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Animal testing for vaccines. Implementing replacement, reduction and refinement: challenges and priorities

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ABSTRACT

Transition to in vitro alternative methods to in vivo in vaccine release testing and characterization, the implementation of the consistency approach, and a drive towards international harmonization of regulatory requirements are most pressing needs in the field of vaccines. It is critical for global vaccine community to work together to secure effective progress towards animal welfare and to ensure that vaccines of ever higher quality can reach the populations in need in the shortest possible timeframe. Advancements in the field, case studies, and experiences from Low and Middle Income Countries (LMIC) were the topics discussed by an international gathering of experts during a recent conference titled "*Animal Testing for Vaccines – Implementing Replacement, Reduction and Refinement: Challenges and Priorities*". This conference was organized by the International Alliance for Biological Standardization (IABS), and held in Bangkok, Thailand on December 3 and 4 2019. Participants comprised stakeholders from many parts of the world, including vaccine developers, manufacturers and regulators from Asia, Europe, North America, Australia and New Zealand. In interactive workshops and vibrant panel discussions, the attendees worked together to identify the remaining barriers to validation, acceptance and implementation of alternative methods, and how harmonization could be promoted, especially for LMICs.

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Abbreviations

3Rs	Replace, Reduce, Refine
AEFI	Adverse Events Following Immunization
AGES	Austrian Medicine and Medical Devices Agency
AHI	Animal Health Institute (U.S.A.)
BET	Bacterial Entodoxin Test
BSP	Biological Standardization Programme
CBER	Center for Biologics Evaluation and Research (U.S.A.)
CEPI	Coalition for Epidemic Preparedness Innovations
cGMP	current Good Manufacturing Practice
CHO	Chinese Hamster Ovary cell
CPP	Critical Process Parameter
CQA	Critical Quality Attribute
DAFIA	Direct Alhydrogel Formulation ImmunoAssay
DCVMN	Developing Countries Vaccine Manufacturing Network
DTaP	Diphtheria Tetanus acellular Pertussis
DTP	Diphtheria Tetanus Pertussis
DTwP-HepB	Diphtheria Tetanus whole-cell Pertussis Hepatitis B
ECBS	Expert Committee on Biological Standardization (WHO)
EDQM	European Directorate for the Quality of Medicines & HealthCare
ELISA	Enzyme-Linked Immunosorbent Assay
EMA	European Medicines Agency
EPAA	European Partnership for Alternative Approaches to Animal Testing
EURL ECVAM	EU Reference Laboratory for Alternatives to Animal Testing
EVI	European Vaccine Initiative
FDA	Food and Drug Administration (U.S.A.)
FeLV	Feline Leukemia Virus
GAVI	Global Alliance for Vaccine Immunization
GMP	Good Manufacturing Practice
GSK	GlaxoSmithKline
GST	General Safety Test
HBV	Hepatitis B Vaccine
HIST	Histamine Sensitization Test
h-PBMC	human Peripheral Blood Mononuclear Cells
HPV	Human Papillomavirus
IABS	International Alliance for Biological Standardization
ICATM	International Cooperation on Alternative Test Methods
IBR	Infectious Bovine Rhinotracheitis
IBV	Infectious Bronchitis Vaccine
IMI 2	Innovative Medicines Initiative 2
IVRP	In Vitro Relative Potency Assay
LABST	Laboratory Animal Batch Safety Test
LMIC	Low- and Middle-Income Countries
MDCK	Madin-Darby Canine Kidney
MAT	Monocyte Activation Test
MIT	Mouse Inoculation Test
MNVT	Monkeys Neurovirulence Test
NC3Rs	National Centre for the Replacement, Refinement and Reduction of Animals in Research (UK)
NCL	National Control Laboratory
NEP	Non-Endotoxin Pyrogen
NGO	Non-Governmental Organizations
NIFDC	National Institute for Food and Drug Control (China)
NIH	National Institute of Health (U.S.A.)
NIIMBL	National Institute for Innovation in Manufacturing Biopharmaceuticals (U.S.A.)
NMPA	National Medical Products Administration (China)
NRA	National Regulatory Authority
OIE	Organization for Animal Health
OMCL	Official Medicines Control Laboratory Network
PCR	Polymerase Chain Reaction
Ph. Eur	European Pharmacopoeia
QC	Quality Control
RIVM	National Institute for Public Health and the Environment (Netherland)
RSE	Reference Standard Endotoxins
RPT	Rabbit Pyrogen Test
SRID	Single Radial Immunodiffusion
TABST	Target Animal Batch Safety Test
TBEV	Tick-borne Encephalitis Virus
TRS	Technical Report Series
VAC2VAC	Vaccine batch to vaccine batch comparison by consistency testing
VLP	Virus Like Particle
WHO	World Health Organization

1. Introduction

The field of vaccines is experiencing significant momentum in the development of alternative methods to animal testing for quality control and release testing, leading to a technical progress in analytical methods and their application that offers the opportunity for Replacement, Refinement and Reduction (3Rs) implementation in specific animal-based tests, and opens the door to the implementation of the consistency approach as vaccine quality strategy [1]. However, scientific progress alone will not be enough to ensure acceptance of 3Rs by all stakeholders globally. Regulations need be updated to embrace alternative methods, and for this to happen, a concerted science driven effort of influencing and inclusion is called for. Influencing, in order to root and establish a perspective that is significantly different from the animal-based one that was ingrained in the sector for decades. Inclusion, so to ensure that the most advanced and newly developed methods do not remain prerogative of only those regions or countries already at the forefront of innovation, and that can be shared and implemented in as many regions as possible. Such an effort, now more necessary than ever, can only be carried out through the open cooperation of all the stakeholders involved. The end result of such a cooperation ought to be a more harmonized vaccine sector, where regulations are as aligned as possible between countries/regions, where obsolete animal tests are expunged from pharmacopoeias and regulations, and where alternative methods are recognized and accepted as quality control instruments, which will enable the reduction time to market of vaccines and increased global access reducing costs [2,3]. The IABS *Animal testing for vaccines - Implementing Replacement, Reduction and Refinement: Challenges and Priorities*, held in Bangkok, Thailand, on 3–4 December 2019, was organized specifically to discuss the current status of the field, and offer an opportunity to multiple stakeholders to share and gather information, to collectively identify the key hurdles and develop a roadmap that would lead to a wider acceptance of alternative methods in lot release for human and animal vaccines. Bangkok, Thailand, was chosen as the venue to further stress the importance of participation to the discussion of LMICs and Global Alliance for Vaccines and Immunization (GAVI) member countries including major stakeholders from Asia. Indeed, participants from many of these countries and from around the globe enriched the lively discussions that happened at the scientific sessions and workshops of this conference.

2. Opening remarks

Nakorn Premsri, Director of the National Vaccine Institute of Thailand, Joris Vandeputte, President of IABS, and Hilde Depraetere, European Vaccine Initiative (Germany), opened the conference welcoming all the participants and encouraged them to actively participating in order to learn more about everyone's perspective.

3. Animal use and 3Rs

This session was chaired by Coenraad Hendriksen, Intravacc, Netherlands, Koji Ishii, National Institute for Infectious Diseases, Japan, and Jim Webster, OIE Collaborating Center, New Zealand. The session collected interventions on ongoing and successful efforts to implement 3Rs, to remove animal testing from legislation, and on the importance of understating Reduction and Refinement when Replacement is still not possible.

3.1. Animals in batch testing: need for 3Rs

In the absence of **Dr Suresh Jadhav**, Executive Director of the Serum Institute of India Pvt. Ltd., his speech was conveyed by **Dr. Sunil Goel**, Additional Director also of the Serum Institute.

Goel's talk focused on a series of actions needed to proceed towards 3Rs, and on the analysis of the key hurdles to be dealt with: first, he suggested a particular attention be paid to pharmacopoeias' monographies, some of which contain reference to unnecessary tests, others imply indirectly that use of animals is allowed even when alternative methods exist, and others are mutually inconsistent – their inconsistencies leave the door open to animal testing. He was also clear in asking for an increased enactment of humane endpoints to improve animal welfare, especially for products like Diphtheria, Tetanus, Pertussis (DTP) and Rabies vaccines due to the severity of the diseases involved [4]. He then discussed key hurdles on the road to the implementation of 3Rs, in particular mentioning the *validation process*, often long and complex, suggesting that Pharmacopoeia Commissions should devise new strategies for validation aimed at ensuring batch-to-batch consistency of the most relevant parameters rather than seeking a correlation with animal methods, and *costs*, referring to costs incurred by manufacturers to submit a variation of a licensed product, which need be multiplied by every country in which the product is licensed. As a remedy to this one-sided burden, he proposed and advocated for a *fee amnesty* to process those license variations that would result in fewer animals being used in quality control for already licensed products.

The greatest hurdle identified is the lack of *international harmonization*, which forces vaccine manufacturers to meet the differing requirements of the different authorities of the countries they export to. While the obvious solution would be the harmonization of test requirements or mutual acceptance of test data, Goel advocated for the Pharmacopoeial Discussion Group to prioritize harmonization of monographs that describe challenge assays that are used as routine batch potency tests. Such result, he stated, would prevent unnecessary animal use and suffering, and would also permit the use of serological and in vitro methods of potency determination in all regulatory regions.

3.2. The (long) journey towards the implementation of the 3Rs – every step counts

Eriko Terao, scientific coordinator at the EDQM, the European Directorate for the Quality of Medicines & HealthCare (Council of Europe).

Her talk focused on the various European efforts towards the implementation of the 3Rs. Terao began by retracing the long-termed commitment of the Council of Europe to animal protection, which as early as the 1960s developed conventions on animals. In 1971, the Council began discussions on animals used for scientific purposes involving representatives from member states, observer states and non-governmental organizations (NGO), integrating the 3Rs principle, in an effort culminating in 1986 with the *Convention on vertebrate animals used for experimental and other scientific purposes*. A brief introduction was dedicated also to the European Directorate for Quality of Medicine & HealthCare's (EDQM) role in fostering scientific cooperation and exchanges towards a harmonized consensus on 3R approaches to the quality control of medicines (and on its other function of coordinating the Official Medicines Control Laboratory Network (OMCL)), briefly presented the European Pharmacopoeia Commission, and gave information on the Biological Standardisation Programme (BSP), a joint activity of the Council of Europe and the Commission of the European Union that supports standardized and harmonized quality control methods for biological medicines, that also coordinates international collaborative studies to generate scientific data corroborating the selection of the most suitable consensus alternative methods.

She showed the different strategies used by the Experts of the European Pharmacopoeia (Ph. Eur.) to apply the 3Rs principle to the elaboration and implementation of Ph. Eur.texts. For method refinement, she gave examples such as humane endpoints for challenge tests in many vaccine products, improvements of methods, and in vivo testing of lower severity replacing previous more severe tests (serology instead of challenge for tetanus, diphtheria, veterinary rabies etc., and the bacterial endotoxin test in place of the rabbit pyrogen test for vaccines and other products).

