3Rs expression in quality control paradigms of human vaccines

Abstract
Communicable diseases remain the leading cause of mortality worldwide in past. Children and adolescent were the most affected individuals. However, the development of vaccine played a vital role in decreasing mortality and increasing life expectancy. Currently most of vaccines are based on utilizing animal pathogens and use of animals. This use of animal in quality testing of vaccines is inevitable. Over the past century, concern about animals’ interests was limited to ensure that animals be treated humanely and not subjected to unnecessary sacrifice and sufferings. However, with the rise of 3Rs concept the global scenario for use of animals is being changed a little. The current review addresses the refinement, reduction and replacement aspects of animal use in vaccine testing. It also dialogues about challenges to implement 3Rs and gadget this key concept for effective quality testing. The acceptability and implementation of 3Rs concept is based on good manufacturing practices, good in-process quality control and validated procedures and processes. It is easier for new vaccines to adopt this concept. However, several difficulties are still experienced in the implementation of 3Rs principles for vaccine potency assays. Thus, insistent exploration is obligatory by both health industries and regulatory agencies for the implementation and validation of robust 3Rs approaches around the globe.

Keywords: vaccination, quality control, 3Rs, potency testing

Introduction
Communicable diseases remain the leading cause of mortality worldwide in past. Children and adolescent were the most affected individuals. However, the development of vaccine played a vital role in decreasing mortality and increasing life expectancy. Vaccines prevented 178 million cases from 1888 to 1924 in United States only. Similarly, 300 million people died of smallpox in the 20th century but no one dies today due to vaccination. Worldwide life expectancy has also been increased. Vaccines are being developed using the principles set by Louis Pasteur since more than 100 years. In last few decades, several technologies have been industrialized to develop vaccines.

The current review addresses the refinement, reduction and replacement aspects of animal use in vaccine testing. It also dialogues about challenges to implement 3Rs and gadget this key concept for effective quality testing.

The vaccines spectrum
Vaccines have saved more lives than any other medicine or medical product around the globe. It has been proved very effective tool of immunization in modern medicine. There are many types of vaccines (Table 1) which are categorized by the antigen used in their preparation.

Quality control testing for vaccines
Biological products are different in comparison to other pharmaceutical products; same is the case with vaccines. They are being derived from activemicroorganisms. However, their composition is complicated to be explained in perspective of chemical of physical means. Moreover, the intrinsic diversity of microorganisms and the potential for contamination of materials use in vaccine production requires special attention for quality control of vaccines. Generalized tests of vaccines for human use include pH, Adjuvant, Aluminium, Calcium, Free Formaldehyde, Phenol, Water, Extractable volume and Bacterial endotoxins. Typically, individual vaccine may contain the following tests:

a. Residual pertussis toxin for vaccines (comprising acellular pertussis factor)

b. Residual infectious virus

c. Sterility test (for live vaccines)

d. Pyrogens/Bacterial endotoxins

e. Total protein content (where relevant)

f. Free saccharide (for conjugate vaccines)

g. Potency test

h. Distribution of molecular size/ molecular weight (for polysaccharide vaccines)

i. Ovalbumin content (where a vaccine is produced in eggs)

j. Bovine serum albumin (where a vaccine is produced in cell cultures)

k. Host cell and vector DNA

l. Host cell protein

m. Residual reagents

n. Vesicle size (virosomal vaccines)

Use of animals in quality control
Research on infections and infestations in veterinary contributed a significant part in the advancement of vaccines for human use.
over past century. Currently most of vaccines are based on utilizing animal pathogens.\textsuperscript{10} Likewise, use of animals in quality testing of vaccines has become an important tool. Animals are used in vaccine development and testing to evaluate safety of vaccine; defense against an infection or illness; decrease in clinical signs & symptoms; death of pathogen; commencement and extent of immunity; category of immune response; routes of vaccine administration; and evaluation of particular immune compartments.\textsuperscript{11} Rats, mice\textsuperscript{12} or guinea pigs are used in potency tests for vaccines. There is a huge diversity of potency tests for vaccines especially those labelled as bacterial. Code of Federal Regulations specified few standards for vaccines like cholera, typhoid, pertussis, anthrax and BCG vaccines while others like tetanus, plague and diphtheria vaccines follow minimum requirements. On contrary, acellular pertussis, polysaccharide conjugate and live oral typhoid are analyzed according to adapted standards.\textsuperscript{13} With the diversity of potency testing, large numbers of animals are used in testing procedures. There are no exact figures available for the use of laboratory animal in quality testing of vaccines. However, there is an estimate of 10\% laboratory animals being used in biomedical research and testing. It includes more than one million rodents and guinea pigs only in European countries.\textsuperscript{14} Various approaches were adopted to reduce number of animals in animal testing. However, these approaches were based on a change in experimental design, a change based on statistical review and changes resulting from harmonization of test requirements.\textsuperscript{15} These approaches accompanied the concept of 3Rs.

