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Report

WHO Informal consultation on the application of molecular methods to assure the quality, safety and efficacy of vaccines

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WORLD HEALTH ORGANIZATION
Quality Assurance and Safety of Biologicals

Abstract

In April 2005, the World Health Organization convened an informal consultation on molecular methods to assure the quality, safety and efficacy of vaccines. The consultation was attended by experts from national regulatory authorities, vaccine industry and academia. Crosscutting issues on the application of molecular methods for a number of vaccines that are currently in use or under development were presented, and specific methods for further collaborative studies were discussed and identified. The main points of recommendation from meeting participants were fourfold: (i) that molecular methods should be encouraged; (ii) that collaborative studies are needed for many methods/applications; (iii) that basic science should be promoted; and (iv) that investment for training, equipment and facilities should be encouraged.

1. Introduction

Vaccines and other biological medicines are derived from living materials. This poses particular problems with regard to assuring their efficacy and safety due to the inherent variability of starting materials, the production processes, and the complex nature of the products themselves. For vaccines and other biological medicines, the key to effective quality control (QC) is rigorous testing and validation applied to the starting materials, to different stages of the production process, and also to the final products.

With the advent of molecular biotechnology, there are increasing uses of molecular methods for the characterization and QC of vaccines whether they are currently used or newly developed. The World Health Organization Expert Committee on Biological Standardization (WHO ECBS) has put an increasing emphasis on products and assays arising from new biotechnology in internationally harmonized activities of quality assurance and safety of biologicals, and of vaccines in particular.

2. Selected topics on state-of-the-art in molecular methods

2.1. Quantitative mutant analysis of viral quasispecies by MAPREC test and MALDI-TOF mass spectrometry. *Dr K. Chumakov*

In the QC of live attenuated viral vaccines, genetic stability is of great concern. The presence of even small quantities of mutants or revertants may indicate incomplete or unstable attenuation that may influence vaccine safety. Oral poliovirus vaccine can be monitored with the use of 'mutant analysis by polymerase chain reaction (PCR) and restriction enzyme cleavage' (MAPREC). Genetic variation in live attenuated mumps virus vaccine has been investigated by using both MAPREC and a platform (DNA massarray) based on matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry. Mumps vaccines prepared from the Jeryl Lynn (JL) strain typically contain at least two distinct viral substrains, JL1 and JL2, which have been characterized by full-length sequencing. Dr Chumakov reported the development of assays for characterizing sequence variants in these substrains and demonstrated their use in quantitative analysis of substrains and sequence variations in mixed virus cultures and mumps vaccines. The results obtained from both the MAPREC and MALDI-TOF methods showed excellent correlation. The result suggested the potential utility of

MALDI-TOF for routine QC of live viral vaccines and for assessment of genetic stability and quantitative monitoring of genetic changes in other RNA viruses of clinical interest. The molecular tests mostly provide a gauge of consistency, and quantification of mutants by MAPREC, MALDI-TOF or microarray hybridization can be used as a surrogate test for viral vaccines. For the future, there is a need for a method to look at the entire genome and be able to detect mutational heterogeneities.

2.2. DNA microarrays: Global gene expression analysis in infectious diseases. *A/Prof N. Curtis*

Two advances in molecular biology over the last 10 years are set to revolutionize the investigation of infectious diseases and host/pathogen interactions. The first is the exponential increase in the number of organisms whose genome has been sequenced, exemplified by the completion of the human genome project. The second is the development of microarrays that allow the simultaneous detection of tens of thousands of nucleic acid sequences by hybridizing fluorescent-labeled target samples to probe sequences immobilized on a glass slide. Microarrays can be used to detect genomic DNA or to detect gene expression represented by the mRNA transcriptome. There are a number of different microarrays platforms including two color spotted arrays (e.g. cDNA microarrays and long oligonucleotide arrays) and single channel arrays (e.g. high density short oligonucleotide arrays), each with their own advantages and disadvantages. In addition to careful experimental design, the use of microarrays involves a heavy reliance on highly specialized statistical bioinformatics for image processing, quantification, and normalization of data within and between microarrays (preprocessing), as well as for the analysis (using clustering and other algorithms) of the large quantities of data derived from microarray experiments. Validation and interpretation of results requires the integration of microarray data with other bioinformatics resources (data mining). Advantages of the use of microarrays include: (i) comprehensiveness (the ability to interrogate the entire genome or global gene expression); (ii) high sensitivity; (iii) the ability to interrogate host/pathogen genomes without prior bias; and (iv) the ability to discover genes or pathways of previously unknown function. Disadvantages include (i) the high (but decreasing) cost of the technology; (ii) the dependence on highly specialized bioinformatics expertise; (iii) issues of reproducibility and reliability; and (iv) concern that the transcriptome may not always accurately reflect protein activity. Despite these problems, there are now countless examples of how microarray-based studies can provide fresh insight into the molecular pathogenesis of infectious diseases. Microarray technology offers many opportunities for future work, in both research and clinical settings, on improving the diagnosis, treatment and prevention of infectious diseases.