For reduction, she cited statistical evaluations that decrease animal cohorts by 20–50%, improvement of methods to avoid invalid tests and re-tests, and an official batch release reduction scheme for established products based on production history & pharmacovigilance.

For replacement, examples included the addition of a validated ELISA as alternative to serology for Hepatitis A vaccines, two Biological Standardisation Program (BSP) projects, one completed on veterinary Clostridium vaccines, and one ongoing on human rabies vaccine. She also touched the European Pharmacopoeia's 5.2.14 chapter, recently (2018) added, aimed at facilitating the substitution of in vivo method(s) by in vitro method(s) for vaccine quality control [5]. Terao also expounded on the idea of a fourth R, standing for Removal, that is the elimination from the European Pharmacopoeia of animal tests. Examples of significant successes are represented by the deletion the Abnormal Toxicity Test (2017) and the Target Animal Batch Safety Test (2013), the replacement of the residual pertussis toxin by an in vitro Chinese Hamster Ovary cell (CHO) assay and removal from the individual monographs of the test for irreversibility of pertussis toxoid and the requirement to test the final lot for residual toxin (2018, a result stemming from the BSP114 collaborative study). Promising ongoing activities were also reported by Terao, like the recently ended BSP130 collaborative study for the replacement of in vivo tests for the *Clostridium septicum* vaccine (in consequence of which the Ph. Eur. Group of Experts is currently revising Ph. Eur. texts), and the ongoing BSP136 collaborative study on Clostridium tetani human and veterinary vaccines, for the evaluation of an in vitro replacement for the residual toxicity test, which led in 2019 to the removal from the Ph. Eur. of the test for irreversibility of toxoid, for lacking scientific bases and due to the absence of batch release data on reversibility [6]. Such endeavor necessarily needs be based on an evaluation of the scientific rationale of the specific test to be removed and on a comprehensive risk assessment, based on data (historical data, including batch release), but it must also consider the context (alternative approaches, redundancy of tests in European Pharmacopoeia tests, regulations, Good Manufacturing Practices (GMPs), pharmacovigilance), and needs be carried out through a concerted effort of engagement of the key stakeholders to secure information (OMCLs, manufacturers, regulators, both European and global, and also taking advantage of discussion groups and workshops).

3.3. Towards deletion of general batch safety tests: recent progress and next steps

Marlies Halder of the European Commission, Joint Research Centre, Italy.

The presentation summarized recent progress and next steps towards the deletion of general safety tests. Halder gave a brief historical background on the abnormal toxicity test (ATT) used for human vaccines. More than 100 years ago, a test in mice was used to detect phenol in diphtheria antisera and a test in guinea pigs to detect contamination with tetanus toxin. Later, these two very specific safety tests were combined into a general safety test for detection of non-specific contaminants in vaccines and other pharmaceuticals for human use. The ATT is also known as General Safety Test (GST) or Innocuity Test. General safety tests for veterinary vaccines are the Target Animal Batch Safety Test (TABST) and Laboratory Animal Batch Safety Test (LABST). The scientific relevance of general safety tests has been questioned since more than 30 years and Halder highlighted the following points: lack of specificity, reproducibility, reliability, and suitability for the intended purpose. She further provided an overview on the progress achieved in various countries and regions; for example, the ATT was removed from European Pharmacopoeia monographs for batch release testing of human vaccines already in 1997 [7], but only recently also for the production step. The US-FDA revoked the GST in 2015, whereas the WHO ECBS announced in 2018 the discontinuation of the Innocuity Test in all future WHO recommendations, guidelines and manuals for biological products published in the Technical Report Series. Moreover, WHO ECBS emphasized that discontinuation should also be applied to previously published WHO Technical Report Series documents [8,9]. She mentioned that many other countries, for example, India, Brazil, Argentina and Africa, deleted the ATT over recent years or allow waivers after demonstration of consistency of production.

Halder reported on the progress achieved for veterinary vaccines [2,3]. Thus, European Pharmacopoeia deleted the LABST already in 1997 and the TABST in 2013. Furthermore, she mentioned the possibility of waiving the TABST if consistency of production was demonstrated as outlined in VICH GL50 and GL55 [10]. This is applicable for VICH regions (Europe, Japan, USA); however, the OIE refers to these VICH guidelines in its Terrestrial Manual. A comparable guideline on waiving possibilities for the LABST (GL59) is close to publication [11].

Next, Halder outlined possible steps and proposals to accelerate the deletion of general safety tests, involving international organizations, National Control Authorities and manufacturers, and underlined that collaboration is key. Referring to the recommendation of WHO ECBS 2018, she suggested that WHO should remove the Innocuity Test from all relevant documents, since it is still mentioned in most of WHO's recommendations. Halder further invited OIE to actively promote the deletion of TABST/LABST at national level, or at least underline the possibilities to grant waivers in the light of VICH GL50, GL55 and the upcoming GL59. National Control Authorities should remove the general safety tests from their requirements for human vaccines as recommended by WHO ECBS 2018. With regard to veterinary vaccines, they should promote the deletion of TABST/LABST or at least allow waivers as outlined in VICH GL50, GL55 and the upcoming GL59. Retrospective analyses of general batch safety data may help to facilitate deletion, as it had been the case in Europe [12,13]. Manufacturers should continue to ask for the deletion of general batch safety tests where they are still required, providing evidence on other safety measures, and referring to the WHO ECBS 2018 statement and VICH guidelines (GL50, GL55, GL59). Halder closed her talk by highlighting the importance of dedicated collaboration between stakeholders being a key element for success. She advocated for increasing the dialogue between authorities and manufacturers, for mutual learning and sharing of information between manufacturers, and called for collaboration of all stakeholders at a global level.

3.4. Monocyte activation test (MAT)

Eliana M Coccia from the *Department of Infectious diseases, National Health Institute of Italy (ISS)*.

She described her teams' effort – within the Vaccine batch to vaccine batch comparison by consistency testing (VAC2VAC project) – to investigate the possibility to apply the Monocyte Activation Test (MAT) to detect pyrogen contamination for a human vaccine against tick-borne encephalitis virus (TBEV). Coccia clarified how the currently prescribed test for TBEV, both in European Pharmacopoeia [14] and in the WHO Technical report series [15], is the Rabbit Pyrogen Test (RPT), but that in reality the MAT is a more **reliable** test. Reasons adduced by Coccia are the following: (1) MAT eliminates the need for animal testing and is considered a suitable (after product-specific validation) substitute for RPT by European Pharmacopoeia; (2) it is more appropriate for testing pyrogens for intramuscularly/subcutaneously administered vaccines (RPT is performed intravenously); (3) it allows testing a vaccine for human use in a human setting, and with its longer incubation time (22 ± 2 h, vs 3 h for RPT) allows the detection of delayed inflammatory response.

Currently, MAT is employed by both OMCLs and manufacturers for the batch release of the *Neisseria meningitidis* group B vaccine *(Bexsero®)* [16] while, by OMCLs only for the *Salmonella Typhi* vaccine (*Typhim Vi®*). RPT is state of the art for multivalent DTwP-HepB vaccine, vaccines against human rabies, pneumococcal and meningococcal polysaccharide vaccine, and TBEV.

Coccia proceeded then to expound on the methodological approach pursued. As the vaccine targeted contains TBEV inactivated by formaldehyde as active substance, and aluminum hydroxide, TRIS buffer, sucrose, traces of tetracycline, gentamicin, neomycin, and formaldehyde as excipients, but shows no intrinsic pyrogenicity, the choice fell on human peripheral blood mononuclear cells (h-PBMC) – for their ability to recognize a wide repertoire of pyrogens and release pro-inflammatory cytokines – and on the Interleukin-6 (IL-6) – chosen as a read-out for its robust production after PBMC stimulation with reference standard endotoxins (RSE) as well as with non-endotoxin stimuli, namely R-848 and FSL-1. The methods employed were MAT Method A (quantitative) and B (semi-quantitative), as described in European Pharmacopoeia chapter 2.6.30. The relative results highlighted the necessity to adapt the validity criteria of both methods to fulfill at best the Ph. Eur. requirements for a vaccine without intrinsic pyrogenicity.

Coccia observed that the experiences and results from her group's work demonstrate the suitability of MAT for product specific replacement for the RPT with the possibility of adjusting it to face a heterogenicity of vaccine formulations, both viral and bacterial, thanks to the possibility to select between primary cells or monocytic cells, and three different methods of analysis, and commenting that MAT could be a useful tool to rule out presence of endotoxins and non-endotoxin pyrogens (NEPs) in vaccines, both during the manufacturing process and in batch release (although changes in the application of Method A and B of the European Pharmacopoeia are probably to be expected for vaccines with no-intrinsic pyrogenicity) [17]. Coccia's final remarks were dedicated to the regulatory status of MAT, noting that while the test did receive good acceptance in Europe, the position of the Food and Drug Administration (FDA) and US Pharmacopoeia (USP) is not as well defined, but that luckily pharmacopoeia harmonization seems to be on a good track, with China having announced MAT implementation in its pharmacopoeia for 2020, and Health Canada and the Japanese National Institute of Health are on the way.

3.5. Rabies potency testing: glycoprotein assay

Koraphong Pinyosukhee of the Institute of Biological Products, Department of Medical Sciences of Thailand's Ministry of Public Health (National Control Laboratory for Biological Products). He presented Thailand's Institute of Biological Products efforts for the validation of a

method for potency testing and consistency of production of rabies vaccine based on the assay of Glycoprotein by ELISA [18] as an alternative to the National Institute of Health (NIH) test.

Pinyosukhee stressed the importance of the rabies vaccine for Thailand, and globally as a means for the World Health Organization's (WHO) *Zero by 30* project for the elimination of rabies by 2030 [19]. In Thailand, the potency of the rabies vaccine is tested by the National Control Laboratory with the NIH method, requiring 168 mice per sample, 30 days, with a cost of 3240 US Dollars, and being very poor from an animal welfare point of view. To overcome all these difficulties, the Institute of Biological Products worked on an alternative method for potency testing based on an ELISA assay to measure glycoprotein – the major protein playing a role in the host's immune response – content in the vaccine. The method was validated in 2015, and is extremely promising, significantly shortening the time needed for testing (30 days for the NIH, just 3 days with this method), being cheaper at 400 US Dollars, and could potentially replace the use of NIH potency test method for both human and veterinary rabies vaccines in the future. Pinyosukhee clarified that at the moment, rabies vaccines in Thailand are still being released with the NIH test by the Institute of Biological Products, which tests 1 every 5 lots. The institute is currently collecting data of glycoprotein content from every batch of every manufacturer, to amass enough information on the various vaccines to make it feasible to stop using the NIH and move to the glycoprotein assay for batch release (with NIH test remaining a fallback in case problems with out-of-range glycoprotein contents were to be identified).

