Developing Countries Vaccine Manufacturers Network

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Survey results: Animal use and 3Rs Interest within DCVMN

The aim of this report is to present the findings of the survey on Animal Use and 3Rs Interest among DCVMN members.

This survey was developed to support the planification of future DCVMN activities aimed at promoting to Reduce, Replace and Refine animal testing (3Rs principles) in vaccine production and release. The information collected through the survey included:

- Current status of animal use among DCVMN Members;
- Members' ongoing projects to implement 3Rs;
- Interest of the Members towards the implementation of 3Rs opportunities.

The survey was circulated to all 41 DCVMN members on November 7th, 2020 and was held open for one month. DCVMN received 28 replies, with the highest response rate from the South East Asia region (based on the WHO Regions).

WHO Region	DCVMN respondents	Percentage (%)
Americas	2	7
Eastern Mediterranean and African	2	7
Western Pacific	10	36
South East Asia	14	50
Total	28	100

Table 1. Geographical distribution of DCVMN respondents.

The survey focused on vaccines produced by the DCVMN members: BCG, Cholera, Diphtheria-Tetanus, Enterovirus 71, Influenza, Haemophilus Influenza type B, Hepatitis A, Hepatitis B, Hepatitis E, HPV, IPV, Japanese Encephalitis, Meningitis, MMR, OPV, Pertussis, Pneumo 23, Rabies, Rotavirus, Typhoid, Varicella, Yellow Fever.

Production and in-process control testing

The survey's first questions enquired about animal use during the first step in the manufacturing process as well as in-process control testing.

Vaccine	Production	
	Species	Number
Diphtheria-Tetanus	Mice/Tetanus	184
	Guinea pigs/Diphtheria	144
Pertussis	Mice	Up to 210
Haemophilus Influenza type B	Guinea pigs	2
	Mice	5
OPV	Monkeys	10/20
Rabies	Mice	400
Japanese Encephalitis	Mice	50
MMR	Chicks	10
	Embryonated eggs	35
	Rabbits	5
Flu	Embryonated eggs	500’000 up to 600’000
Yellow Fever	Chicks	10

Table 2. Use of animals in vaccines’ production, as reported by DCVMN respondents.

Vaccine	In process		
	Test	Species	Number
Diphtheria-Tetanus	Diphtheria Detoxification test	Guinea pigs	1
	Diphtheria Specific toxicity	Guinea pigs	5
	Diphtheria irreversibility	Guinea pigs	10
Pertussis	Mouse Weight test	Mice	20

Table 3. Use of animals in vaccines' in-process control testing, as reported by DCVMN respondents.

Based on the replies, some members reported that products like DTP, Hib, OPV, Rabies, Flu, MMR, Japanese Encephalitis and Yellow Fever require the use of animals in their first manufacturing step (Table 2). When evaluating the quantity of animal used the results of the survey indicate that the most significant use of animals is for flu vaccines (embryonated eggs). The results also indicate that 6 species of animals are used for all surveyed vaccines with monkeys being exclusively used in the manufacturing of OPV vaccines.

For in-process control testing, animals are still used, although with limited numbers for DTP vaccines (Table 3).

Potency and Safety Testing

Potency Testing

From the 22 vaccines the DCVMN queried, in vivo potency testing is performed for 13 vaccines. Animals used for potency testing include mice, guinea pigs, rats and rabbits. The survey results showed that in vivo potency tests are *not* conducted on OPV, Rotavirus, MMR, Typhoid, BCG, Meningitis, Pneumo 23, Varicella and Yellow fever.

Safety testing

Among the respondents, Rotavirus and Cholera vaccines are the only products that do *not* undergo safety testing (only exception: Cholera residual toxin activity). Animals used to conduct safety tests include mice, guinea pigs and monkeys.

Summary results are shown in Table 4.