3. Test methods for seed lot characterization and in-process control

3.1. Evaluation of reverse genetics-derived pandemic influenza reference viruses. *Dr J. Robertson*

Human influenza vaccine reference strains are prepared as required when an antigenically new strain is recommended by WHO for inclusion in the vaccine. Currently,

for influenza A, these strains are produced by a double infection of embryonated hens' eggs using the recommended strain and the laboratory strain PR8, which grows to high titre in eggs, in order to produce a high-growth reassortant (HGR). HGRs are provided by WHO reference laboratories to the vaccine manufacturing industry which uses them to prepare seed virus for vaccine production. The use of "reverse genetics" in preparing vaccine reference strains offers several advantages over the traditional method: (i) the reverse genetics approach is a direct rational approach compared with the potentially hit-or-miss traditional approach; (ii) reverse genetics will decontaminate a wild-type virus that may have been derived in a non-validated system, e.g. a cell line not validated for vaccine purposes, or that may contain additional pathogens; (iii) at the plasmid stage, the HA can be engineered to remove pathogenic traits. The use of reverse genetics in deriving HGRs has been demonstrated by several laboratories, including its use in deriving a non-pathogenic reassortant strain from a highly pathogenic virus. Dr Robertson and colleagues reported advances in the use of reverse genetics by making use of a cell line acceptable for human vaccine production, by demonstrating directly the short time frame in which a reassortant virus can be derived, and by deriving a non-pathogenic pandemic vaccine reference virus in cells validated for vaccine production and under quality controlled conditions. The value of reverse genetics includes the rational design of viruses for novel vaccine development, current vaccines, and pandemic vaccines. An added value of reverse genetics includes the removal of pathogenic traits. Highly pathogenic avian strains have an insertion of 3-5 basic amino acids at the HA cleavage site and these can easily be deleted at the plasmid DNA stage, rendering the subsequently rescued virus non-pathogenic.

3.2. Tests of polio vaccines. *Dr P. Minor*

Molecular basis of the attenuation of the type 3 Sabin vaccine was determined through a stepwise approach: (i) identification of a virulent strain closely related to the vaccine strain (actually the precursor used by Sabin); (ii) cloning, sequencing, and making recombinants to identify attenuating regions; and (iii) confirmation by site-directed mutagenesis to deattenuate the vaccine strain. This approach required an animal model (monkeys or transgenic mice) and "reverse genetics" to recover virus from cloned genomes. Results in animals showed what changes attenuated the viruses for a particular route of inoculation. The results were consistent with isolates from real cases of vaccine-associated diseases, i.e. all vaccine-associated paralytic poliomyelitis (VAPP) isolates have reverted amino acid (aa) at 472 and suppressed the effects of VP3 91. Type 3 virus excreted by healthy vaccinees was known to increase in virulence. This related to aa changes at 472. Vaccine passaged by manufacturers increases in virulence for monkeys, so that passage levels are tightly controlled. MAPREC showed an excellent correlation between 472 content, passage level and monkey virulence. It has been used in establishing production conditions by new manufacturers and monitoring production lots. But general applicability of the approach used for oral polio vaccine (OPV) is not necessarily possible for other vaccines. Polio has a good animal model, vaccine-associated cases, easy reverse genetics, isolates from vaccine associated cases and healthy babies, a single set of vaccine strains used world wide, well studied pathogenesis and fifty years of experience. On the other hand, measles has no good animal model, no vaccine-associated cases, difficult reverse genetics, several vaccine strains that are related

but distinct, and poorly understood pathogenesis. In terms of polio pathogenesis in humans, attenuation at 472 is lost so rapidly in vaccinees that it is difficult to believe that it really has an attenuating effect in the normal course of events. If this is so, then the safety of the vaccine strains is due to the other mutations which are lost later on, typically around 11 days or later. Therefore, MAPREC or other molecular markers should be considered as a consistency measure. Safety of vaccines is based on clinical experience. This assumes that vaccine production is consistent. Various measures of consistency may be used. Such measures do not necessarily have to be related to virulence/attenuation. If there are mutations that change on virus growth under production conditions, they can be used to monitor consistency even if they have no biological effect. This approach requires the ability to detect and measure small subpopulations of mutants.