3.6. Potency testing and 3Rs: general overview

Sylvie Uhlrich, Sanofi Pasteur, France, presented a general overview of vaccine Potency Testing and 3Rs. After a brief historical introduction on potency testing, showing the progression from the first challenge tests, to toxin neutralization tests and then to immunogenicity assays, finally to reach the stage where in vitro assays are established [4], Uhlrich proceeded to highlight the reasons that make transitioning to alternative methods important. In vivo models act as a "black box", with at times questionable relevance to humans, while at the same time suffering often from poor robustness and high variability inherent to the use of live individuals. Moreover, due to historical reasons, specifications and potency tests for human vaccine batch approval often differ for various parts of the world, resulting in either duplication of animal testing or partial implementation of 3Rs for some vaccines when distributed worldwide. A problematic issue, considering that 90–95% of the animals used by manufacturers is employed in batch control testing, with ulterior quantities employed in independent batch release testing by National Control Laboratories. Considerations other than those of scientific character also play an important role. Uhlrich mentioned (1) animal welfare considerations, with large quantities of animals subjected to severe pain, and a societal response increasingly concerned by the use of animals; (2) legal considerations, because in Europe, Directive 2010/63 clearly states the duty to use, wherever possible methods or testing strategies not entailing the use of live animals; and (3) economical, because in vivo tests are expensive, require long times, and their inherent variability can lead to the unjustified rejection of what are actually safe and efficacious vaccines and to delays in market release which may turn into shortages of vaccine. To showcase how alternative methods can influence the sector for the better, Uhlrich discussed the case of an ELISA as a potency assay for Hepatitis B vaccine. Comparing it to the Immunogenicity assay (described in WHO TRS 978), which requires 10 to 20 mice, at least 3 dilutions of vaccine, bleeding of the mice after 4–6 weeks, and then an ELISA assay for HBsAg antibodies, while the in vitro potency assay (IVRP) is based on a sandwich ELISA using 2 monoclonal antibodies H35 and H57 targeting the "a" determinant of HBsAg, and the IVRP of each formulation is then determined against a homologous reference. The in vitro assay proved to be much more consistent, and more discriminant to detect subpotent batches, than the immunogenicity assay [20]. The presentation moved then to discuss the current limitations to 3Rs implementation. They are of regulatory and scientific kinds. Regulatory ones are (1) lack of harmonization of regulatory requirements; (2) caution on the side of health authorities to accept deviations from established guidelines and monographs; (3) general risk-adverse attitude to forego in vivo assays used for decades and considered a "gold standard"; and (4) the complexity of regulatory changes that do not generate strong incentive to develop and implement alternatives to animal testing. Scientific ones are (1) historically, in vivo assays were not validated according to current ICH Q2(R1) principles [21], (2) change in assessment of product attributes very likely when switching from an in vivo to an in vitro method, (3) one-to-one comparison is usually very challenging and not necessarily justified (an example of this is the fact that challenge tests have limited discriminative power to detect sub-potent lots, so, for them, no concordance would be possible between in vivo and in vitro assays). Uhlrich shared her opinion that the time is ripe to consider a change in perspective, retiring the idea of a one-to-one replacement. In its place, an approach that permits an existing in vivo method be substituted by more than one in vitro method to control key qualitative and quantitative attributes, putting the focus on understanding the critical quality attributes of the product, and leveraging an integrated perspective on product quality that leads to the *consistency approach* as a methodology to ensure safety and efficacy of vaccines without need to use animals [1]. An approach of this kind is already implemented in the case of polysaccharide conjugate vaccines such as Haemophilus Influenza type b vaccine (Hib) for which appropriate control of conjugate composition, integrity, content and size at different manufacturing stages led to removal of in vivo test on final product. Uhlrich's conclusion expressed the need for a worldwide regulatory harmonization, and for the involvement all stakeholders (regulators, scientists, animal welfare organizations, the public and decision-makers) in the communication of best practices.

3.7. When animals are still needed for reduction and refinement

Coenraad Hendriksen, Chair of the Scientific Committee for this conference, from the Institute for Translational Vaccinology (Intravacc), the Netherlands, focused the attention on two components of 3Rs – reduction and refinement – that are less glamorous than replacement, but that are of pivotal importance, in those cases where animals remain essential, for improved animal welfare and for better science. Hendriksen noted how a variety of barriers (science, regulations, tradition, etc.) can slow down the implementation of non-animal methods, noting how efforts should be encouraged toward refinement and reduction, which represent low hanging fruits (less innovative, easier to develop, and relatively easy to implement in the regulatory setting) in those cases where scientific tools for replacement are not (yet) available, also in the light that about 15% of all animal use in Europe often undergo severe suffering [22]. Discussing reduction, Hendriksen noted that much can be achieved through improvements in standardization (an example was made of the high inter- and intra-laboratory variation leading to poor reproducibility and invalidity for the Kendrick Test [23], and of the influence of species and mouse strain on potency testing of the Tetanus toxoid), through use of Standard Operating Procedures, improved staff training, and richer information background on the animals (lowering the numbers of animals of sample sizes by correctly factoring in the animal related variability, physical and environmental noise, and the impact of husbandry and animal care). About refinement, Hendriksen stressed the importance of limiting pain and distress, highlighting the need for pain management – through anesthesia when needed, monitoring of animals, improved animal living conditions, and application of humane endpoints to be weighed against one or several markers (clinical, pathophysiological, behavioral, hormonal, haematological and micro-biological). An example of humane end-points application was supplied, regarding whole-cell Pertussis vaccine potency testing: clinical signs (through observation), body weight (daily), and body temperature

(monitored by temperature sensitive probe). In the conclusion, Hendriksen stressed again the fact that animal replacement has to be the ultimate goal, but that in the meantime, efforts must be made to implement reduction and refinement, as they lead both to better testing, better science, and better animal welfare.

3.8. Implementation of 3Rs in quality control testing of vaccines

Sunil Goel, Additional Director of the Serum Institute of India (SIIPL) Pvt. Ltd, described its organization's commitment to the development, introduction, validation, and implementation of 3Rs and consistency-based approaches, and how such activities are helped by the fact that Indian Pharmacopoeia always proved supportive and receptive to such endeavors.

The first progress described was related to the DTP group of vaccines, for which the Institute secured successes in both replacement and reduction. Replaced was the conventional lethal challenge on guinea pigs/mice for potency testing by two assays, a Vero cell assay for the potency testing of the diphtheria component, and a T-ELISA for the potency testing of the tetanus component. Both assays required about 3 years from development to final acceptance by National Control Laboratory (2006 and 2007 respectively), with a first approval requiring 1 in 10 batches potency testing, and since 2017 an approval requiring 1 in 25 batches to be tested (or once in six months, whichever was to be the earliest). The replacement reduced the number of guinea pigs used per batch from 232 to 30 (~85%). On the reduction front, the SIIPL secured authorization, by sharing data on a large number of batches, to switch from a multi-dilution assay to a single-dilution assay, receiving approval first from the National Control Laboratory (NCL)/National Regulatory Authority (NRA) and the WHO.

Refinement was achieved by securing approval in 2017 to switch from the lethal challenge test in guinea pigs for tetanus potency to a paralytic challenge test in mice. Goel proceeded to describe further results obtained in-house. For the Hepatitis B vaccine, leveraging WHO TRS 787 and 889 (which suggest the release of the final lot with a validated in vitro assay), data on in vitro and in vivo assays was submitted to the NCL for review and replacement of the in vivo assay and the in vitro assay was accepted by the NCL in 2006 (requiring in vivo testing for 1 in 5 lots). In 2017, the NCL approved a complete waiver, resulting in zero animals used for lot release.

Goel also described how SIIPL secured approval to discontinue the test for specific toxicity of the tetanus toxoid for two polysaccharide conjugate vaccines (Hib and Meningococcal Conjugate A), a result achieved in both cases by establishing consistency of a number of lots and sharing results with the National Regulatory Authority (NRA), and then receiving from that Authority (and later from WHO) authorization to discontinue the test. The elimination of the test spares 5 guinea pigs per bulk conjugate lot, and thus eliminates the need for the long (21 days) test. Discussing the Abnormal Toxicity Test, Goel first reminded of how the test causes substantial unjustified use of animals without any benefit with regards to demonstrating product safety, and how it clashes with animal welfare and the 3Rs principle, lacking as it is a sound scientific rationale and justification, and then shared that the SIIPL, following Indian Pharmacopoeia, implemented its deletion for most of its vaccines through the route of Post Approval Changes/Variations [24], although regulatory harmonization remains very much needed, as ATT is still required for product registration in different countries. Progress in 3Rs was met also with the replacement, for Hib and Meningococcal A vaccines, of the Rabbit Pyrogen Test (RPT) with the Bacterial Endotoxin Test (BET), with ongoing efforts to implement the same for Rabies and Hepatitis B vaccines.

Goel gave then a description of the significant results obtained in implementing 3Rs for the Rabies vaccine, for which alternative methods were used for characterization of the vaccine along with in vivo methods, suitable correlations were developed, and then monitored for a number of batches laying emphasis on data monitoring of critical parameters and trend analysis, which together allowed the implementation of non-animal methods. Specifically, in process in vivo tests were replaced by the Fluorescent Antibody Test, final bulk NIH potency test was replaced by the Single Radial Immunodiffusion (SRID) Test and in vivo Mouse Inoculation Test (MIT) challenge replaced with a Fluorescent Antibody Test, which led to a reduction in test duration (from 14 to 4 days), and of the animals used, from 32448 for 96 lots produced in a year to 0.

Also mentioned was the application of 3Rs in stability studies of various vaccines, replacing in vivo testing with in vitro methods for maximum time points, and performing the in vivo testing only at the terminal stability time point. In the case of Rabies vaccine, the SRID Test was implemented for the 3, 6, 9, 12, 18- and 24-months' time points, leaving the NIH potency only at the terminal (36 months) time point or either at annual time points, with a significant replacement and reduction of animal usage.

In closing the intervention, Goel touched on the importance of the consistency approach for routine lot release of vaccines, specifically noting how its approach, based on the identification of critical indicators of safety and efficacy and of parameters that indicate product consistency, can lead to the application of newer concepts, such as quality by design, and highlighting how the Rabies vaccine discussed before can represent an interesting case study of the combined outcome of 3Rs and consistency approach.

4. Product development and in vitro production/analysis

This session was chaired by Yeowon Sohn, Seoul National University, South Korea, and Robin Levis, FDA/CBER, U.S.A. The session was dedicated to ongoing and successful examples of non-animal approaches to product development and production.

4.1. Production of Japanese encephalitis vaccine using the vero cell-line

Tuan Dat, VABIOTECH, Vietnam, described its organization' success in shifting the production of Japanese Encephalitis vaccine from culture in mice to Vero cells. The previous vaccine, *Jevax*, was produced through virus inoculation in mouse and successive harvest from the mouse's brain, with about 1 million creatures needed to produce 6 million doses of vaccine per year, and a 3 months production process, for a vaccine that can cause allergy and acute disseminated encephalomyelitis in vaccinated subjects, and showing poor immunogenicity requires multiple doses and boosters. Thanks to a switch to Vero cell culture, Dat explained, the new vaccine, *Jecevax*, can be produced in only 2 months, replacing mice in production, while at the same time overcoming the disadvantages in terms of adverse reactions of the vaccine produced with mouse-brain tissue.

