

Technical Workshop on International Collaborative Studies on HPV Reagents for Laboratory Diagnostic Procedures: A progress report

Immunization, Vaccines and Biologicals



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Abbreviations

ECBS	WHO Expert Committee on Biological Standardization
Geq	genome equivalent
Hep B	hepatitis B (vaccine)
HPV	human papillomavirus
HR	high risk
LiPA	line probe assay
NAT	nucleic acid amplification techniques (assays)
NIBSC	National Institute for Biological Standards and Control
QSB	Quality Assurance and Safe Biologicals, IVB
rHPV	recombinant HPV (genomes)
RIA	radioimmunoassay
UNM	University of New Mexico
VLPs	virus-like particles
VQC	viral quality control

Executive summary

A technical workshop, International Collaborative Studies on HPV Reagents for Laboratory Diagnostic Procedures, was held on 24–25 September 2003 at the World Health Organization (WHO), in Geneva.

This meeting discussed the standardization of HPV reagents and the results of a collaborative international study which evaluated a set of reference reagents in a number of participating laboratories. WHO presented its plans to facilitate evaluation of HPV vaccines, and the establishment of a network of HPV DNA laboratories was also discussed.

Molecular reagents: recommendations

The panel discussion lead by Dr E.M. de Villiers concentrated on a review of experimental results with the goal of making recommendations to WHO on developing HPV DNA standards by consensus opinion of experts, as follows:

- International standards (IS) for HPV 16 and 18, followed by HPV 31, 33, 35, 45, 52 and 58 should be developed.
- Reagents (i.e. HPV DNA) should be from recombinant plasmids.
- Reagents should be monovalent.
- Human genomic DNA purified from C33a cells should be used as diluent/stabilizer.
- Pilot studies of lyophilization of reagents are required.
- Six to seven WHO contact laboratories will initially test rHPV IS.
- A future collaborative study for standardization is to be planned.

Serological standards: recommendations

The panel discussion on serological standards was led jointly by Dr I. Frazer (University of Queensland, Australia) and Dr J. Schiller. A proposal was presented for the further development of WHO HPV reference antisera into international standards.

- Monotypic reference antisera developed for HPV types 16 and 18 should become international standards.
- Other oncogenic monotypic antisera (for HPV types other than 16 and 18) would become increasingly important in the future and might be needed.

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- Selected pools of antisera would be combined, filled and lyophilized, to be distributed to test laboratories for determination of the titres and will constitute the international standard.
 - The potency for each monotypic antisera pool would be defined with respect to sera characterized in the initial studies. It was noted that the actual number assigned is arbitrary and should be given a potency value that is functional with respect to results of the serological tests that the sera will be used for.

1. Molecular reagents for HPV detection

Dr L. Villa (*Instituto Ludwig de Pesquisa sobre o Cancer, Brazil*) welcomed the participants and opened the session.

WHO's plan to facilitate introduction of human papillomavirus (HPV) vaccines was presented by Dr M. Aguado (*WHO Secretariat*) indicating that the challenge we face now is to avoid a time lag between availability of new vaccines and deployment globally. Such a lag occurred for hepatitis B (Hep B) and *Haemophilus influenzae* type b (Hib) vaccines. For example, implementation of hep B vaccine in developing countries did not begin until 13 years after their commercial availability in industrialized countries, and more than 20 years was required to achieve 50% coverage of the target populations. This was due to combinations of delays at several levels: from cost and logistics of distribution, to acceptance and compliance issues. WHO is working to advance HPV vaccines using a parallel track approach so that the knowledge and experience acquired in first-generation efforts in safety, immunogenicity, efficacy against HPV infections and HPV-related cervical lesions, and correlates of protection, will be immediately transferred to facilitate the development of second-generation vaccines more suitable for public health use. Work is currently being done to fill in gaps in the knowledge about the country-specific prevalence of HPV types in cancer, to analyse the cost-effectiveness of vaccination, and to initiate advocacy for HPV vaccines. Standard HPV diagnostic reagents and assays are needed immediately. In 2001, WHO began to discuss HPV vaccine issues. From September 2002 to February 2003, WHO convened a series of workshops in India, the Philippines and Republic of Korea to explore the possibility of HPV vaccines as a tool to prevent cervical cancer. Finally, in May 2003, HPV and cervical cancer experts met to discuss appropriate outcome measures of HPV vaccine efficacy, especially in developing countries.