3.3. Application of molecular methods to BCG vaccine characterization. *Dr S. Mostowy*

The Bacillus Calmette-Guérin (BCG) family of vaccines currently used to prevent tuberculosis (TB) consists of clonal bacterial strains independently shaped by nearly a half-century of evolution. Derived from virulent *Mycobacterium bovis*, the causative agent of TB in cows, daughter strains of BCG were additionally passaged under the same laboratory conditions that resulted in their original attenuation. Genomic loss of RD1 (Region of Difference 1) has been demonstrated to coincide with attenuation from virulence, while deletions occurring after the loss of RD1 are speculated to be responsible for BCG's 'over-attenuation'. As a result, current BCG strains are genomically different from one another, and from the original BCG vaccine initially used in 1921. Apart from genomic deletions, other types of genomic changes such as gene duplications or substitutions have also been reported. A more complete catalogue of genetic changes in un-sequenced BCG strains can be obtained from comparison to the upcoming sequence of BCG Pasteur 1173-P2, and by whole-genome expression profiling via RNA/RNA microarray analysis. Studies of expression profiling can suggest a phenotypic consequence of BCG evolution, and compare the transcriptional capacity of different BCG strains. For example, expression of the antigenic mycobacterial proteins MPB70 and MPB83 have been recognized to vary considerably across BCG strains, although the reason for this phenotypic difference was unknown. By immunoblotting, BCG could be separated into high- and low-producing strains. By quantitative reverse transcription PCR (qRT-PCR), it was determined that transcription of the antigen-encoding genes, *mpb70* and *mpb83*, follows the same strain pattern with mRNA levels reduced over 50-fold in low-producing strains. Transcriptome comparison of the same BCG strains by DNA microarray revealed two gene regions consistently downregulated in low-producing strains compared with high-producing strains, one including *mpb70* (Rv2875) and *mpb83* (Rv2873), and a second that includes the predicted sigma factor, *sigK* (Rv0445c). DNA sequence analysis revealed a point mutation in the start codon of *sigK* in all low-producing BCG strains. Complementation of a low-producing strain, BCG Pasteur, with wild-type *sigK* fully restored MPB70 and MPB83 production. Microarray-based analysis and confirmatory qRT-PCR of the complemented strains revealed an upregulation in gene transcription limited to the *sigK* and the *mpb83/mpb70* gene regions. From this, it could be concluded that a mutation of *sigK* was responsible for decreased expression of MPB70 and MPB83 in low-producing BCG strains. For future directions of BCG studies, it is hoped that specific gene sets can be associated with clinically important variation.

The outcomes from these studies will be exploited to assist in the direction of future TB vaccination programs. Additional study is directed towards identifying potential methods or assays that can be applied to the control of BCG vaccine production, such as improving a BCG identity test and measurement for consistency of production.

4. Test methods for potency or potential hazards in vaccines

4.1. Potency estimation of live virus vaccines using infectivity PCR. *Dr M. Schalk*

The potency of live attenuated virus vaccines is determined by counting or titrating viable viruses in cell cultures (e.g. plaque test or $CCID_{50}$). These classical potency tests have the drawback that they are time consuming and laborious and show a high lab to lab variation. Dr Schalk and colleagues developed an infectivity PCR to measure the potency of mumps, measles and rubella virus in trivalent measles, mumps, rubella (MMR) vaccines. Infectivity PCR is a combination of virus propagation and quantitative PCR (qPCR). Vero cells are infected with serial dilutions of a trivalent vaccine or a trivalent reference with known potency, viruses are allowed to replicate, and subsequently replicated virus is quantified by quantitative PCR using the LightCycler technology. The potency of vaccine samples is estimated against reference preparations using parallel line analysis. The potency of measles and mumps virus is estimated within one assay after one day of cell culture. The potency of rubella virus is estimated in a separate assay after two days of cell culture. Compared with conventional $CCID_{50}$ and plaque assays, the infectivity PCR assay has the advantage in being fast, because the assay is not dependent on the formation of cytopathic effect, while accuracy and intermediate precision were similar. Furthermore, assay design is simplified: serological neutralization can be omitted because PCR is virus-specific, and under the conditions used, the individual virus components of trivalent MMR vaccines do not interfere with each other.