4.2. Development of cell-based pandemic influenza vaccine for national security

Parichat Duangkhae, from Thailand's Government Pharmaceutical Organization (GPO) – a State enterprise operating under the Ministry of Public Health and active in pharmaceutical products, including vaccines – described the development in Thailand's Government Pharmaceutical Organization of a cell-based influenza vaccine for national security and emerging preparedness, as Asia is at risk of becoming the epicenter of a future influenza pandemic. GPO began producing and egg-based Live Attenuated Influenza Vaccine including H1N1 strain in 2015, but recognizing the various limits of the egg-based technology (not last, limited egg supply at the time of pandemics), the decision was taken to create the next vaccine, targeting the H7N9 strain, with a cell-based process based on the Madin-Darby Canine Kidney (MDCK) cell line, for which 3Rs alternative quality control tests are being investigated (a plaque assay and quick real-time polymerase chain reaction (PCR) in lieu of eggs for infectivity, and high performance liquid chromatography in lieu of the single radial immunodiffusion (SRID) for the influenza

hemagglutinin quantity). The cell-based vaccine will offer increased possibilities to scale up production in case of national security needs.

4.3. HPV vaccine in vitro/in vitro release test history and current situation

Rober Sitrin (PATH) presented a case study on the implementation of an in vitro potency assay for a human papilloma virus (HPV) vaccine, Gardasil® (a Merck recombinant quadrivalent – HPV 6, 11, 16 and 18 – vaccine. Gardasil®, Sitrin explained, was specifically developed with in vitro potency [25] as the sole release testing in mind, a result achieved in 2006 when the product was licensed both in the US and in the EU with in vitro potency test only. The choice to pursue a non-animal potency test for release was motivated both by animal welfare considerations – 80 mice sacrificed per sample tested – and by practical ones – relying on mice adds cost, variability, lengthens the release cycle as much as 6 months and shortens the vaccine's shelf life. The HPV vaccine is based on the immunogenic properties of icosahedral virus like particles (VLP) produced by recombinantly expressing the major HP protein, L1, for each type of virus, in *saccharomyces cerevisae* yeast. The strategy was to use in vivo in early development, gather data and then transition to in vitro before approval. To measure potency, an in vitro relative potency (IVRP) assay was developed, a sandwich enzyme immunoassay measuring the amount of antibodies bound to neutralizing epitopes for each HPV type. The IVRP was used both in monovalent bulks and on final container samples. The assay proved able to provide a direct comparison between the antigen content of each VLP type in a given test sample and the content of a batch that was shown to be efficacious in humans, and its results also did show correlation with immunogenicity measured through a traditional mouse assay, so its result is considered predictive of immunogenicity in humans. Discussing the feasibility of in vitro only release testing, Sitrin stressed the importance of a good vaccine characterization and known monoclonal reagents as pre-requisites to develop in vitro potency assays that correlate with in vivo data, and the fact that human data can be leveraged to supplement existing data to gain additional concordance. Concluding the talk, Sitrin mentioned that in vitro potency was listed as an acceptable assay for HPV vaccines in the corresponding WHO TRS report (TRS 962), but also remarking that some countries, like China, still insist on an in vivo assay format, which is a negative byproduct of the uneven international regulatory framework.

Li Shi, of the Shanghai Zerun Biotechnology Co., Ltd, offered information on vaccine release using non-animal testing in China, focusing on HPV vaccines and the manufacturer's ongoing efforts. Shi reported a significant comment from an unnamed officer from China's National Institute for Food and Drug Control (NIFDC), dating 2019, stating that "in vitro testing is encouraged to replace in vivo testing", with the proviso that substitution can be granted in case of successful systematic verification of in vitro methods, and the fact that the Chinese Pharmacopoeia, in its 2015 edition, advocates the use of in vitro methods instead of animal experiments to identify the quality of biological products to reduce use of animals for experiments, while it also contains in vitro relative potency methods for recombinant Hepatitis B vaccine and for inactivated Hepatitis A vaccine. About those two vaccines, Shi explained, first a correlation between in vivo relative potency in mice and in vitro relative potency was fully established, national vaccines standards set, and highly valid testing kits were approved for both. Discussing the status of HPV non-animal vaccine release in the Country, Shi explained that there is no clear timeline for the transition to in vitro release, because, even though all the HPV vaccine releases for clinical studies are currently using both in vitro and animal testing, data on the correlation between the two methods is being collected (albeit not systematically yet), and while the country's NIFDC is working on establishing national vaccine standards in terms of antibodies and antigens, there is still no recognized or approved valid testing kit for HPV batch release test. Shi moved then to Zerun Bio's work on in vitro release for HPV vaccine, discussing both the work on the in vitro relative potency test and the correlation studies with the in vivo test (for strains 16 and 18), and then introducing the internal work on a Direct Alhydrogel Formulation ImmunoAssay (DAFIA) [26] developed internally (to determine antigen content, identity and integrity directly on the aluminum adjuvant). The DAFIA method was shown internally to produce maintain high specificity for the HPV16 and 18 strains, good repeatability, and correlation with both the mouse potency test and the ELISA.

5. Improved product characterization using non-animal methods

This session was chaired by Gautam Sanyal, Vaccine Analytics, LLC, USA, and Denis Lambrigts, GSK Vaccines, Belgium. The session was dedicated to ongoing efforts to characterize legacy vaccines to enable transition to alternative methods and to the successful implementation of the consistency approach.

5.1. Product characterization by non-animal methods: general overview and the VAC2VAC project

Hilde Depraetere, Director of Operations of the European Vaccine Initiative, spoke next, introducing the VAC2VAC (vaccine batch to vaccine batch comparison by consistency testing) project [27]. VAC2VAC is a wide-ranging collaborative research project funded by Innovative Medicines Initiative 2 (IMI 2) programme, formed by 22 partners in a public-private consortium involving experts from veterinary and human vaccine industry in a partnership with OMCLs, regulatory authorities, academia, translational research organizations, and vaccinology alliances. Its overall objective is to demonstrate proof of concept of the consistency approach for batch release testing of established vaccines by developing sets of in vitro and analytical methods and approaches. In describing the consistency approach, Depraetere underlined how its key tenet is not ensuring product quality through release testing only, but rather by ensuring that each batch produced is consistent with a (historical/clinical) batch already proven to be safe and efficacious. Such approach leads to a radical paradigm shift in vaccine quality control: from the current premise of the uniqueness of each produced batch and on the fundamental relevance of quality control testing on the final product, the consistency approach posits that each batch must be considered as one of a series, shifting quality control from testing on the final product to strict control of every step of the production process, and within this perspective, a vaccine is of demonstrable quality and efficacy if non deviation from consistency can be demonstrated [1]. Such approach, Depraetere added, increases the in-depth knowledge of the product, makes it possible to simplify the standardization of methods, which can lead to a global streamlining of batch release methods, and it can bring about beneficial consequences for animal welfare (with significative reductions of animals employed), plus overall savings of both time, and costs. These goals require the creation of new – or the optimization of – non-animal methods for consistency testing, which must be developed, pre-validated, and accepted by the regulatory authorities. VAC2VAC is currently focusing on a series of veterinary (Rabies, Canine Leptospira, Infectious Bovine Rhinotracheitis (IBV), Infectious Bovine Rhinotracheitis (IBR), Feline Leukemia Virus (FeLV), *C. Perfrigens*, *C. chauvoei*, Tetanus) and human (TBEV, and Tetanus, Diphtheria, acellular Pertussis in DTaP combinations) vaccines, for which work is ongoing on physicochemical, immunochemical and cell-based (notably, monocyte activation test for TBEV was pre-validated as replacement for the rabbit pyrogen test) methods. These activities, Depraetere added, do not happen in a vacuum: the consortium started, through a workshop in 2017 [28], an open discussion with all stakeholders – vaccine manufacturers of major human and animal health companies, competent authorities, OMCLs, EDQM etc. –, holds regular meetings with European regulatory agencies (with a strong interaction with EDQM), and crosses the European borders by executing outreach activities toward the international regulators and organizations, so that it results can be leveraged internationally.

5.2. Three samples of product characterization

5.2.1. Tick borne encephalitis vaccine (TBEV)

Dieter Pullirsch from the Austrian Medicine and Medical Devices Agency (AGES), the Austrian Official Medicines Control Laboratory (OMCL). He discussed AGES activities within VAC2VAC for the pre-qualification of an ELISA test for potency testing of the Tick Borne Encephalitis vaccine (TBE). AGES had initiated the development of non-animal test methods already in 2011, and then proceeded within VAC2VAC as TBE vaccines were one of the topics selected for investigation in the project. Within VAC2VAC AGES initiated a cooperation with a manufacturer of TBE vaccine (only two registered vaccines in Europe for TBE, both based on inactivated whole virus, aluminum adjuvanted, produced by two manufacturers); since 2019, both manufacturers became project partners of VAC2VAC. In Europe, each vaccine batch must be tested for potency by the manufacturer and additionally by an OMCL – AGES is the only one performing it – and it currently consists of a lethal challenge assay on mice (Eu. Ph. 1375). Pullirsch explained that within VAC2VAC, immunochemical test methods were developed based on ELISA using structure specific monoclonal antibodies. TBEV antibodies were characterized with different methods (western blotting, pH treatment, freeze-thaw cycles, detergent treatment, thermal alterations) using ELISA plates coated with the inactivated virus non-adsorbed antigen, showing that one antibody is the most sensitive one in forced degradation experiments. Different ELISA formats and DAFIA were also tested, to demonstrate that all the antibodies are capable of recognizing the antigen in the presence of the aluminum hydroxide adjuvant. Currently recovery, specificity/selectivity, precision, robustness, structure/stability indications are qualified, while work is ongoing on the validation pertaining accuracy, response function/calibration cure, and intra-laboratory precision and transferability. Pullirsch also commented on the other ELISA method, developed autonomously (although with periodic scientific interactions with AGES) by the second manufacturer of TBEV vaccine. The method was transferred to the AGES laboratory and good interlaboratory precision was shown between the manufacturer and AGES. A comparison with the animal challenge test was initiated. Preliminary data show comparable mean results between the ELISA and the challenge assays performed by AGES and the manufacturer. In closing the intervention, Pullirsch expressed the conviction that these methods have the potential to accurately quantify the viral target antigens in TBE vaccines and to detect structural changes, but that to introduce these potency assays in the European Pharmacopoeia further work is needed, centered on implementing a small scale transferability study for both methods, on further investigations for stability testing, and on defining the specifications to replace the test for the Official Control Authority Batch release testing.