Dr S. Pagliusi (*WHO Secretariat*) reminded participants that the objective of WHO is the attainment by all peoples of the highest possible level of health. The functions of WHO, as elaborated in the constitution, include the standardization of diagnostic procedures and the establishment of international standards. The mission of the WHO Department of Immunization, Vaccines and Biologicals (IVB) is to achieve a world in which all people at risk are protected against vaccine-preventable diseases. Thus it was appropriate for the Department to convene this meeting to pursue collaborative studies on the international harmonization of HPV laboratory diagnostic procedures. This effort began in March 2001 at a meeting convened by WHO in Heidelberg and was followed by the September 2001 workshop in Florianopolis. As a result of these meetings, international collaborative studies to evaluate reference reagents for type-specific HPV DNA detection, type-specific HPV serological assays, and cell-mediated HPV immunity were launched in September 2002.

The current meeting would discuss the experimental results of these studies, the advisability of publication of the results in peer-reviewed journals, and the establishment of a network of HPV DNA laboratories to facilitate international harmonization of assay results.

Results of the first international collaborative studies

Dr C. Wheeler (*University of New Mexico, USA*) reviewed the study design of the international collaborative studies on HPV detection assays using a candidate WHO reference panel. The goals of the study were to determine the participating laboratory's ability to correctly identify HPV types in a background of human DNA in the presence of other HPV types or alone, as well as to determine the analytic sensitivity of assays for HPV 16 and 18. Plasmid cloned HPV genomes were agreed to be the optimal choice for reagent standards. This decision was made recognizing that this would not allow HPV types cloned within the L1 region to be included, as this region is targeted by many HPV detection assays. The intellectual property issues surrounding these HPV clones were clarified with the involved parties by WHO, and authorizations were obtained before reagent preparation. Dr Wheeler's laboratory prepared and purified plasmids for HPV types 6, 16, 18, 31, 33, 35, 45 and 52. The optical density and picogreen DNA quantitation established for each preparation agreed within 2–3 fold among laboratories. Human cellular DNA was extracted from cultures of cell line C33A. These materials were sent to the CLB-Sanquin laboratory, where a panel of 24 samples was prepared. The reagent-panel included: plasmids with cDNAs of single HPV types (HPV 6, 16 and 18) at 100 genome equivalents (low copy number) or 10 000 genome equivalents (high copy number) in a background of 10 000 human genomes per 10 microlitres; the same materials spiked into a mixture of plasmids with cDNA of high risk HPV types (HPV 31, 33, 35, 45 and 52), in a background of human DNA, and a dilution series of HPV 16 and 18 (0.01, 0.1, 1.0, 10, 100, 1000 and 10 000 genome equivalents) in the background of human DNA as mentioned above.

The panel was tested in two reference laboratories (University of New Mexico, USA, and Delft Diagnostic Laboratories, the Netherlands) prior to distribution to the participating laboratories. Results on the panel from both reference laboratories were in general agreement (see Table 1). Then the aliquots were sent on dry ice with instructions for use and data collection forms for summary information on methods and results. Laboratories were encouraged to run the panel-assays at least twice on different days. To assure laboratory confidentiality, completed forms were to be returned to the neutral office (CLB-Sanquin) for summary and analysis of results. Each laboratory was to receive an evaluation report in which their own results were to be presented in relation to the aggregate results. It was expected that these results would be prepared for publication.