4.2. PERT assay for adventitious retroviruses detection in viral vaccines. *Dr F. Fuchs*

The detection of adventitious retroviruses has always been critical for assessing the safety concerns associated with viral vaccines. Assays for the enzymatic activity of reverse transcriptase (RTase) are used as general methods for the detection of both known and unknown retroviruses. PCR-based RTase (also called product-enhanced RTase, PERT) assays were reported to be a million times more sensitive than previous RT assays. Soon after their description, studies using the PERT assay demonstrated RTase activity in live viral vaccines grown in chicken cells. Dr Fuchs and colleagues assessed the performances of such an approach for the quantitative detection of RTase activity in vaccines. Sensitivity, linearity, and reproducibility of a PERT assay were studied on purified RTase and viral vaccines treated to release RTase from potentially contaminant retroviruses. The level of RTase activity detected in chicken cell-derived vaccines was higher for live attenuated vaccines compared with inactivated ones. Contrary to other studies, RTase activity was found in some mammalian cell-derived vaccines. It was known that RT activity could be attributed to some cellular DNA polymerases. In order to discriminate specific retroviral activity from nonspecific activity, sensitivity analysis using the triphosphorylated form of the nucleoside analogue 3'-azido-3'-deoxythymidine

(AZT) as inhibitor of RTase activity and competitor DNA as DNA polymerase inhibitor was performed. Given the conditions of the assay we established, low level of RT activity was seen in vaccines tested. However, the results and their significance were difficult to interpret because decrease of the signal was within the range of variability of the method. The laboratory thus further developed a real-time PERT assay. Sensitivity studies using this assay are under way. Definition of an acceptable threshold of the assay will be needed to correlate RTase activity with the risk of viral transmission using in vitro and in vivo models.

4.3. Testing for extraneous agents. *Dr H.-P. Ottiger*

Certification of freedom from extraneous agents is an essential quality requirement of biological products including vaccines for veterinary use. Vaccines can be contaminated by various materials used for production and during manufacturing process. The European Pharmacopoeia (EP) requires poultry vaccines to be free of adventitious agents and this includes monitoring for at least 16 viruses. PCR has been recognized to be a valid alternative testing method for adventitious agent testing for such vaccines. A number of generic PCR assays for final product testing have been recently developed and validated. However there is a need for further development. Some methods are well established and validated, but others are still under development. Further collaborative studies and investment in training, equipment and facilities are needed to promote and enhance these developments. The quality standards have an important implication also for animal welfare. Therefore a close coordination between science, legislation and quality management for implementation schemes is needed. This will ensure that molecular methods can be taken up rapidly by vaccine manufacturers and control authorities. Examples are available of the design and development of PCR methodology. In particular the process of standardization and validation requires special emphasis

4.4. Tests for host cell DNA: Safety issues on residual mammalian cell DNA in vaccines*. Dr K. Peden*

Vaccines and other biological products manufactured in cells contain contaminating residual DNA derived from that production cell substrate, with the amount and form of this DNA depending mainly on the type of vaccine. The potential risk of this cellular DNA has been debated for over 40 years without resolution. Opinions on residual DNA have varied from it being considered an inert contaminant, and thus its presence should not be deemed to be a risk to vaccine recipients, to it being considered an important risk factor, particularly for vaccines manufactured in certain cell substrates, such as cells derived from tumors or cells that are tumourigenic. The major risk for residual cell-substrate DNA has been considered to be due to the oncogenicity of the DNA, particularly if the cell substrate is tumourigenic or is derived from a human tumor. DNA oncogenicity has been considered to arise mainly through the introduction of a dominant oncogene, as oncogenesis arising through insertional mutagenesis has been considered to be an improbable event. However, another potential safety concern is the risk that the genome of an infectious virus is present in this DNA, either as an integrated genome or as an extrachromosomal element, and that, once inoculated, this genome could generate an infectious agent that could then establish a productive infection in the vaccine recipient.

Such an infection could have pathogenic consequences. Thus, to assess potential risks associated with residual cell-substrate DNA, both biological activities (oncogenicity and infectivity) need to be considered and addressed. An evaluation of risks should be based on quantitative experimental data. Furthermore, as long-term safety data from vaccinated people are usually unattainable, it is prudent to make risk assessments based on the most sensitive model system. To this end, sensitive quantitative assays are being developed to assess the oncogenicity of DNA in various animal model systems and the infectivity of DNA in cell culture. Preliminary results from the DNA oncogenicity studies have demonstrated that: (i) Expression plasmids for human T24-H-*ras* and murine c-*myc* are oncogenic at 12.5 μg of each plasmid; (ii) Newborn normal mice are more susceptible than adult mice; (iii) A dual expression plasmid for both oncogenes is more active than the separate plasmids. Preliminary results from the DNA infectivity studies have demonstrated that: (i) An in vitro assay can detect as little as 1 pg of a plasmid containing the HIV provirus; (ii) Treatment with either benzonase or β-propiolactone (BPL) can reduce the infectivity of DNA; (iii) Using the proportion of HIV DNA that would be present in infected cells and the level of 10 ng per vaccine dose, the amount of DNA permitted by the WHO for residual DNA derived from cell lines, extrapolations suggest that the safety factor is 60 (in the absence of chemical inactivation or enzymatic digestion); (iv) With chemical inactivation or enzymatic digestion, safety factors of more that 10^7 can be obtained for the clearance of DNA.