5.2.2. DTaP vaccine

Paul Stickings, from the United Kingdom's National Institute for Biological Standard and Control (NIBSC), presented work on the development of monoclonal antibody immunoassays to measure the relative amount and quality of antigens in Diphtheria-Tetanus-acellular Pertussis (DTaP) vaccines. This approach is based on the use of well characterized and relevant monoclonal antibodies to ensure the quality and consistency of vaccine batches. Such tests have the potential to play a key role in a control strategy no longer including an in vivo potency test [2].

The first part of this project focused on the thorough characterization of monoclonal antibodies (44 in total) directed against one of the antigens present in DTaP vaccines [29]. Antibodies were evaluated in terms of their ability to bind the native, detoxified and adsorbed antigen, and antigen that was altered following exposure to elevated temperature. For some antigens (D and T), neutralization tests were available and used to identify antibodies that target a relevant functional epitope on the antigen. Finally, affinity measurement and epitope competition studies were performed to identify pairs of high affinity antibodies that could be used in a sandwich ELISA format. Stickings presented the results obtained so far focusing on tetanus as an example. The developed monoclonal antibody capture ELISA for tetanus proved able to detect antigen in a wide range of tetanus vaccines for human and veterinary use, including antigen detection in the final lot in the presence of non-aluminum and aluminum-based adjuvants. It is specific, quantitative, and able to identify changes in antigen content for a vaccine that was deliberately formulated to contain a graded series of tetanus toxoid doses. The assay, is able to detect antigenic changes following exposure of non-adjuvanted toxoids to elevated temperature, although studies are still ongoing to determine whether similar changes will be detected in final lot vaccines containing adjuvant and whether the assays will be able to provide indications on stability. Further work will focus on desorption investigations to cover all aluminum containing vaccines available in the consortium to understand what proportion of the total antigen content is being detected by the ELISA when applied to final lot samples. The final part of the project will focus on validation studies according to ICH/VICH guidelines [21] and transferability studies, while efforts from other partners will focus on the development of a multiplex approach to measure of all the DTaP antigens in the same assay. In closing, Stickings shared some final considerations on what could affect the regulatory acceptance of an antigen immunoassay *in lieu* of in vivo potency testing. An antigen ELISA is a quantitative assay, but only in relative terms, which makes it unlikely that results could be expressed in units traceable to an International Standards, a fact that would give rise to the need to set product-specific specifications. Also, results obtained so far in the VAC2VAC project suggest that products would need be controlled with a specific reference vaccine (a common reference may work for some vaccines, but probably not for all). Lastly, for highly adsorbed aluminum containing vaccines a monoclonal antibody ELISA would detect only a proportion of the antigen present in the vaccine, and it will need to be demonstrated that the proportion of antigen being detected is representative for the quality of the vaccine as a whole.

5.2.3. Towards the end of the NIH test for rabies vaccines

Jean-Michel Chapsal, from the European Partnership for Animal Alternative Approaches to Animal Testing (EPAA), made an overview of the current status of the validation of an ELISA for potency assay of human rabies vaccines within the Biological Standardisation Programme 148 (BSP148). Summarizing the current potency testing method, the NIH method, he noted its several criticalities (including a very high variability, and the safety factor of requiring use of a live virus [30]), and the extreme severity of the procedure's effects on the animals, to highlight the consensus on the need to find a suitable in vitro alternative, and then proceeded to trace back the steps that led to the current BSP148 project for the validation of a G-protein ELISA for potency testing [31]. He mentioned the 2012 EPAA workshop at Arcachon (France), where the decision to create an International Working Group for the creation of an G-protein ELISA replacement to NIH test was taken, to be based on the evaluation of three competing ELISA assays, a study which eventually produced a candidate ELISA (developed at Sanofi Pasteur) based on 2 monoclonal antibodies (accessible to all laboratories, and commercially available worldwide from two suppliers) specific to the conformational trimeric form of the glycoprotein G, which does not react with non-immunogenic soluble glycoprotein, that recognizes most rabies strains used worldwide for human vaccines, and is able to discriminate sub-potent vaccines altered by a variety of methods including over-inactivation by b-propiolactone, the viral inactivation agent. Based on these results [32], the international collaborative study BSP148 was launched by the Biological Standardisation Programme of the Council of Europe and the EU Commission to further validate the transferability and robustness of the selected ELISA, supported also by numerous stakeholders worldwide, including the World Health Organization, with a view to revise the relevant

European Pharmacopoeia to include a standardized ELISA, and propose ultimately a global replacement of the in vivo NIH test. The program is divided in three phases: Phase 1 of the study was focused on logistical support for the procurement and testing of additional vaccines, the study protocol preparation for participants and on reaching the commercial distribution of both the capture and the detection antibody, with production of batches for exclusive use of BSP148. Phase 2, which is foreseen in 2020, will see participants (about 30 laboratories worldwide, 9 manufacturers and 21 official control laboratories) use a standardized protocol to test a common set of samples covering various virus strains and potencies, and EDQM will be tasked with analyzing the generated data. Lastly, the Phase 3 (foreseen 2020-21) of the study will consist of testing of as many routine commercial batches as possible using the standardized ELISA protocol, with results reporting to EDQM, with the aim of using the results to support the evaluation of the applicability of the method to routine testing and of the potency requirements in view of the revision of compendial texts, including Ph. Eur. monograph 0216. In concluding the overview of the project, he mentioned that the BSP148 is expected to be able to produce its results in time for a revision of the Ph. Eur. monograph by the European Pharmacopoeia Group 15 in 2022-23.

6. Harmonization: challenges & opportunities

This session was chaired by Richard Hill, International Alliance for Biological Standardization (IABS), and Wassana Wijagkanalan, BioNet-Asia, Thailand. The session was dedicated to the discussion of the changes achieved, and those needed in the regulatory environment to make 3Rs implementation a concrete global achievement.

6.1. What did we learn from the past?

Arnoud Akkermans, from Netherland's National Institute for Public Health and the Environment (RIVM), talked of the lessons learned on the road leading to the replacement of in vivo testing. Animal testing, Akkermans reminded, had a crucial role in vaccine development and vaccine quality control at the initial developing stage of vaccine development, starting from the late XIX century when mice took the role of biological test tubes to identify phenol preservatives. But the developments of the recent decades in the applied technologies of vaccine production and also in the way vaccines can be controlled, and their application, contributed to a shift from traditional vaccine control technologies to more focused monitoring of critical quality attributes indicating consistency of vaccine production. Akkermans retraced some of these steps through few key articles [23,33–36], through activities, symposia and workshops which brought together and fostered the exchange of information between different stakeholders, and on how these activities produced tangible changes in the European Pharmacopoeia (like the revised 5.2.14 article of the Ph. Eur. on the substitution of in vivo methods by in vitro methods for the quality control of vaccines). The process of introducing innovative quality control tests for traditional vaccines took many years: replacing classical tests with an alternative test proved complex, with results often lacking correlation between in vivo and in vitro methods, while securing acceptance of an innovative test method by regulators (and industry) often proved difficult. Akkermans noted that the current highly defined vaccine production processes, online production controls, and critical quality indicating in vitro test methods enable extensive controlling of the production process for newly produced vaccine lots. But that alone won't bring about the change. What is needed, instead, is for a thorough scientific assessment of these in vitro models, and sharing of data between industry and regulators, and sharing of information on limitations, possibilities, focusing on the critical quality attributes for characterization and stability indication, and following these steps, success is possible. Akkermans showed examples of such successes, like the possibility to waive the in vivo potency test for poliomyelitis vaccine (inactivated), releasing the vaccine through an in vitro D-antigen ELISA, the introduction of a risk assessment as cornerstone of testing strategy for the test of extraneous agents in viral vaccines for human use *in lieu* of the test on adult mice and guinea pigs, which were deleted (Ph. Eur. 2.6.16), the replacement of the test for irreversibility of the toxoid for pertussis (acellular component) vaccine (adsorbed) with a CHO cell-clustering assay for residual pertussis testing (Ph. Eur. 2.6.33), deletion of test of tetanus specific toxicity for DTaP adsorbed vaccines, and the ongoing discussion on the removal of the test for specific toxicity of diphtheria from the General Provisions sections of the Ph. Eur., all successes, Akkermans concluded, made possible by successful interaction, cooperation and data sharing on the in vitro models between industry, National Control Laboratories and regulatory authorities.

6.2. Replacement of in vivo assays, from one to one replacement to the evolution of strategy on new products and beyond

Jean-Francois Dierick's talks focused on Glaxo Smith Kline's (GSK) successes in enacting replacement for old and new products, on the internal strategy to reduce animals for quality control (QC) testing, and on how the company sees the replacement of in vivo tests for established commercial products through the consistency approach. First, Dierick described how the company tackled the replacement of in vivo potency test for four (unspecified) products. For three of them, parallel in vitro and in vivo testing were carried out during the various clinical phases, to accumulate a comprehensive set of data to demonstrate comparability (or superiority) of the in vitro assay, and the release packages included assays addressing antigen conformation, integrity and aggregation. The fourth product was an already marketed vaccine: in this case too, parallel in vivo and in vitro assays were run to create data sets demonstrating solid performance, and the replacement was performed in close collaboration with European authorities.

Dierick concentrated then on the strategy to replace in vivo potency testing for new products, which pivots on embedding replacement principles already at the beginning of development, on good characterization of the product, and on making the best use of clinical phases. Quality by Design is applied to identify critical quality attributes (CQA) and critical process parameters (CPP) supporting potency, while in vivo testing is used to confirm the identification of the critical quality attributes for potency. Relevant in vitro assays are developed that must be able to show solid performance both in assay validation and routine use, and superiority in detecting product evolution and alteration; making best use of clinical phases to support in vitro testing for the release and collect relevant data. Dierick moved then the focus to the legacy products, for which several exist (no in vitro potency assays addressing potency made at the time of clinical phases, products might be of low characterization and of high complexity) and key questions need be answered to transition to in vitro assays, examples of which being how to justify the relevance, and the comparability of an in vitro assay, how to establish a link to clinical data, and how to accumulate evidences required to justify replacement. Enablers and challenges were listed both for the removal of an in vivo assay from a control strategy, and for one-to-one replacements which is made possible when both assays measure the same CQA. In that case, key enabler would be the use of quality by design, and collaboration between manufacturers and release authorities towards harmonized solutions, while key challenges identified are product complexity – which can make it difficult to apply one in vitro assay to test potency at drug product level –, the need to have a deep understanding of the mechanisms of action of the product, difficulties in comparison and correlation between in vivo and in vitro due to the in vivo inherent variability and possible synergistic effects on the immune system, and, in the words of Dierick, the power still hold by the dogma that "in vivo assay sees it all", which can only be challenged by science-based approaches. Dierick moved then to discuss the Consistency Approach for legacy products, outlining different possibilities for replacement of in vivo potency testing at drug level: replacement by measurement of CQAs demonstrated to participate in potency (i.e.

antigenicity, antigen content, etc.) at Drug Product level, or with a mix of measurements at Drug Product and Drug Substance levels (in cases when the in vitro assay doesn't work at Drug Product level, and there is support from deep process understanding and solid data), and replacement with in vitro assays (at least one at Drug Product level) plus measurement of the CPPs that were demonstrated to ensure the delivery of a potent product. In closing the intervention, Dierick listed enablers and challenges specific to the consistency approach, listing as key enablers (1) Quality by Design principles and its repercussions on CPPs and quality attributes, (2) ICH's Q12 guideline allowing an evolution of regulatory approaches and design of control strategies, (3) sharing of knowledge between manufacturers and regulators on a same assay or product, (4) big data to transform historical data into product knowledge, while, as key challenges, (1) the need to take into account the process variability that will emerge by measuring CQAs and CPPs related to potency on legacy products, and that was not see in the in vivo testing, (2) how to establish product specifications considering that some assays were not applied on clinical batches, and (3) the fact that this approach will need to be proved in some successful applications before it can be widely applied. Still, Dierick concluded, the Consistency Approach is the best approach to date to deal with products for which attributes supporting potency are multiple, and complex [1].