Table 1: Results of HPV DNA testing by reference laboratories

Sample code	Geq HPV	UNM	DDL	Sample	Geq HPV 18	UNM	DDL
24	100 000	16	16	22	100 000	18	18
8	10 000	16	16	1	10 000	18	18
21	1 000	16	16	20	1 000	18	18
16	100	16	16	11	100	18	18
19	10	Neg	Neg	15	10	Neg	18
23	1	Neg	Neg	18	1	Neg	Neg
9	0.1	Neg	Neg	12	0.1	Neg	Neg
5	0.01	Neg	Neg	4	0.01	Neg	Neg

UNM = University of New Mexico, Roche line probe assay

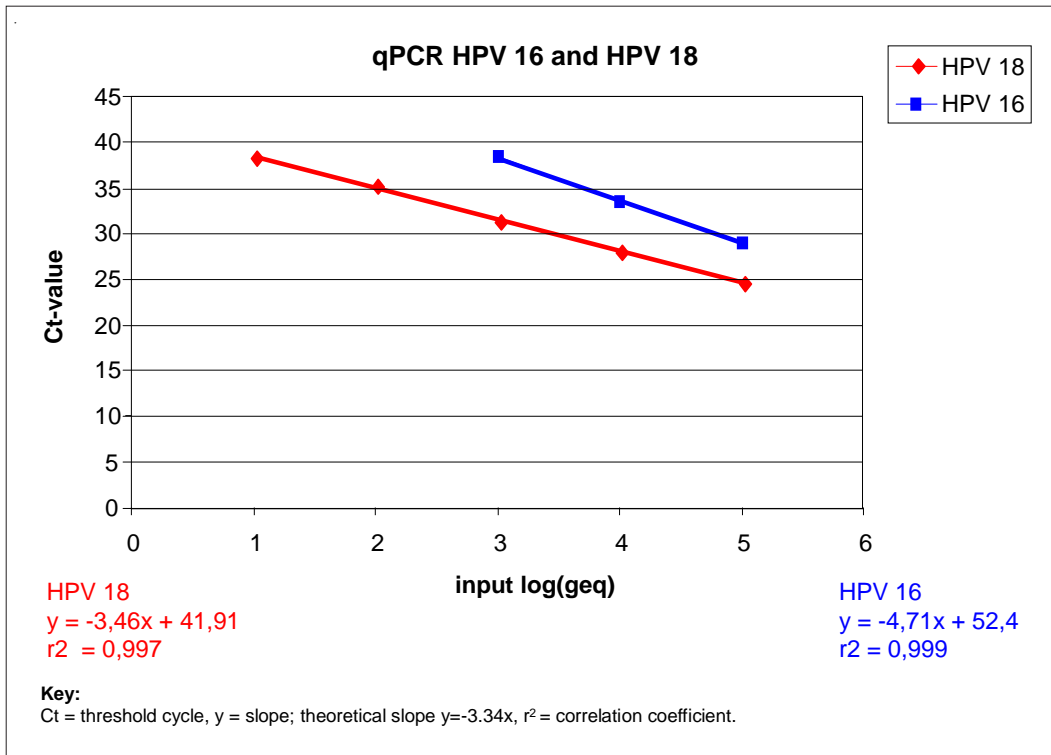
DDL = Delft Diagnostic Laboratory

SPF10 = short PCR fragments

LiPA = Line probe assay; Geq = genome equivalent

Dr N. Lelie (*Viral Quality Control – VQC – international laboratory, the Netherlands*) described the preparation of the WHO candidate HPV-DNA reference panel in more detail. After preparing the panel of samples in the background of C33A human genomic DNA, the VQC laboratory used a β -globin quantitative PCR (qPCR) to verify that this matrix DNA was equally distributed in all samples in the panel. The panel was delivered to the two groups serving as reference laboratories for the study, the University of New Mexico and Delft Diagnostic Laboratory (DDL). The UNM laboratory used PCR with Roche line blot detection kit and DDL used line-PCR assay kit (LiPA) and a type-specific qPCR assay. The qPCR on the dilution series of HPV 16 and 18 showed the HPV 16 detection limit was approximately 2 logs less sensitive than the HPV 18 detection limit. The amplification efficiency was ideal for HPV 18 but was lower for HPV 16. Low copy number HPV 16 could not be detected in the background mixture of high risk HPV types. The difference between HPV 16 and 18 detection could reflect differences in the assay performance characteristics, the physical state (i.e. supercoiled, nicked, etc.) of the HPV 16 and 18 plasmids affecting amplification, or in the template concentration (Figure 1). The reagent panel was distributed to 29 laboratories.