Vaccine	Potency Test	Safety Test
Diphtheria-Tetanus	x	x
Pertussis	x	x
Haemophilus Influenza type B	x	x
Hepatitis A	x	x
Hepatitis B	x	x
Hepatitis E	x	x
OPV		x
IPV	x	x
Rabies	x	x
Japanese Encephalitis	x	x
Rotavirus		
MMR		x
Typhoid		x
Cholera	x	
BCG		x
Enterovirus 71	x	x
Flu	x	x
Meningitis		x
Pneumo 23		x
Varicella		x
Yellow Fever		x
HPV	x	x

Table 4. Use of animals in vaccines' potency and safety tests.

Table 5 and 6 show product-specific potency and safety tests, species of animals used and the average number of animals used per batch.

Vaccine	Potency Test	Species	Average number of animals
Diphtheria-Tetanus	In vivo challenge assay	Mice	142
		Guinea pigs	132
	Serological assay	Mice	100
		Guinea pigs	29
	Cell assay	Mice	72
Pertussis	In vivo challenge assay	Mice	176
	Serological assay	Mice	80
Haemophilus Influenza type B	Serological assay	Mice	40
Hepatitis A	Serological assay	Mice	105
Hepatitis B	In vivo challenge assay	Mice	160
	Serological assay	Mice	80
Hepatitis E	Serological assay	Mice	50
IPV	In vivo challenge assay	Rats	40
Rabies	In vivo challenge assay	Mice	149
Japanese Encephalitis	Serological assay	Mice	37
Cholera	Serological assay	Rabbits	4
Enterovirus 71	In vivo challenge assay	Mice	40
HPV	Serological assay	Mice	70
	In-vivo relative potency test	Mice	130

Table 5. Animals and species used per vaccine, per type of potency test.

When examining the average number of animals used per vaccine, mice are the predominant animal model of choice for potency testing, followed by guinea pigs. The

highest average use of mice (176) for potency testing is for pertussis vaccines, and the highest average use of guinea pigs (132) for potency testing is for DT vaccines.

For some products listed in Table 5, **3Rs opportunities can be considered**, particularly DTP-containing vaccines (e.g., single dilution assays and serology). Similarly, for Hepatitis A and B, there is the possibility to use in vitro assays. Besides, for IPV, there is the possibility to waive the in vivo assay based on the validation of the D-antigen assay, and on the consistency of the production. For HPV, replacement opportunities include using an ELISA or the radio-immunoassay (RIA), which uses monoclonal antibodies specific for protection-inducing epitopes of the L1 protein. For rabies vaccines, an international collaborative study is ongoing to validate an ELISA to replace the intracerebral challenge (c.f. EDQM BSP 148).

Vaccine	Safety Test	Species	Average number of animals
Diphtheria-Tetanus	Abnormal Toxicity Test	Mice & guinea pigs	7 & 3
	Specific Toxicity Test	Guinea pigs	5
	Irreversibility test	Guinea pigs	15
Pertussis	Specific Toxicity Test	Mice	24
	Abnormal Toxicity Test	Mice & guinea pigs	8 & 4
Haemophilus Influenza type B	Abnormal Toxicity Test	Mice & guinea pigs	6 & 2
	Specific Toxicity Test	Guinea pigs	5
Hepatitis A	Abnormal Toxicity Test	Mice & guinea pigs	6 & 3
Hepatitis B	Abnormal Toxicity Test	Mice & guinea pigs	5 & 2
Hepatitis E	Abnormal Toxicity Test	Mice & guinea pigs	10 & 3
OPV	Abnormal Toxicity Test	Mice & guinea pigs	5 & 2
	Monkey Neurovirulence Test	Monkeys	26 (type 1), 38 (type 3)
IPV	Abnormal Toxicity Test	Mice & guinea pigs	8 & 3

Rabies	Abnormal Toxicity Test	Mice & guinea pigs	6 & 2
Japanese Encephalitis	Abnormal Toxicity Test	Mice & guinea pigs	5 & 2
MMR	Monkey Neurovirulence Test	Monkeys	14
Typhoid	Abnormal Toxicity Test	Mice & guinea pigs	6 & 2
BCG	Abnormal Toxicity Test	Guinea pigs	5
Enterovirus 71	Abnormal Toxicity Test	Mice & guinea pigs	8 & 3
Flu	Virus Inactivation test	Eggs	100
	Virus Inactivation test	Mice	10
	Abnormal Toxicity Test	Mice & guinea pigs	5 & 2
Meningitis	Abnormal Toxicity Test	Mice & guinea pigs	5 & 2
Pneumo 23	Abnormal Toxicity Test	Mice & guinea pigs	10 & 4
Varicella	Abnormal Toxicity Test	Mice & guinea pigs	8 & 3
Yellow Fever	Monkey Neurovirulence Test	Monkeys	20
HPV	Abnormal Toxicity Test	Mice & guinea pigs	15 & 4

Table 6. Animal and species used per vaccine, per type of safety test.