6.3. Regulatory acceptance for the substitution of In vitro for In vivo vaccine potency and safety assays: science versus the fear factor

Dean Smith, Health Canada's Center for Biologics Evaluation, discussed the barriers to the development and authorization of in vitro assays for legacy vaccines, citing the European Pharmacopoeia (Ph. Eur.) General Chapter 5.2.14, as an important tool to address these barriers and drive the approval and implementation of alternative methods. Smith began the presentation illustrating a complex "catch-22" that regulators and manufacturers are locked in. As long as regulators are convinced of the superiority of in vivo methods, and are unwilling (or fearful of) changing long held (but unsupported) assumptions regarding the performance and value of animal assays, manufacturers will have no incentive to invest and develop innovative in vitro assay alternatives. However, Smith noted that Quality Control (QC) without in vivo testing for vaccines is already well established for several products. He gave the examples of two of highly effective types of human products: 1) Human Papilloma Virus (HPV) vaccines (based on recombinant viral-like particles), and 2) Meningococcal and Pneumococcal Bacterial Conjugate vaccines (based on polysaccharides conjugated to carrier proteins). With both groups of vaccines, the key quality attributes necessary to ensure the safety and efficacy profile of the products are accurately and robustly controlled using a combination physical/chemical and in vitro methods (i.e., without in vivo assays).

Smith emphasized that under cGMP, quality is built into the production process for all vaccines through in process controls and extensive consistency monitoring, which can best be achieved using more accurate, robust and rapid vitro methods, as noted above. Yet, for legacy products, such as Rabies and DPT vaccines, despite the demonstrated technical capability of manufacturers to successful implement vitro-based QC strategies, there is still a reluctance by some regulators to accept in vitro assays for these products. Smith asked, how can this be scientifically justified?

Importantly, Ph. Eur. 5.2.14 is explicit regarding the limitations of in vivo assays, when compared to appropriately in vitro alternatives. These include the inherent variability of in vivo assays, which typical lack of ICH Q2 (R1) [21] or VICH GL2 [37] validation. Such assay variability has resulted in failures of multiple international collaborative studies that required a one-to-one comparison between the vivo method (i.e., the in vivo NIH rabies potency test) and a more-consistent, validated in vitro method. These study failures were because of a lack of concordance between the two methods, due to the variability of the NIH test. Ph. Eur. 5.2.14 also recognizes that vivo methods for human vaccines "do not necessarily predict the actual responses in the target population". Smith notes that it is therefore more appropriate to consider in vivo assays as merely highly variable bioassays, with no special properties in a QC context, and several liabilities. Additionally, 5.2.14 notes that because in vivo and in vitro methods may assess the same quality attribute differently, one-to-one agreement between the two methods "is generally not scientifically justified and should not always be not always be expected".

Smith presented the assay substitution approach provided in Ph. Eur. 5.2.14, which is intended to facilitate the implementation of in vitro methods. Substitution is proposed "where a typical one-to-one assay comparison is not appropriate, unrelated to the suitability of one or more of the vitro methods". This approach came into effect in as of January 2018, through the work of the EDQM Groups 15 (human vaccines) and 15V (veterinary vaccines), which includes representatives from Health Canada and US/FDA CBER. Additional key statements in Ph. Eur. 5.2.14 note that "the inherent variability of in vivo assays can make them less suitable than appropriately designed in vitro assays for monitoring consistency of production and for assessing the potential impact of manufacturing changes. As a result, it is essential to continually challenge the scientific value and relevance of these in vivo test methods." "The use of appropriate in vitro methods … enhances the predictability of the release of safe and effective vaccine lots for use." Further considerations in 5.2.14 regarding the implementation of in vitro alternative methods include: (1) the importance of their scientific relevance, (2) that while international collaborative studies can be used to implement new methods, but this is not a requirement, and (3), in some cases, more than one in vitro method may be required to characterize a vaccine's key qualitative and quantitative attributes as measured by the existing in vivo test. Concluding, Smith commented how the new regulatory perspective described in Ph. Eur. 5.2.14 has provided additional support for industry to invest in in vitro assay development (e.g., the VAC2VAC Consortium). Additionally, it has greatly accelerated the discontinuation of longstanding animal-based tests, which are now understood to be scientifically unjustified. Examples include the recent discontinuation of the General Safety Test (GST)/Innocuity Test and the Histamine Sensitization Test (HIST) from the Ph. Eur., as well as the subsequent recognition by the WHO regarding lack of scientific justification for the GST. Many similar changes are now anticipated in the Ph. Eur [6].

6.4. 3Rs assessment of WHO guidelines and recommendations for biologics

Anthony Holmes, from the UK National Centre for the Replacement, Refinement and Reduction of Animals in Research (NC3Rs), made a presentation on a new partnership between the UK's National Centre for the Replacement, Refinement and Reduction of Animals in Research (NC3Rs) and the WHO to review the animal testing requirements described in WHO guidance documents for biologics to identify opportunities for the integration of the 3Rs, with the aim of enabling vaccines manufacturers and regulators to apply the latest non-animal testing approaches and strategies to support faster access to cheaper vaccines by the global communities who need them most urgently. Holmes explained that no systematic review of established WHO guidelines for 3Rs had ever been made before, so there is no definite information on the amount of animal testing recommended or required by them for the manufacture and batch release testing of biologics. Due to this, non-animal methods already validated and approved within some regulatory jurisdictions are not yet included in the WHO recommendations; the opposite is also true, with non-animal methods present in the recommendations not being implemented by some regulatory authorities.

Holmes noted that there is a global movement – including cosmetics, pharmaceuticals and chemicals – for 3Rs driven by scientific, ethical, regulatory and economical rationales. To date there was little guidance on how to ensure a global harmonization of 3Rs tests and methods as they become available for biologics development. This results in missed

opportunities to embed the latest technologies into the development pipeline of biologics, leaving expensive and often poorly predictive animal tests in use. It is important, therefore, for the WHO to better understand the extent of animal testing requirements in their guidelines and recommendations and to assess where there are already opportunities to apply non-animal testing approaches.

The project envisioned in the NC3Rs and WHO's cooperation, Holmes explained, will be articulated to address three major areas: (1) what is the extent of animal testing included within the collection of WHO recommendations for biologics and, of those, are there alternative methods already validated and approved elsewhere that should be included in the recommendations; (2) evaluate whether a WHO guideline for the adoption of 3Rs principles into the quality control and lot release of licensed vaccines could be useful for harmonization of non-animal methods and for guidance to WHO member states; (3) analysis of the barriers hindering the adoption of 3Rs principles.

Holmes concluded extending an invitation to collaborate in the project, as a project so large and complex relies on the engagement of the global biologics' community.

6.5. Statements from various international organizations

Eriko Terao (EDQM) did not present the EDQM which activities and endeavors for the 3Rs were presented earlier during the meeting but highlighted 3 main challenges for the 3Rs: coordinated actions, data supported candidate methods and availability of samples & reagents for validation studies. She stressed the importance of coordinated actions and of a real global involvement of stakeholders in the dialogue, not only to increase confidence in harmonized alternative methods, but also to ensure wide implementation by ensuring applicability to a wide range of products and method accessibility.

Jim Webster (Ruakura Research Centre, Hamilton, New Zealand) reported on the World Organization for Animal Health's (OIE) effort in promoting and guiding animal welfare, good animal husbandry, good housing practices, and 3Rs (all contained in section 7 of the Terrestrial Animal Health Code) [38]. Webster mentioned also the organization's Global Animal Welfare Strategy to promote animal welfare and 3Rs, adopted by 145 countries in 2017, members of the organization, based on development of animal welfare standards, capacity building and education, communication between governments, organizations and public, and implementation of animal welfare standards and policies [39].

Robin Levis (FDA, U.S.A) introduced the United States' Center for Biologics Evaluation and Research (CBER) and the role of its scientists in supporting regulatory policy and their many collaborations in the last years, in particular with VAC2VAC. Levis traced an overview of the projects supporting alternative methods that were carried out in CBER, citing the deletion of the General Safety test (Revocation of general safety test regulations that are duplicative of requirements in biologics license applications, FDA Federal Register, 07/02/2015), refinement of the neurovirulence test in monkeys (MNVT) for the mumps vaccine [40].

Laura Viviani presented the efforts of the Humane Society International (HSI) and the activities initiated – promotion of global regulatory alignment for the deletion of the general safety test [3] and the use of non-animal based methods to replace the rabbit pyrogenicity test – through the engagement of key regulatory and industry stakeholders of various countries. Ms. Viviani also presented the project of a dedicated database for non-animal based methods, their implementation status and the current existing collaboration opportunities.

Speaking on behalf of the Bill & Melinda Gates Foundation (the foundation), **Gautam Sanyal** gave an overview of the foundation's strong commitment to delivery of life saving vaccines to the developing world at an affordable cost. The Foundation actively supports efforts to (a) accelerate development timelines, (b) reduce cost of manufacturing, (c) secure supply for GAVI, the Global Alliance for Vaccines and Immunizations, and (d) ensure appropriate product profiles, including new combinations and novel vaccine formulations, as needed for different geographies. The foundation's commitment to 3Rs is driven by several practical considerations, including: low precision and high variability of in vivo assays, long turn-around time, high cost, difficulty in sourcing and maintaining animals, and different requirements from different regulatory bodies. In a collaborative initiative with the National Institute for Innovation in Manufacturing of Biopharmaceuticals (NIIMBL) [41], the foundation has established a Global Health Fund to support development of in vitro assays aimed at replacing or reducing use of animals in potency and safety testing. Priority areas include replacement of monkey neurovirulence test, in vivo adventitious viral agents tests, the Kendrick test, etc. NIIMBL will also be looking for novel technologies that may potentially remediate manufacturing gaps in Developing Countries Vaccine Manufacturing Network (DCVMN). The foundation welcomes collaboration opportunities with private, public, governmental and regulatory groups across the globe in accelerating data driven reduction of animal testing.