Figure 1: Linearity of dilution series HPV 16 and HPV 18 plasmid DNA



Dr Wim Quint (*Delft Diagnostic Laboratories, Netherlands*) presented a summary of the 33 data sets returned by 24 laboratories. Although not all of the returned questionnaires were fully completed, the methods used in the 32 data sets included the most commonly used assays. Some methods were only represented by one data set. The methods are summarized in the following table.

Table 2: Different typing methods used in the international collaborative study to detect HPV DNA and frequency of their use

Method	Number of data sets
PGMY-line blot	8
SPF10-LiPA	5
Digene hybrid capture	3
qPCR HPV 16 and 18	3
Roche MWP assay	1
Deg GP5+/6+ reverse line blot assay	1
Deg GP5+/6+ Biomed DNA chip	1
Other PCR methods	11

MWP = multiple well plate

The criteria for proficiency of results were correct identification of the negative sample and a logical pattern of detection in the HPV 16 and 18 dilution series. On this basis, 26 of the 33 data sets (79%) were deemed qualified for analysis of correct detection of HPV 16 and 18 in the presence of the high risk (HR) HPV mixture and correct identification of the other HPV genotypes. Probit analysis determined the PCR detection limits of HPV 16 and 18 in the dilution series to be 3×10^{-6} and 1.8×10^{-8} respectively, indicating a difference of 52 between the detectability of HPV 16 and 18.

The Digene and Roche Multiple Well Plate (MWP) datasets were not included in the analysis of the impact of the mixture of other HR HPV types on the detection of HPV 16 and 18. While fewer data sets detected low copy number HPV 16 spiked into the HR HPV mixture, the difference did not achieve statistical significance. Dr Quint suggested that the difference in detection of HPV 16 and 18 should be further investigated. Results of detection of the HPV types within the mixture demonstrated that some PCR typing assays did not consistently detect HPV 31 and 35. Further analysis of the current results will require that laboratories complete the method data collection forms. Wider use of proficiency and reference panels is warranted.

The strengths and weaknesses of the reagent panel were discussed. As currently constructed, the panel does not address the efficiency of sample extraction. The DNA clones used for the study were the reference clones HPV 16 and HPV 18 as isolated at the HPV Reference Center in Heidelberg. It was suggested that cell culture DNA might not be a clinically relevant human DNA background, and pools of "clinical cervical samples" collected in varying transport media could be considered as alternatives. The mixture of HPV types could be expanded. The difference in performance between the HPV 16 and HPV 18 samples was not clear and could be attributed to errors in dilution, DNA degradation, physical state of the HPV plasmids, differences in assay detection limits or differences in the assay efficiency. Additional panel materials based on plasmid DNA could be prepared to compare the stability of lyophilized versus frozen materials.

It was noted that panel sample volume in aliquots received by some laboratories was not consistently the intended volume of 115 μ l. As aliquots were sent on dry ice, problems with the temperature, tubes and/or transportation may have occurred. It was noted that performance of polypropylene tubes varies. Based on the sensitivities reported in this study, the question was raised as to how many HPV 16 positive women would be potentially missed by these assays and whether an acceptable lower limit of sensitivity should be specified. There was consensus that specifying an acceptable clinical sensitivity for HPV 16 should not be addressed at this workshop. Instead it was agreed that it is important to have a standard for HPV copy number and that translation of results to detection of HPV in clinical samples could be established in a second step.

While data collection was performed in a neutral laboratory, analysis of results was carried out in cooperation with a reference laboratory. While the hard work of statistical analysis was recognized and appreciated, there was consensus that future and further analyses should be carried out by a neutral laboratory only. It was mentioned that some variations in the results could be due to differences in the assay performance, differences in laboratory experience, or both. One of the limits of this study is that some assays were performed only by one laboratory.