Mice and guinea pigs are primarily used for Abnormal Toxicity or specific toxicity testing in relatively low numbers. There is a significant use of monkeys for OPC, MMR and Yellow Fever vaccines. For the listed safety tests, there have also been recent developments related to 3Rs: the abnormal toxicity test is no longer requested by WHO (c.f. *Dianliang Lei, et al., 2020)* and has been removed by Europe, USA, Canada, India, Brazil, Argentina, Cuba, and South Africa (c.f. *Laura Viviani et al., 2020*). Other countries are also considering waiving it or allowing product-specific waivers (e.g., Japan). The specific toxicity for DT could be removed as well based on the validated detoxification

process, which is recommended in Europe. Lastly, international efforts have been initiated to replace the polio vaccine animal-based neurovirulence test with next generation sequencing approach.

Pyrogenicity Testing

The rabbit pyrogenicity testing (RPT) was assessed in a separate section of the survey. 15 members out of 23 reported conducting pyrogenicity testing for their products. Tests on rabbits are used for 11 vaccines, while the bacterial endotoxin test (BET or LAL) is used to test 17 vaccines, as shown on Table 7.

RPT Tested Vaccines	BET/LAL Tested vaccines	
Rabies	Rabies	Typhoid
Hepatitis B	HPV	JEV
JE	Influenza	HiB
Meningococcal polysaccharide Vaccine	Dengue	DT
Hepatitis E	Hep A	EV71
Typhoid	Hep B	COVID-19
HPV 16/18	Hepatitis (general)	
Hepatitis A	Hep E	
Hib	MMR	
23-valent PPV	Meningococcal	
13-valent PCV	Pneumococcal	

Table 7. Vaccines tested with RPT and with BET or LAL

Of the 15 laboratories conducting the pyrogenicity test using both LAL and RPT methods, only 3 manufacturers are *not* aware that the Monocyte Activation Test (MAT) could replace the test on rabbits. In addition, only 4 members currently employing LAL

were *not* aware that the synthetic recombinant Factor C technique, which enables highly specific endotoxin testing (due to no Horseshoe-crab blood interference), could be used as a replacement to LAL.

In the survey, the interest in replacing RTP is also explored: of the 15 manufacturers who perform pyrogenicity testing;12 manufacturers indicated they would be interested in learning more through the DCVMN, 1 manufacturer stated interest in the rFC assays and only 1 manufacturer indicated *no* interest in 3Rs approaches.

Regional implementation of 3Rs

Finally, the survey aims to inquire from the 28 surveyed members whether 3Rs initiatives are already implemented. At the forefront of this effort, the survey identifies members in South East Asia, that not only minimize animal testing but also employ new approaches in pyrogenicity, batch potency and safety testing (Table 8).

Vaccine	WHO Region	Type of R	Type of test
Diphtheria-Tetanus	Region of the Americas	Replacement	Potency by ToBI test for DT fraction
	South-East Asia Region	Reduction	One dilution serological assay for potency, ATT removed
Pertussis	Region of the Americas	Refinement	Challenge under anesthesia
	South-East Asia Region	Refinement and Reduction	PSPT (ongoing)
		Reduction	Single dilution assay
Hepatitis B	Region of the Americas	Replacement	Potency test by ELISA
	South-East Asia Region	Replacement	For Antigen content determination Use ELISA method
		Refinement	Pyrogenicity with BET
		Removal	Removal of Abnormal toxicity test