4.5. Testing issues for residual cell DNA in vaccines. *Dr A. Beck*

Since the 1980s, a number of studies raised potential safety issues with regard to residual DNA. Residual DNA from continuous mammalian cell lines may transfer activated oncogenes and infectious provirus DNA. The United States Food and Drug administration (FDA), WHO, and European Medicines Agency (EMEA) published guidelines on residual DNA. In 1985, FDA set the upper limit of 10 pg of residual DNA per medicinal dose. In 1986, a WHO study group considered a number of issues associated with the acceptability of new cell substrates for the production of biologicals and concluded that the risk is negligible when the amount of such DNA is 100 pg/dose [1]. In 1996 WHO ECBS further increased levels up to 10 ng per purified dose as acceptable [2]. The new upper limit was not for products orally given or derived from microbial, diploid or primary cell cultures. In 1997, EMEA defined residual cell DNA as impurities to be eliminated during the downstream processing for safety and tolerance reasons. According to EMEA guidance, further data for DNA from continuous mammalian cell lines suggest it poses less risk than previously thought. For live viral vaccines or less purified products (e.g. intact virions produced by lysis of the cell substrate) compared to purified biologicals, it may not be possible to comply with the upper limit of the total DNA, i.e. 10 ng per dose. As a proposal for a dose limit, key factors to take into account include: total quantity of DNA, number of doses to be given, size of DNA fragments, number of copies of potentially infectious or oncogenic sequences per cell, state of DNA (e.g. association with chromatin, integration into the cellular genome, linear, circular, etc.) and treatment with virus-inactivating agents (e.g. beta-propiolactone). Of the various quantification methods used for residual DNA, three methods are commonly used: (i) DNA hybridization and dot blot analysis; (ii) Total DNA threshold assay; and (iii) qPCR. Each method has its strength and weakness in terms of

sensitivity, specificity, minimal size of target DNA for detection, robustness, running time, material safety/toxic waste, reagent stability and costs. A key issue would be that DNA standards for the host cells are commercially unavailable. There are various approaches to the initial sample treatment for DNA purification. Requirements for detection sensitivity of methods are very low (e.g. picogram range sensitivity in milligrams of recombinant vaccines), so that method validation would be a critical factor.

4.6. New analytical approaches for residual DNA quantification and size evaluation: example of residual Vero DNA for inactivated vaccines. *Dr L. Mallet*

There are several approaches available for DNA quantification with different spectra of analytical sensitivity and specificity. Approaches with broader specificity include UV spectrophotometry, colorimetry, picogreen and threshold method, while hybridization and qPCR are specifically applied for quantification of Vero cell DNA. The threshold method is an immuno-enzymatic method. It is very sensitive, and its accuracy is greater than hybridization. It allows the determination of DNA clearance and purification during the production process of vaccines. It has, however, some limitations and disadvantages. It requires a sterile sample. The reaction is inhibited by DNA fragments shorter than 80 bp in size. Operating conditions are difficult to optimize and are sample dependent. Its quantification range is relatively narrow (5-150 pg/well) and throughput is low. There is only one supplier, and no information can be obtained on the DNA size distribution. The best choice of an approach for both residual DNA quantification and DNA size evaluation would be qPCR. It also allows the detection of cellular DNA while excluding viral DNA. It is a sensitive, high throughput technology with large range of detection (1 pg to 100 ng per reaction). Disadvantages of qPCR would be that it does not quantify DNA fragments below the size of the PCR product (59 bp for Vero cell DNA) and that there may exist biased estimates of the whole DNA, since it is based on quantification of a specific gene. In January 2002, a research group in Sanofi Pasteur initiated development of qPCR methods for both Vero cell DNA content and Vero cell DNA size evaluation in products under development in line with FDA recommendations (FDA letter, 12 March 2001). Five primer sets were designed to amplify overlapping fragments of Vero cell DNA (beta-actin gene region) with 5 different sizes of amplified fragments (59-, 108-, 240-, 407- and 620-bp). The relative proportion of each population of DNA fragments was evaluated. Study results suggested that qPCR could provide both quantitative and qualitative information for evaluation of residual cellular DNA in vaccines produced from Vero cells. Also it was noted that qPCR would be more appropriate than Threshold method for vaccines treated with deoxyribonuclease (DNase) or containing small DNA fragments following purification and/or inactivation process. Experimental data demonstrated that only small-size fragments are found in the manufacturer's Vero cell-derived, purified and BPL-inactivated rabies vaccine (≤ 620 bp). There is a need for a WHO collaborative study to evaluate these new quantitative and qualitative approaches and to set up appropriate DNA specifications related to these new methods in order to monitor consistency of production.