Ideally, a WHO international standard would be lyophilized. It was mentioned that commercial clinical assays (subject to US Food and Drug Administration – FDA – approval) might be very different from assays used to evaluate clinical trials. It was agreed that the **WHO standards would be primary reference standards used to calibrate in-house samples and standards**. The primary reference standard would not be included in every assay, whereas the secondary quality control (QC) standards would be.

Establishing an international standard

Dr D. Wood (*Quality Assurance and Safe Biologicals – QSB – WHO Secretariat*) described the role of the WHO Expert Committee on Biological Standardization (ECBS). WHO has a constitutional responsibility – mandated by the 192 Member States through the World Health Assembly – to ensure biological standardization. An expert panel and committee on biological standardization implement this process through the development of written guidelines for the production and quality control of vaccines and other biological medicines, by creating reference preparations for vaccines and biological medicines and by providing scientific guidance in the conduct of research on quality and safety issues. WHO – as part of its normative function – publishes guidance for national regulatory authorities and manufacturers on the technical specifications for the production and quality control of vaccines. This guidance, published in the technical report series, defines international expectations for safety and efficacy of vaccines, and Member States may use this information in their immunization programmes.

In addition, WHO establishes international biological standards and reference reagents for substances of biological origin. These standards are established in circumstances where the biological substance cannot be adequately assessed by chemical and/or physical means alone, and when they are used in prophylaxis, therapy or diagnosis of human disease. WHO international reference material can be used to create international standards, which are established to enable the activity of biological preparations to be globally expressed in a standard format (IUs). These international standards are then used as primary calibrants in the calibration of in-house controls. WHO international reference materials can also be used as reference reagents, where they provide a means of checking the specificity of diagnostic reagents or are used in the comparison of research data for assays in the fields of prevention, treatment and diagnosis of disease. These reagents can serve as tools for comparison of results between different assays (biological measurement), to support harmonization of international regulations, to underpin appropriate clinical dosage, and to facilitate the development of diagnostic and therapeutic products.

Generally, prior to establishing an international standard, there first needs to be an assessment of need, to be established by an independent scientific body or group of experts. Thereafter, a high quality source of the reagent is distributed in final containers. The current batch size that the National Institute for Biological Standards and Control (NIBSC) can produce is 3000 vials but this capacity will soon increase to 10 000 when a new filling device becomes available. The quality of the final containers is critical for ensuring the long-term stability of the reference material. Heat-sealed ampoules are preferred to rubber-stopped vials and the coefficient of variation for the fill process should not exceed 0.25%. Most international standards are lyophilized to ensure that the required 10-year shelf life is attained.

Real-time and accelerated stability testing using the Arrhenius equation are performed to determine and validate the shelf life of the product. Once a candidate international standard preparation has been developed, international collaborative and blinded studies are conducted to determine the suitability of a preparation. Ideally, these collaborative studies should be conducted using a standard protocol and participants should be from more than one country. Data collected through the collaborative studies will be analysed and a written report created. The WHO Expert Advisory Committee reviews this report and makes a final recommendation regarding the standard.

Dr Wood articulated that it is still very early to define the HPV standard, and although he appreciates the need for an HPV DNA standard, he urged the group to come to a decision on HPV genotype and on the required specifications of that standard. It was concluded that WHO has considerable experience and well-established procedures for standardization of biological assays. WHO international standards are widely used to facilitate comparison of results between laboratories, especially for regulatory purposes. A series of nucleic acid standards for HIV (human immunodeficiency virus), hepatitis C virus (HCV), hepatitis B virus (HBV) and hepatitis A virus (HAV) have been created for nucleic acid tests (NAT), as any NAT assay is considered a biological assay. Regarding the international standards for HPV, Dr Wood requested that a progress report on HPV standardization be presented at the next ECBS meeting in November 2003. The ECBS can advise the group on the need for a reference reagent versus an international standard, but it will not provide a recommendation on what assays should be used for NAT detection. These standards can also be used by in vitro diagnostics manufacturers to determine the sensitivities of their assays.