OPV	South-East Asia Region	Refinement and Reduction	Next Generation Sequencing, Maprec, transgenic mice
	Western Pacific Region	Replacement	Cell culture to reduce monkey use production; new method for trypsinization to increase available cells.
		Replacement	Next generation sequencing to replace the polio virus neurovirulence test
Rabies	South-East Asia Region	Removal	Removed ATT
		Replacement	SRID and ELISA
Japanese Encephalitis	South-East Asia Region	Refinement	Pyrogenicity with BET
		Removal	Removed ATT
MMR	Region of the Americas	Removal	MNVT for MMR are discontinued for quality control
	South-East Asia Region	Replacement	Cell-culture effective dose Method
Typhoid	South-East Asia Region	Replacement	Rocket immunoelectophoresis
		Removal	Removed Abnormal toxicity test.
Cholera	South-East Asia Region	Replacement	Inhibition ELISA
Flu	South-East Asia Region	Replacement	Single Radial immunodifussion method
Meningitis	South-East Asia Region	Replacement	Rocket immunoelecrophoresis
Varicella	South-East Asia Region	Removal	Removal of Abnormal toxicity test
Yellow Fever	Region of the Americas	Reduction	Increase production of working seed lot to increase time interval for the next MNVT.
HPV	Western Pacific Region	Replacement	Relative antibody titer to replace in-vivo relative potency test

Table 8. 3Rs opportunities implemented per vaccine per region.

Final Remarks

DCVMN thanks all the members that have dedicated time to respond to this important survey. Based on these results, DCVMN and the 3Rs Working Group will work to identify the 3Rs opportunities that are relevant to members, prioritize them and help to plan future activities in vaccine manufacturing processes and batch release testing (potency, safety and pyrogenicity testing). Simultaneously DCVMN will encourage and endorse the adoption of 3Rs opportunities among as many DCVMN members as possible with the aim to reduce vaccine batch release costs and speed up their release to the population.

Background Information About the DCVMN 3Rs Working Group and its current activities and priorities

During the DCVMN CMC workshop in Hyderabad held in June 2019, the DCVMN participants agreed on the creation of a specific 3Rs Working Group. Its main objective is the promotion of 3Rs (Replacement, Reduction, Refinement of animal testing) through the interaction with leading expert laboratories worldwide to follow the development and validation of harmonized alternative methods for testing legacy vaccines.

The working group met for the first time for a face to face meeting in Bangkok on December 2nd 2019, where the participants agreed to make the WG a quarterly appointment aimed to discuss about 3Rs opportunities (ongoing projects, education and trainings) on DTP-containing vaccines, Rabies and in particular on the replacement of the Rabbit Pyrogenicity Test (e.g. Monocyte Activation Test).

Since then, the working group has been growing and it has now representatives from **14 companies** from Bangladesh, Brazil, China, India, Pakistan, Indonesia, South Korea, and Vietnam.

DCVMN has collaborations with the Istituto Superiore di Sanità (ISS) in Italy to promote knowledge on the Monocyte Activation Test (MAT) as replacement for the Rabbit Pyrogenicity Test and on 3Rs Opportunities for Tetanus, Diphtheria and Pertussis; with Humane Society International (HSI) to promote the global alignment on regulatory acceptance of alternative methods (non-animal based) for vaccine batch release testing, notably the deletion of the abnormal toxicity test (ATT).

In addition, DCVMN is running a project called *International assessment of the Pertussis Serological Potency Test,* supported by the NIIMBL PC3.1G Funds.The aim of the project is to carry out an independent multi-laboratory assessment of the Pertussis Serological Potency Test (PSPT) as an alternative to the intracerebral challenge stipulated by the Mice Protection Test of Kendrick test to measure the potency of whole-cell Pertussis containing vaccines.

The project derives from the interest expressed by DCVMN companies on the need to improve whole-cell Pertussis batch testing after the fruitful contribution with both Istituto Superiore di Sanità (Italy) and Intravacc (The Netherland) which have been informing and updating the companies on the PSPT opportunity through the DCVMN workshops in the last two years. The work is being performed by a consortium of 11 laboratories, including national control laboratories and is expected to provide results on the assessment of the PSPT at the beginning of 2022.