4.7. Determining an acceptable safety limit for residual DNA from a continuous cell line in an oral vaccine: Studies of Vero DNA uptake by the oral and intramuscular routes. *Dr J. Lebron*

Although there is a WHO guidance for a limit on residual DNA for parenteral vaccines produced on continuous cell lines, there is no corresponding guidance for oral vaccines. To help determine an oral limit, we performed a study in rats in which the relative uptake and persistence of DNA from the Vero continuous cell line (an African green monkey kidney cell line) administered orally was compared with its uptake when delivered intramuscularly. Uptake for each route of administration was quantitated on a variety of tissues using a TaqMan® qPCR assay specifically developed to target the 171-bp long α-DNA repetitive sequence, which is present in the African green monkey genome at approximately 5 million copies per genome. Since this qPCR assay has an exceptionally sensitive limit of detection of 1-10 attograms (10^{-18} gm), the results of this study allowed the generation of an empirically derived intramuscular versus oral factor (10^6) representing the relative inefficiency of DNA uptake by oral administration. This factor was then applied to the WHO recommended parenteral limit of 10 ng/dose to determine a corresponding upper limit on the level of residual DNA for an oral vaccine of 10 mg. As a conservative approach, this empirically determined limit was reduced 100-fold to 100 µg. Thus, the results of this animal study, together with additional evidence in the literature, support a residual DNA safety limit of 100 µg per dose for an oral vaccine produced on a continuous cell line.

5. Methods being applied to epidemiological or clinical studies

5.1. Rotavirus typing methods and algorithms. *Dr T. Fischer*

Vaccination is the current strategy for control and prevention of severe rotavirus infections, a major cause of acute, dehydrating diarrhoea in young children worldwide. Public health interventions aimed at improving water, food and sanitation are unlikely to adequately control the disease. Development of vaccines against severe rotavirus diarrhoea is based upon homotypic or heterotypic protection provided against either a single common G serotype (monovalent vaccines) or against multiple serotypes (multivalent vaccines). Rotavirus strain surveillance has a high priority in disease control programs worldwide. The continued identification of the most common G and P serotypes for inclusion in vaccines is an important priority. And subsequent to the introduction of a vaccine candidate, not only monitoring of circulating strains is recommended, but also surveillance of potential reassortment of animal rotavirus genes from the vaccine into human rotavirus strains is critical. Conventional methods used in the characterization of rotavirus strains, such as enzyme immunoassay serotyping and reverse-transcription PCR-based genotyping often fail to identify uncommon and newly appearing strains. The application of newer molecular approaches, including sequencing and oligonucleotide microarray hybridization, may be required to characterize such strains. Improved detection and characterization of incompletely typed strains will help develop a comprehensive strain surveillance that may be required for tailoring effective rotavirus vaccines.

5.2. Molecular methods for evaluation of human papillomavirus (HPV) vaccines. *Dr S. Pagliusi*

At the outset of the development and implementation of strategies for the use of new HPV vaccines against cervical cancer, it is important to assure the quality of laboratory data collected in the context of epidemiological and clinical studies worldwide. A WHO international collaborative study was initiated to assess the feasibility of generating HPV DNA standard reagents, and the suitability of cloned DNA corresponding to viral genomes for this purpose. A total of 29 laboratories in 12 countries participated in the study to assess their performance in detecting and quantifying human papillomavirus DNA. A panel consisting of 24 coded samples was distributed to participants. The samples included dilution series for the most common HPV types associated to cervical cancer, HPV 16 and 18, alone or in combination with five other high-risk HPV types including HPVs 31, 33, 35, 45 and 52, the low-risk HPV type 6 as well as a negative control. Participating laboratories used a variety of commercial and in-house assays (hybridization, PCR, PCR-hybridization, DNA chip technology, etc.) to detect HPV DNA in the panel samples. Results showed that all laboratories correctly identified the negative sample. The results of qualitative assays were generally consistent across laboratories, and most of the invalid results reflected a lack of test sensitivity. Overall, there were no false-positive results for HPV 18; in contrast, HPV 16 detection demonstrated 25% false-positive results. Inaccurate results were mostly clustered in individual laboratory, and it was suggested that standard operating procedures would help improve laboratory quality. Methods with high sensitivity and specificity would be useful for evaluation of HPV vaccine efficacy in conjunction with its clinical outcomes. International standard reagents would provide a helpful tool for assurance of high quality HPV detection and typing.