### Table 1: Types of vaccines with examples

| Class | Type | Nature | Example |
|-------|------|--------|---------|
| I     | Live attenuated vaccines | contain whole bacteria or viruses which have been “weakened” so that they create a protective immune response but do not cause disease in healthy people | Rotavirus vaccine, MMR vaccine, Nasal flu vaccine, Shingles vaccine, Chickenpox vaccine, BCG vaccine against TB, Yellow fever vaccine, Oral typhoid vaccine (not the injected vaccine) |
| II    | Inactivated vaccines | contain whole bacteria or viruses which have been killed, or small parts of bacteria or viruses, such as proteins or sugars, which cannot cause disease | Inactivated polio vaccine or IPV, Some inactivated flu vaccines which are described as ‘split virion’, Hepatitis A vaccine, Rabies vaccine, Japanese encephalitis vaccine |
| IIA   | Whole killed’ vaccines | contain whole killed viruses | |
| IIB   | Subunit vaccines (sometimes called ‘acellular’) | do not contain any whole bacteria or viruses at all. (‘Acellular’ means ‘not containing any whole cells’) Instead contain polysaccharides (sugars) or proteins from the surface of bacteria or viruses. These polysaccharides or proteins are the parts that our immune system recognizes as ‘foreign’, and they are referred to as antigens | Diphtheria vaccine, Tetanus vaccine, Pertussis (whooping cough) vaccine |
| IIB-1 | Toxoid vaccines | Some bacteria release toxins (poisonous proteins) when they attack the body. The immune system recognizes these toxins in the same way that it recognizes polysaccharides or proteins on the surface of the bacteria. Some vaccines are made with inactivated versions of these toxins. They are called ‘toxoids’ because they look like toxins but are not poisonous | |
| IIB-2 | Conjugate vaccines | In most conjugate vaccines, the polysaccharide is attached to diphtheria or tetanus toxin protein. The immune system recognizes these proteins very easily and this helps to generate a stronger immune response to the polysaccharide | Hib vaccine, MenC vaccine, PCV |
| IIB-3 | Recombinant vaccines | made using bacterial or yeast cells to manufacture the vaccine. A small piece of DNA is taken from the virus or bacterium against which we want to protect. This is inserted into other cells to make them produce large quantities of active ingredient for the vaccine (usually just a single protein or sugar) | Hepatitis B vaccine, MenB vaccine, inactivated flu vaccines described as ‘surface antigen’, PPV, Injected typhoid vaccine (a polysaccharide vaccine) |

#### 3Rs concept in vaccine quality control

William Russell and Rex Burch formulated the principles for humane technique firstly in 1959.\textsuperscript{16} A milestone was achieved as 3Rs concept in quality control of vaccines in an international forum in London in the year 1985. This concept of 3Rs can be translated in the following context:

| a. Replacement | b. Reduction | c. Refinement |
|----------------|--------------|---------------|
| means implementing methods which avoid or replace the use of animals | means changing the test design in order to minimize the number of animals per experiment | means moving to methods that minimize suffering and improve animal welfare (e.g. replacing challenge tests by immunogenicity assays) |

Europe converted this concept into a legal requirement and documented it in European medicines agency guidelines 1997.\textsuperscript{17} It was followed by a directive published in 2001 for both veterinary

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Prominence of 3Rs in vaccinology

The development of robust assay in vaccine demands the mechanism for induction of safe immune response and action of pathogen or pathogenic entity in causing disease. Furthermore, this assay development necessitates the insight of virulence factors that exert their pathogenic effects. Development of assays considering these principles will result in complete replacements of animal models. However, lack of scientific knowledge at present limits the development of such mechanism-based assays. Currently, the concept of vaccine quality control is being shifted from classical model to consistency approach. Thus, the foremost attention should endure in monitoring of consistency rather than to establish the factual efficacy of a vaccine. Four tiers are being used to implement and justify the concept of 3Rs in quality control of vaccines (Figure 1). However, Figure 2 shows few examples of 3Rs for human vaccines potency testing.