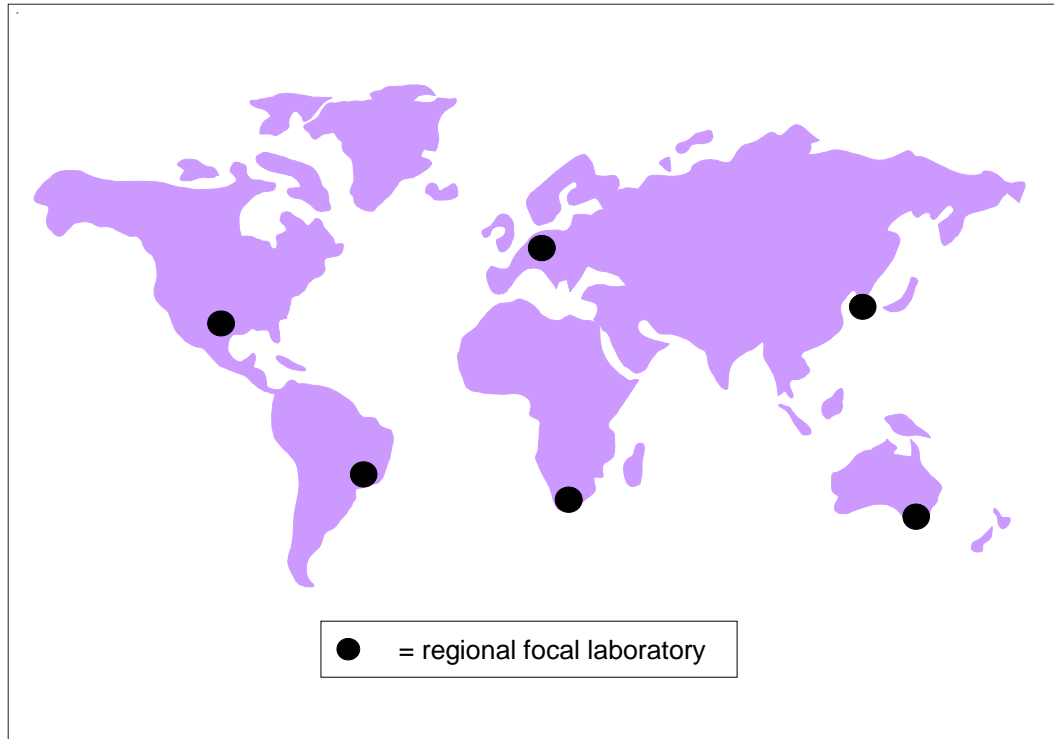
The participants agreed that it is important to look at the relevance of the proposed standards for applications in both vaccine studies and clinical diagnosis. In summary, it was agreed that there is a need for international standards in the field of HPV and the group should proceed with their development. The genotype and units of expression (copy numbers or viral load) of these standards need to be further discussed and defined. Although these standards may have applications to in vitro diagnostics, WHO's primary focus would be to accurately measure the efficacy of an HPV vaccine.

Proposal on laboratory network for HPV DNA detection

Dr S. Garland (*Women and Children's Hospital in Melbourne, Australia*) presented a proposal for the creation of a laboratory network for HPV DNA detection. This network would provide technical advice and annual training in HPV DNA assays to other laboratories involved from the same geographical region. The main objective would be to disseminate good practice on HPV DNA detection methodology and advocate for high quality standards, independent of assay format used. Laboratories within the network could serve as repositories for standard reagents, if necessary, to enable inter-laboratory and intra-laboratory comparisons of NAT assays. It was proposed that six well established HPV laboratories be selected among the study participants, distributed so as to assure global coverage (Figure 2). Although the establishment of a network is an important activity, every effort should be made to link with existing private and government organizations rather than creating new centres. It will be key to first establish the technical capabilities of laboratories. It was agreed that a small task force would be created to define the

mission of the network, to further discuss the selection criteria for training laboratories, and to define the activities and objectives of a given laboratory. There is a need for a strengths, weaknesses, opportunities and threats (SWOTs) analysis to better define the overall objectives of the network and to establish evidence-based decisions on the selection of appropriate laboratories.