Gautam Sanyal also spoke on behalf of the Coalition for Epidemic Preparedness and Innovations (CEPI), an innovative global partnership between public, private, philanthropic, and civil society organizations. CEPI's mission is to accelerate development of vaccines against emerging infectious diseases and to enable equitable and timely access to these vaccines for at risk populations, regardless of their ability to pay. The goal is to end an outbreak or curtail an epidemic. CEPI funds, coordinates and actively engages with partners in this process and builds capabilities as needed for rapid response to new or anticipated epidemic threats.

This presentation described CEPI's support of platform technologies in vaccine development to expedite delivery of "Just in time" vaccines. Shortly after this conference and as SARS-CoV-2 emerged, CEPI substantially expanded its investments in such platform technologies to accelerate the development of vaccines against this virus. Vaccine development efforts must be complemented with high quality in vitro or analytical assays that are precise, accurate, reproducible, sensitive and have short turn-around time. Analytical characterization based on critical quality attributes of vaccines are key to ensuring consistency and comparability between batches used in different clinical development phases, which often require transition from relatively small to large-scale manufacturing processes. Such analytical bridging is especially important in rapid response situations as it can eliminate the need for clinical comparability studies between batches, thereby reducing the development cost and timeline for regulatory approval. CEPI recognizes that pre-clinical safety and immunogenicity research often requires the use of animals, although results may not necessarily translate to response in humans. For CEPI-sponsored projects, CEPI will support animal studies if the potential health benefits are compelling, appropriate welfare standards are met, and where there are no alternatives. CEPI adheres to and requires its partners to fully comply with 3Rs as mandated by UK National Centre for the Replacement, Refinement, and Reduction of animals in research.

Sunil Goel described DCVMN's commitment to facilitate the implementation of 3Rs among its members. DCVMN is a voluntary, non-governmental, nonpartisan, not-for-profit, public health driven alliance of vaccine manufacturers, research and policy organizations from all over the world, representing 43 manufacturers from 14 countries. The current interest of the 3Rs Working Group from DCVMN is focused on DT-containing vaccines, whole-cell pertussis and rabies [42].

6.6. Collaboration and communication of regulatory bodies and industry: panel discussion

A panel discussion was dedicated to the collaboration and communication between regulatory bodies and industry, and it was kickstarted by two questions: (1) what the regulatory expectations are when a company wishes to change a testing method? and (2) what is the

effective interaction between industry and regulators?

Robin Levis cited the human rabies vaccine as an important showcase of the complexity of implementing change from the perspective of regulatory authorities. While a new method or assay must be able to secure the quality/safety/potency of a product, other guarantees must also be in place, including for example the proved availability of needed reagents for both manufacturers and regulatory, and an effective experience of manufacturers in the implementation of the consistency approach.

Richard Hill (International Alliance for Biological Standardization (IABS), U.S.A) commented on the need to encourage a change in the regulators' approach, which is often strongly favoring a conservative perspective based on old codified methodologies. As an example of the possible complexities involved in transitioning away from in vivo testing, Hill brought up the complex case of veterinary rabies vaccines [31] – a multi-species product that is tested and licensed separately for many species, each of which needs to be tested to validate efficacy. Veterinary vaccines comprise over 200 different antigens which are available in many combinations. An important scientific effort will be needed to define the best alternative testing strategy to address this complex issue, which can be simplified only through engagement of regulators and the biologics industry.

Guang Gao (PATH, China) reported personal experience of China's activities, noting the existence of delays in the application of 3Rs in vaccines due to some historical reasons related to product safety issues. Gao commented on the a very recently (June 2019) introduced, and very severe, new law on vaccines administration, which mandates stricter vaccine management and threatens grave penalties to ensure vaccine safety. This legislation, Gao commented, put a significant burden on regulators, which, together with the life-time consequences it threatens in case of emerging safety issues, could render enacting change a longer and more complex process, which will require engaging the regulators in dialogue as early as possible.

Sunil Goel voiced the effect of the absence of harmonized requirements for the testing strategies has on manufacturers, that is forcing them to continue to use very variable required tests instead of in vitro assays already established in the product's development (e.g. SRID, ELISA) to monitor the product before the release.

William McCauley (Animal Health Institute (AHI), U.S.A.) expanded on Goel's critical report, stating that the lack of harmonized difficulties also affects the U.S. veterinary vaccine manufacturers, and informed on an initiative of AHI to petition the US Department of Agriculture, expressing support for the Department's activities, but also to openly ask for more concrete actions.

Dean Smith commented on the perception that some manufacturers seem to have regarding the process of securing approval from regulators for a new method. Smith noted that that there is nothing mysterious about the process, which is in well and publicly articulated in ICH compliant regulatory environments. However, speaking as a regulator, Smith also made it clear, that in his opinion, there is still work to be done to overcome the misplaced value awarded to animal assays in a quality control context with some regulators, and their suspicion/fear of novel methods, even in Europe and North America is still an issue. Smith's comments raised a question by **Robert Sitrin**, who asked about what could concretely be done to change the current paradigm, for example in countries like India and in China?

Sunil Goel reported the current situation in India, and explained that a constant dialogue between the Indian Pharmacopoeia Committee, manufacturers and other organizations is in place and that 3Rs are getting into the agenda of the various Indian stakeholders.

Li Shi commenting on China, remarked that the country is inclined to follow the example of other key regulatory agencies and that if the US FDA were to approve an alternate to the NIH test, Chinese regulators would be under less pressure to stay with the status quo. Li Shi also noted that it is important for organizations like WHO and the Bill & Melinda Gates Foundations to be engaged and active to accelerate change.

Jean-Michel Chapsal put forth a suggestion that engaging more manufacturers from China in international collaborative studies ought to facilitate an exchange of information and data that could lead to regulatory changes.

Gautam Sanyal brought back the discussion on U.S. and Canada, asking what is effectively holding the respective regulators from accepting alternative methods.

Both **Robin Levis** and **Dean Smith** confirmed that some regulators showed openness to such changes for a number of years. However, manufacturers appear to be hesitant to make requests and submit the relevant data for assays that they propose as fit for purpose.

Arnoud Akkermans (RIVM) enquired on what the influence of EDQM working groups of experts on vaccines had in other countries.

Sunil Goel following indirectly Akkermans question commented on how manufacturers, in the presence of a too varied regulatory landscape (in which too many regulatory scenarios, including the WHO requirements, are to be considered), needs to take decisions based on the most efficient way to release its products.

Guang Gao brought the focus again on China, sharing information on the new version of the Chinese Pharmacopoeia expected in 2020 where no changes are expected in the vaccines' chapters, so she asked how Europe and U.S. could concretely help to rekindle discussion on alternative methods in China.

Marlies Halder commented that China is open to alternative methods and, in particular, for cosmetics testing. For example, the National Medical Product Administration (NMPA) approved two non-animal tests for the skin sensitization. China is observer of the International Cooperation on Alternative Test Methods, ICATM.

Supaporn Phumiamorn informed that also Thailand is interested in implementing alternative methods, surmising that a working strategy could be based on increased dialogue and alignment between the National Regulatory Authority and the National Control Laboratory (providing with scientific data).

The panelists were finally asked to conclude the session with key statements to summarize their position and the discussion. This resulted in a consensus on the fact that regulatory alignment and harmonization should be a priority from a global perspective, which should be enacted through the active participation of each stakeholder (manufacturers, regulatory authorities, international organizations and charities), common guidelines, and continuous collaboration.

7. Workshops

After the panel, two distinct, parallel workshops – on *Validation, acceptance, implementation & harmonization*, and on *Needs of emerging economies* – were organized to focus discussion on the specific themes, elicit direct contributions and foster exchanges between the participants, explore the possible consensus on different issues, and propose future actions.

7.1. Validation, acceptance, implementation & harmonization

Moderated by: Laura Viviani, Humane Society International (HSI), Switzerland, Marlies Halder, European Commission Joint Research Centre, EURL ECVAM, Italy, Jim Webster, OIE Collaborating Center, New Zealand, Hilde Depraetere, European Vaccine Initiative (EVI), Germany, Denis Lambrigts, GlaxoSmithKline, Belgium.

The aim of the workshop was discussion on the concrete meaning and the various aspects of validation, acceptance, implementation and harmonization of alternative methods.

The workshop moderators presented definitions of the four terms and invited participants to discuss the relevant drivers and barriers for each one; to foster participation, participants interacted through a live survey platform.

Validation: Defined, in agreement with the participants, as a process which should demonstrate that *a method is relevant and reliable for the*

given purpose. Further details were introduced about validation studies – defined as either as single-lab validation study for a given product (e.g. according ICH/VICH Validation guidelines, ICH Q2 R1 [22],VICH GL1, GL2 [37]) or as multi-lab validation study (e.g. ring trial or collaborative study to establish a new method to be used for a class of products). European Pharmacopoeia's new 5.2.14 chapter *Substitution of* in vivo *methods by* in vitro *methods for the quality control of vaccines* was mentioned since it outlines how the barrier of one-to-one replacement could be overcome, in particular, in light of the high variability of animal tests and the inherent fundamental differences of in vivo and in vitro tests.

Participants – all familiar with the concept and the dynamics of validation – expressed interest in participating to collaborative studies and in learning more about chapter 5.2.14 of the European Pharmacopoeia.

Participants considered the following as main barriers to the validation of alternative methods:

- risk averse attitude of regulators;
- lack of materials and/or reagents;
- how to design validation studies;
- business cost to plan with uncertain regulatory acceptance;
- lack of regulatory harmonization, and lack of innovation in investing in new approaches/products.

Their suggestion to overcome some of those difficulties were the following:

- more training, case studies and guidelines,
- but also, to be more proactive in creating or participating to international collaborative studies (only few of the workshop participants had participated in at least one collaborative study),
- and it was proposed creating a reagent bank, for consideration of future international conferences.

Acceptance: the group agreed upon that acceptance implies that a regulator considers a method appropriate to be used in a regulatory context (e.g. batch release testing).

Participants listed the following main barriers to the acceptance of 3Rs methods:

- the majority of alternative methods are not included in compendial text, i.e. not officially part of pharmacopoeias or regulatory requirements and participants identified this as the most important hurdle;
- manufacturers considered information on data requirements about alternative methods and documentation to be provided to the regulators as insufficient, and, therefore, the dialogue is perceived as inefficient.

Regarding suggestions on incentives and support regulators could provide to manufacturers to encourage them to invest in non-animal testing, the participants listed various possibilities:

- making license variations faster, easier, and less expensive.
- the introduction of a standardized dialogue between manufacturers and regulators (along the lines of the scientific advice procedure [43] introduced by European Medicines Agency (EMA)).
- national and international agreement on the type of data requested for the submission.
- rendering successful cases available for others to study/follow.
- increasing collaborations between manufacturers to approach regulators with a common agenda and more data.