Figure 1 Four tiers of 3Rs.
### 3Rs Expression in Quality Control Paradigms of Human Vaccines

| Vaccines                        | 3Rs Expression | References |
|---------------------------------|----------------|------------|
| Acellular pertussis             | ![Reduction] | [22]        |
| Traditional: The histamine sensitization test | | |
| Alternate: Combination of enzyme coupled-HPLC (E-HPLC) & carbohydrate binding assays | | |
| Acellular pertussis Component   | ![Refinement] | ![Reduction] | [23]    |
| Traditional: Multiple-dilution mouse serology | | |
| Alternate: Immunization (mice) and ELISA | | |
| Whole cell pertussis            | ![Refinement] | ![Replacement] | [24, 25] |
| Traditional: Lethal Challenge potency test | | |
| Alternate: Evaluation phase and validation phase with humane endpoints | | |
| Anthrax                         | ![Reduction] | ![Refinement] | [26]    |
| Traditional: In-vivo mouse immunogenicity test | | |
| Alternate: Toxin neutralization assay | | |
| Cholera                         | ![Refinement] | ![Replacement] | [27]    |
| Traditional: Multi-dilution vaccination & serology | | |
| Alternate: ELISA | | |
| Diphtheria                      | ![Reduction] | ![Refinement] | [28-30] |
| Traditional: Lethal/intradermal challenge test, Residual toxicity in diphtheria | | |
| Alternate: ELISA, Vero cell assay | | |
| Diphtheria component            | ![Refinement] | ![Replacement] | [31, 32] |
| Traditional: Guinea pig lethal challenge test | | |
| Alternate: Erythema score following intradermal challenge | | |
| Haemophilus type B Conjugate    | ![Refinement] | ![Replacement] | [33]    |
| Traditional: Multi-dilution vaccination & serology | | |
| Alternate: High throughput SBA for anti-Hib antibodies | | |
| HBsAg                          | ![Replacement] | ![Refinement] | [34]    |
| Traditional: In-vivo method | | |
| Alternate: In-vitro method for HBsAg content using Auszyme EIA kit | | |
| Hepatitis A                     | ![Replacement] | ![Refinement] | [35]    |
| Traditional: Mouse serology | | |
| Alternate: Antigen quantification | | |
| Hepatitis B                     | ![Replacement] | ![Refinement] | [35]    |
| Traditional: Potency test | | |
| Alternate: Serological antigen quantification | | |

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| Vaccine Type | Traditional Method | Alternate Method | Notes |
|--------------|-------------------|-----------------|-------|
| Human papillomavirus | Mouse serology | Antigen quantification | [36] |
| Japanese encephalitis | Mouse immunogenicity assay followed by plaque reduction neutralization (PRN) test | ELISA | [37] |
| MMR | CCID50 and plaque assays | Quantitative PCR after cell culture | [38] |
| Poliomyelitis (Inactivated) | Serological potency test | Antigen quantification | [35] |
| Polio (Oral) | Oral polio neurovirulence test | Mutant analysis by PCR and restriction enzyme cleavage (MAPREC test) TgPVR21 Mouse Neurovirulence Test | [39-41] |
| Rabies | Lethal challenge test | Single dilution assay | [35] |
| Rabies virus (Inactivated) | Lethal challenge test, NIH mouse protection test, In-vivo rabies vaccine potency | Humane endpoints for rabies potency testing, Multi-dose serological assay, Time resolved fluoroimmunoassay (TRFIA) | [42, 43] |
| Rotavirus | In-Vivo potency assays | Cell-based viral replication followed by quantitative reverse-transcription polymerase chain reaction (RTQPCR) analysis | [44] |
| Rubella virus | In-Vivo potency assays | In-vitro cytopathic effect (CPE) with rabbit kidney epithelial (RK-13) cell culture | [45] |
| Smallpox virus | Titration onto CAM assay | Vero cell culture titration assay | [46] |
| Tetanus toxoid | Lethal/paralytic challenge test | ELISA, Toxin Binding Inhibition test | [47-49] |

**Figure 2** Matrix for 3Rs expression of potency testing (human vaccines).
Challenges to implementing 3Rs

World health organisation directed to national quality control laboratories to apply the concept of 3Rs. However, there are two main challenges to implementing 3Rs. First is scientific and second is regulatory. Scientific challenge involves the inherent variability of in vivo assays, validation issues of in vivo assays as per ICH guidelines and the attributes of product quality. On the other hand, the regulatory challenge implicates the lack of harmonization in regulatory standards around the globe, complexity of regulatory changes and discretion of health authorities to consent deviation from established guidelines. Therefore, a one-to-one comparison is often challenging and not necessarily justified.

Conclusion

The acceptability of 3Rs concept is based on good manufacturing practices, good in-process quality control and validated procedures and processes. However, it is easier for new vaccines to adopt this concept. Significant developments have been made in the improvement, maintenance, and upgrading of in-vitro potency assays like ELISA, Gel electrophoresis, Cell culture etc. which minimize the animal use and suffering. However, several difficulties are still experienced in the implementation of 3Rs principles for vaccine potency assays. Thus, insistent exploration is obligatory by both health industries and regulatory agencies for the implementation and validation of robust 3Rs approaches around the globe.

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Conflicts of interest

Authors declare no conflict of interest.

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