Figure 2: Proposed global laboratory network of HPV DNA detection.



Panel recommendations on HPV DNA standards

- 1) **Assessment of need.** Three areas of activity will benefit by the availability of standards:
 - Vaccine studies require the assessment of cervicovaginal HPV DNA to document prevalence in a trial population, potential responses to vaccination in terms of HPV infection incidence and natural history, and subsequent distribution of HPV DNA types in lesions and tissue subsequent to vaccination. Standardization will allow comparison of vaccine effectiveness across studies and geographic areas.
 - Clinical diagnostic laboratories currently use a variety of commercial, regulated and independently developed assays. The availability of standards will allow laboratories to validate their assays and determine analytical sensitivity.
 - Epidemiological prevalence studies and surveillance studies have a need to compare and contrast HPV DNA results over time between different geographic locations, populations, and anatomic sites.

2) Reference materials or international standards?

It was unanimously recommended that the world would be best served by development of international standards (IS). The sensitivity of different HPV assays used by laboratories and investigators would be determined through development of “in house” standards based on international standards. The IS are not produced to serve as day-to-day reagents but for laboratory collaboration and comparison. It was felt that the reference reagents that were developed and tested in the collaborative study will not meet the analytic needs of the research, clinical, vaccine and industrial communities.

3) Composition of international standards

- a) **HPV types.** Eight HPV DNA types were recommended by majority vote for development into international standards. Priority and rapid development of HPV 16 and 18 IS were uniformly recommended. Choice of HPV DNA types was based on prevalence in cervical cancer and includes: HPV 31, 33, 35, 45, 52 and 58. The majority favoured parallel development of these reagents to avoid potential delay as subsequent HPV-type vaccines are developed. If a priority for development in sequential order is necessary, consideration of global prevalence in cervical cancer should be given priority. Dr M. Doeberitz (University of Heidelberg, Germany) volunteered to reclone any HPV genomes if necessary to avoid disruption of the L1 (open reading frame). There was also discussion of whether HPV 6/11 IS should be developed since these HPV types are currently in vaccine trials. Since these types do not contribute to global mortality, there was little enthusiasm for inclusion in the IS panel at this time.

Majority opinion was to develop monovalent or individual type standards. This will allow unequivocal calibration of type-specific assays. There was some discussion on developing 16 and 18 individually and mixing the others. However, the lack of analytic sensitivity in the collaborative study and the complexity and possible interference of multiple types in PCR assays were viewed as reasons to move forward with individual type-specific IS.

- b) **Diluent/stabilizer.** A solution/material is needed to dilute the recombinant HPV genomes (rHPV). This material should have the properties of stabilizing dilute amounts of rHPV that are prone to loss over time, be readily available for present and future use, lack infectious materials and possibly simulate conditions of clinical HPV samples.

Four materials were discussed:

- **C33a:** genomic DNA from a cervical cancer cell line. This material was successfully used in the collaborative study (i.e. tested negative for HPV DNA in 97% of laboratories) and builds upon reagent testing procedures. It is readily available from commercial sources, renewable and practical. Although not discussed, the growth of cells in serum-free medium should be considered to reduce transmission of potential infectious agents present in bovine serum. Use of C33a DNA was agreed to be the best choice.
- **Exfoliated cells collected from cervicovaginal lavage (CVL) negative for HPV DNA:** This was not favoured because potential infectivity, difficulty in validating presence/absence of HPV DNA and logistic procedures would make it impractical.