Participants were then asked what actions a manufacturer could take to increase the acceptance of non-animal based methods. The answers once more confirmed that collaboration with other manufacturers, increase of scientific data sharing (validation data, trend/analysis of historical and stability data), openness with regulators, and early development of new methods, could all, if implemented, contribute and enhance the overall acceptance of 3Rs.

Implementation: agreed upon that implementation refers to the use of a validated and accepted method by a manufacturer or control authority for the quality control of a given product.

Most of the participants confirmed that 3Rs are routinely implemented within their institutions, although the process of securing their implementation usually proved challenging due to the lack of global harmonization of the testing requirements, technical difficulties in the product-specific validation, and its cost.

The following major difficulties related to implementation were pointed out by the participants:

- lack of interest of some regulatory authorities in alternative methods (still the case in some countries, although positive and encouraging examples were presented during the conference);
- the not-consistent and not-constant availability of reagents;
- the lack of interest from the manufacturers' management in investing in alternative methods (which can be the case both for manufacturers in developing countries and for multinational companies);
- and the lack of scientific and technical expertise.

Global Harmonization: participants were invited to discuss and define global harmonization of testing requirements through concrete cases, such as the deletion of the general safety test for human vaccines that was advanced by Europe, USA, Canada and recently recommended by WHO, and the international collaborative studies to replace the NIH test for Rabies (EDQM-BSP148), and the Vero cell based assays to replace tests on mice to determine toxicity of toxin/toxoid and antigenicity of *C. septicum* vaccines (EDQM-BSP130).The majority of the participants agreed on the key role international collaborative studies play as an instrument to promote harmonization.

- The discussion also focused on whether a universal assay would be preferable to product specific assays, and which of the two approaches would be more efficient in facilitating harmonization. No agreed position was reached.
- To promote harmonization, participants recommended the creation of a common process for submitting variations and the implementation of mutual acceptance of release data across regions.
- The participants were all interested and supportive of NC3Rs-WHO's project on reviewing and implementing alternative methods in the WHO requirements for vaccines and biologicals (which had been presented by A. Holmes on the 1st day of the conference), as a first crucial step to laying the basis for global harmonization and acting as an example for the stakeholders from the developing economies.

7.2. Needs of emerging economies (training, reagents, materials, etc.)

Moderated by: Sunil Goel, the Serum Institute of India Pvt Ltd., India, Wassana Wijagkanalan, Bio-Net Asia, Thailand, Yeowon Sohn, Seoul National University.

The working group focused on understanding the difficulties of the implementation of 3Rs in the developing economies, with participants clearly stating the need for support to move ahead for implementation of 3Rs in their respective countries.

The group started the activity by laying out the situation about the 3Rs acceptance in some of the represented countries, with the general safety test (or abnormal toxicity test, or innocuity test) and some potency tests taken as examples, and then introducing country specific conditions.

- In Thailand, the National Control Laboratory welcomes in general the 3Rs approach and, specifically, the deletion of both the general safety test and of the pyrogenicity test is under discussion, based on the review of historical data; in addition, the single dilution assay for the acellular pertussis potency test is being considered.
- In India, the Indian Pharmacopoeia Committee has been allowing waivers for the general safety test [24] even before the recent WHO endorsement for its deletion [8]. To obtain the waiver, manufacturers have to submit consistency data (3 batches during pre-licensure), and in case of adverse events following immunization (AEFI) has the right to perform investigations on the safety of the product. The Indian regulatory authorities also accept the single dilution assay for the potency tests, as well as the specific toxicity for D and T Components with single dilution assay. Close communication and the regular meetings between manufacturers NCLs were reported. In Japan, ATT is still a requirement, but there is the interest in its removal, and there is a gradual implementation of 3Rs for some vaccines (e.g. Hepatitis B vaccine - (HBV)) based on data review in the framework of the consistency approach.
- In Indonesia, for the HBV the replacement of the in vivo potency test with the in vitro method was accepted by the national regulatory authorities and a change request to delete the general safety test was put forth by the local manufacturer.

After the exchange of information on the specific local conditions, participants discussed the key hurdles hindering transition to 3Rs in emerging economies, agreeing on the following list:

- lack of harmonization among pharmacopoeias, including the WHO requirements;
- different speed in acceptance and implementation of 3Rs;
- perceived lack of effective communication between manufacturers and regulators, and both suffer to resistance to the change;
- limited resources (equipment, funds, materials, personnel, knowledge on new method);
- lack of concrete case studies and scientific evidence that could be gained with investment on alternative methods in the early stage of development of the product;
- accessibility to IP protected methods, materials, reagents.

Discussing the hurdles, the group proposed the following activities as solutions to the above-mentioned difficulties:

- organization of a forum or conference dedicated to the harmonization of guidelines and/or pharmacopoeia on a regular base;
- increase of collaboration and effective communication (even at the early stage of a project) among stakeholders like NCL, industry, academia and global initiatives;
- inclusion of developing economies' stakeholders in collaborative study that could provide and serve as training and to improve their global network, knowledge, experience and also sharing of difficulties, problems but also solutions;
  - o allowing the possibility to have common protocol(s) and procedures, standards,
  - o favoring the interaction with large manufacturing companies for advising the new methods to other companies;
- definition of roadmaps for 3Rs implementation based sufficient scientific evidence;
- in interacting with WHO, establish small group to discuss about proposals for new methods;
- facilitation of access to methods, data (publication), critical reagents/reference standard;
- increase of the learning opportunities from other sectors;
- investments in communication, through IABS meetings for example, about WHO/pharmacopoeia changes toward the 3Rs implementation;
- promotion of the involvement of other NRAs when WHO or EU agencies begin the implementation of alternative methods.

In closing, the group agreed on additional discussion points that should be considered for future conferences, such as.

- reinforcing the concept and related evidences that in-vitro tests are not an addition to in-vivo tests, but valid replacements.
- the importance of common quality standards, ands of common methods with the same reference standards to remove inconsistency.
- application of 3Rs for stability testing (e.g. degradation testing to simulate the vaccine shelf life, stability indicating parameters).

8. Conclusion

The closing remarks to the congress were tasked to **Coenraad Hendriksen**, who began with sharing with the audience a prediction: that 25 years from now, animals will not be used anymore for the quality control of vaccines.

In his view, we are at a time when progress practically halted on animal methods, with all their issues with relevance and reproducibility unsolved, while non-animal models and techniques keep being developed and refined, proving fruitful and efficacious. Such a needed transition would, in his opinion, ferry vaccine quality control from its current empirical nature – where batches either pass or fail testing – to a more scientific approach that finally delves deeper into *why a batch is not doing what it should do.* Such transformation would free the sector from one of its most sedimented axioms, that a vaccine batch is to be considered a *unicum*, embracing a new perspective in which a batch is seen as *one* of a continuum in a series originating from the same master seed lot, which is the perspective embodied in the *consistency approach*.

But, he warned, this transformation will not happen without difficulty. A change of attitude in the community will be required, one that will make it possible to bring the role of animal testing in the context of facts, and away from the sedimented context of beliefs and tradition in which it has historically been enveloped. There's substantial reason to believe that vaccine quality was secured not thanks to animal testing, but because of our ability to consistently produce vaccine batches of high quality, but for this to become ingrained in the vaccine community much effort will be needed: routine, and long termed acceptance of the status quo desensitized the professionals working in the field to a state of conservatism, and, at times, even of ignorance of alternative methods.

And professionals must also be convinced that 3Rs cannot be marginalized merely as a question of ethics pertaining animal welfare. On the contrary, 3Rs represent first and foremost *better science*, overcoming the many issues (like poor relevance and reproducibility) of animal tests while also securing improved animal welfare.

Hendriksen proceeded then to list a series of remarkable changes in the field starting from the growing interest and commitment to 3Rs from many organizations; like DCVMN that set steps to start up activities in this direction; the global progress being made in the rabies project, with the involvement of NRA's, industries and NGO's; the Bill & Melinda Gates Foundation supports in this field; the EDQM that continues to be on the forefront with its Biological Standardisation Program; several vaccine manufacturers set up in-house centers to promote the 3Rs; and like the new initiatives from WHO that should started soon.

Another important project is VAC2VAC, which is a unique effort because it has a clear focus on replacement, it is based on a paradigm shift in vaccine quality control based on the principle of consistency testing and it involves all relevant stakeholders: manufacturers, academia, national control laboratories and regulatory authorities.

Other important progresses to highlights is how the vaccine quality control became an interdisciplinary collaboration, for example, in the field of bio-informatics, analytical techniques and in vitro methods.

In the conclusion of his speech, Hendriksen confirmed what was the *leitmotiv* of the conference: the need of collaboration between all the

stakeholders. Quoting Albert Einstein, that « *You can never solve a problem on the level on which it was created*», he proposed a new paradigm, based on the axiom that a vaccine batch is not unique, but one of several batches being produced of the same master seed lot. Such new paradigm would run in parallel to the development of new innovative technologies, which are the bases of the new paradigm. This change could take place under the aegis of what he defined as the 3Cs: *Commitment*, with every stakeholder accepting responsibility for 3Rs, *Common Sense*, as an appeal to being realistic on the prejudices affecting the field, with the view of overcoming them, and *Communication & Collaboration*, that is, defusing the "catch 22" situation that locks manufacturers and regulators in a difficult equilibrium, where manufacturers don't dare innovate for risk of rejection, and regulators unwelcoming of alternative methods as they have too little data to base their decision upon, something that might be addressed for example through a "safe harbour" mechanism, but also to include developing economies stakeholders in a constant dialogue and in concrete projects. Hendriksen closed the intervention with a quote by Mark Twain meant as an encouragement to look beyond the level of the problem, to a new level that can offer solutions: «They did not know it was impossible, so they did it».

9. A way forward

The conference *Animal testing for vaccines - Implementing Replacement, Reduction and Refinement: Challenges and Priorities* proved successful in engaging key global international stakeholders, including representatives from low- and middle-income countries. Many of the challenges faced the last 10 years by industry, regulatory authorities, public research institutions and not for profit organization in the implementation of 3Rs in the vaccines' field were discussed and acknowledged. But the attendees also reported on significant progresses being made, and on new multi-stakeholders'collaborations initiated that are producing meaningful results with regards to the development, validation and implementation of 3Rs opportunities.

The participants agreed that a way forward in the sector must rest on the cornerstone of a constant and continuous dialogue between the stakeholders, through more frequent conferences and meetings, more educational opportunities, more and better communication on the advancements in the field (including successful case studies), and an increase in the engagement in new collaboration opportunities of stakeholders from LMICs industry and regulatory authorities. Agreement also emerged on the welcoming of international organizations and non-for-profit organizations as facilitators and promoters of those initiatives.

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Disclaimer

This manuscript and the views expressed herein are those of the authors and do not necessarily reflect the views or policies of the various regulatory authorities and organizations.

Declaration of competing interest

None.

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