6. Perspectives from regulators and industry

6.1. State Center for Medicine Control of Cube, Rep.. *Dr R. Cristia*

The Republic of Cuba is one of the countries where local vaccine manufacturers exist. Projects for developing next generation vaccines and other biological products using new technologies are in progress. Molecular methods are being applied and found to be very attractive for product characterization and regulatory evaluation. Molecular methods are expensive but very sensitive. Molecular methods are currently put in place and implemented for regulatory evaluation and control of biological medicines by the Cuban national regulatory authority (NRA) and national control laboratory (NCL) with the adequate support for experts, equipment, reagents and reference materials. The Cuban NRA/NCL envisages to play a reference-centre role in promoting the use of molecular methods in other Latin American countries.

6.2. Korea Food and Drug Administration (KFDA). *Dr Y. Sohn*

KFDA implements science-based regulations compatible with global standards. Diverse projects aimed at developing a new cutting-edge QC method and their introduction into regulatory practice and industry have been performed in the area of vaccines, blood plasma derivatives, recombinant DNA products, and cell/gene therapy products. Examples include a PERT assay for retrovirus detection, PCR methods for mycoplasma detection and methods for quantification and detection of residual host cell

proteins and DNA. An international collaborative effort for standardization and validation of newly emerging molecular methods using international reference preparations would be of important value for introduction and proper use of molecular methods. It would also be very useful if an international standard methodology is recommended by WHO in addition to reference preparations. It would be very useful for WHO to develop an appropriate training program pertaining to molecular methods.

6.3. US FDA. *Dr K. Chumakov*

Evaluation should be done on a case-by-case basis on the ground of the proposed use of the data, phases of regulatory process, preclinical investigation, and studies in support of investigational new drug applications and biologics licensure applications. Molecular methods for the purpose of QC would be the replacements of existing tests and additional tests for consistency and post-marketing surveillance. Encouragement should be given to incorporate molecular approaches into vaccine development at the earliest possible stage, to facilitate collaborative evaluation of alternative surrogate methods, and to promote basic research to establish rationale for molecular testing.

6.4. Developing Country Vaccine Manufacturer's Network (DCVMN). *Mr D. Daout*

As an example of the use of molecular methods assuring the quality, safety and efficacy of vaccine, characterization of hepatitis B vaccine (HepB) manufactured by members of DCVMN were considered. For the production of HepB, the guidelines from WHO's Technical Report Series (TRS) no. 786 were applied. The conditions in the TRS were all complied by DCVMN members. In addition to HepB, additional characterization of the mumps vaccine virus has been done using molecular methods. Essentially, genetic sequence was studied in different passage levels of mumps vaccine virus to show that sequence identity is maintained in seed strains. There was no change in the haemagglutinin-neuraminidase gene sequence of the L-Zagreb mumps vaccine virus at various passage levels, indicating high level of genetic stability of the vaccine seed.

6.5. International Federation of Pharmaceutical Manufacturers Associations. *Drs J. Lebron, L. Mallet and S. Spreng*

Molecular methods are used in every stage of vaccine development, from basic research to post-licensure. In basic research, they are used as a tool in the development of new vaccines. During preclinical development, they are used as part of the analytical testing, which is performed to ensure the quality attributes of the vaccine and as part of the safety testing, which is performed to ensure the absence of adventitious agents. Characterization science, which is also usually performed during preclinical development, also relies on the use of molecular methods. During clinical development, these methods are mainly used to ensure the safety and efficacy of the vaccine. After licensure, the use of molecular methods is equally important in the continued assurance of the quality, safety, and efficacy of the vaccine. In conclusion, molecular methods from an industry perspective:

- have already demonstrated their usefulness in all aspects of vaccine development,
- may allow replacement of various classical analytical and safety testing methods,

- would allow higher throughput, as many molecular methods are amenable to automation, and
- would allow reagent conservation, as many are also amenable to micro scaling.

