation. However, as a matter of human safety, for certain biologicals like immunoglobulins, sera, and vaccines, such safety tests are still required. Even international regulatory agencies have developed non-animal alternatives for most of these products or are under the validation process. But still, many countries' pharmacopoeias are still using *in vivo* methods of safety testing due to a lack of efforts on standardization and implementation of these *in vitro* tests as developed by regulatory agencies.

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