7. Regulation of vaccines: Strengthening the science base. *Dr J. Milstien*

Despite the appearance of new vaccines in the past few years, many vaccines are based on old technologies and are still subject to regulatory practices devised many years ago. Vaccine regulation began with a foundation on vaccine testing, and only in response to tragedies associated with vaccine use did new concepts begin to be defined. Vaccine regulation now includes a range of functions that cover the entire continuum of vaccine development and use. However, some regulatory practices, such as the continuing dependence on outdated animal tests, have not kept pace with these changes. Other practices, such as the continual raising of the standard of good manufacturing practice (GMP) compliance, or the move to increasingly larger phase 3 clinical trials, appear to be based more on perceived risks than on firm scientific principles. The future of effective regulation for vaccines that will allow innovation while protecting the public health must be based on three guiding principles: (i) a firm science base for policies and decisions, (ii) a risk-based approach to implementation of regulatory oversight, and (iii) support for regulatory research to inform these activities. These should be implemented in a setting of international harmonization.

8. Review of current standards and potential needs. *Dr P. Minor*

Nucleic acid standards for attenuated poliomyelitis vaccine virus type 3 and type 2 were developed for the control of OPV during production process. There are many international standards and working reagents with various formulations for the purpose of testing either each virus species or mixed species, such as hepatitis B virus (HBV), hepatitis C virus (HCV), hepatitis A virus (HAV), human immunodeficiency virus (HIV), and parvovirus B19. Also genotype or subtype panels are available for HIV and HCV. There are various standards of viral and bacterial vaccines such as BCG, polio, measles, mumps, vaccinia, yellow fever, etc. New possibilities for establishing standards and collaborative studies are: (i) standards for PERT assay; (ii) quantitation standards for vaccines; (iii) diagnostic standard (e.g. HPV); and (iv) residual DNA standards.

9. Conclusions and recommendations

Various molecular methods such as cloning, Southern blots, dot/slot blots, sequencing, PCR (diagnostic, PERT, infectivity), microarrays and MALDI-TOF are currently being developed or applied to the development, characterization, manufacture and control of vaccines. Some methods are well established and validated, while others are being applied to virus and vaccine characterization at a research level.

The participants of the meeting were in full agreement that basic science and molecular methods for the characterization, standardization, and control of vaccines should be strongly promoted, that collaborative studies are needed for many of the molecular methods and applications, and that investment in training, equipment and

facilities is needed to promote and enhance these developments. The further studies/action needs were proposed:

- For developmental level studies
  - Vaccine potency determination by PCR-based infectivity assays shows good potential and is worth pursuing, continuing with the studies already started with MMR vaccines.
  - Assays to assess risks associated with residual host-cell DNA – these are urgently needed, and the studies described at the meeting are highly welcomed; it was proposed that a working group should ensure the continuation of such studies, assess the implications of the data, and encourage the involvement of other laboratories.
  - MAPREC, MALDI-TOF mass spectrometry and microarray analyses are being developed for the identification of very small unknown mutant populations in a vaccine seed; a current example of this is its use in batch-release testing of the polio vaccine. WHO can assist in this area by collating samples and disseminating information.
  - Microarray technology for the investigation of the molecular pathogenesis of infectious diseases, vaccine and cell substrate characterization, the evaluation of clinical immune responses, and adverse event monitoring has great potential. Further development of the use of this new technology, particularly its use in characterizing vaccines and the immune response to vaccines, is recommended.
  - Further development of generic PCR for virus families is recommended.
- For collaborative studies and working or international reagents
  - PERT assay: An international reference material for use in PERT assays would be useful. This would enable comparisons of assays being run in different laboratories, something that would be useful to those assessing such data, e.g. NCLs. This would require a collaborative study. The assay can also be subject to false positives - a problem that needs attention.
  - DNA quantification: More information is needed by survey/review. A variety of methods are being developed, used and validated to assess the level of residual host-cell DNA, some of which are cell specific; A comparison of methods via a collaborative study would be helpful, and this should be discussed by a WHO working group proposed.
  - PCR assays for extraneous agent testing: PCR is now being used extensively as a diagnostic tool in assuring the freedom of cell substrates and vaccine seeds from extraneous agents. This is good use of the technology and a list of priority agents, e.g. mycoplasma and pestivirus, would be helpful.
  - PCR assays for HPV DNA: PCR technology is being used to determine the infectious status of individuals taking part in clinical trials of novel vaccines

against human papillomavirus. This is an ongoing project within the WHO and should be pursued towards the development of international standard reagents.

Finally centres of excellence were proposed. These centres can take the lead either for trouble shooting, for training, for provision of materials, or for organization of studies. WHO will work in close collaboration with the existing WHO reference laboratories and collaborating centres to assist national regulatory authorities for a broader application of molecular methods to regulation based in science.

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