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- ***E. coli* DNA:** This material was renewable, but most laboratories had less experience with this. In addition, this material is not likely to be appropriate since it does not represent a human source for background DNA that is critical to sensitivity and specificity determinations using assays that are based on target amplification.
 - **White blood cells:** DNA extracted from this cheap, readily available material was used as diluent in the ALTS trial (ASCUS/LSIL Triage Study), but concern was raised about carriage of infectious agents and lack of experience by most groups.
- c) **Production of HPV recombinant DNA.** There was discussion on whether the plasmids should be produced by contract laboratories, and it was concluded that the optimal production would be in a contract GMP facility, with an academic laboratory as second choice. Technical details of preparations were not discussed.
- d) **Concentration of HPV DNA.** There was brief discussion on concentration, whether dilution, absolute weight or genome numbers should be used as the standard nomenclature of the IS. A recommendation of 10 000 genomes per 10 µl (i.e. 10⁷ genomes/ml) was made. This area deserves further clarification.
- e) **IS form – lyophilization.** Comments from NIBSC indicated that standard procedures were to lyophilize the reagent. It was noted that pilot testing of these procedures would be necessary, especially with the use of human genomic DNA as diluent.
- f) **Calibration of IS.** At least three levels of calibration/standardization were identified: (i) subsequent to plasmid purification and prior to individual aliquoting and lyophilization; (ii) subsequent to lyophilization and prior to distribution to large numbers of laboratories for characterization and standardization, (iii) characterization and standardization.

It was recommended that the six WHO focal laboratories and a reference laboratory, seven in total, would be sufficient for (i) and (ii) above.

Formal standardization/characterization of IS in 20–30 or more laboratories distributed globally and using multiple-testing procedures would be decided at a later date. Development of this study should be aimed at validation of quantity and quality of the IS. Procedures, methods and study design should be developed in collaboration with experienced groups such as the Centers for Disease Control and Prevention (CDC) Public Health Laboratory Practices group.

Other comments

The WHO candidate HPV DNA reference panel testing described by Dr Quint was deemed a successful pilot study, documenting that reagents could be prepared, distributed and tested by several laboratories and using various methods. There was overall consensus on HPV type specific sensitivity. Further discussions on how the process could be improved will follow.

Dr J. Weir and Dr R. Levis (*Food and Drug Administration, USA*) commented on their activities in HPV vaccine monitoring for vaccine licensure. They have no laboratory activities but supported the need for international standards to compare clinical trials. IS are not required for agency requirements.

Dr E. Unger (*Centers for Disease Control Prevention, USA*) described two ongoing activities, which included prevalence studies through self sampling and sentinel surveillance of samples obtained by health care providers. However, many other groups at the CDC had an interest in HPV including: National Immunization Section, the Chronic Disease Program and the Public Health Laboratory Practices groups.

Other issues which were discussed included:

- the need for creative funding to finance development and future use of HPV IS;
- the impact of “intellectual property” rights and use of recombinant HPV DNA;
- nomenclature, and international unit of standard was not discussed this time.

2. Serological reagents for HPV detection

International collaboration

As there are no serum standards for HPV serology, an international collaboration was set up to test sera for HPV 16, 11 and 18 antibodies as a starting point in the process of providing standard reagents. An international serum standard would improve inter-laboratory and intra-laboratory assay reproducibility for measurement of antibody levels, and establish whether assays worked in the presence of antibodies against other HPV types. HPV serology has a number of applications within the vaccination context that include screening volunteers for the vaccine trials for previous exposure to HPV, screening vaccinees for HPV seroconversion and using epidemiology to determine the prevalence of HPV seropositive people within a given population. The latter will provide background information for HPV vaccine trials.

Dr J. Dillner (*Lund University, Sweden*) gave an outline of the criteria used for the selection of HPV positive and negative sera. Briefly, monotypic sera against HPV 16, HPV 18 and HPV 6 were selected from women with natural infection who reported one lifetime partner. These types were selected because they are the types currently being tested in vaccine trials. The negative control serum came from a virginal woman whose serum tested negative with radioimmunoassay (RIA) and enzyme-linked immunosorbent assay. A panel of sera was selected from women with natural infections and from women participating in HPV vaccine trials. Of the 16 sera samples sent to three reference laboratories, 12 were chosen to be sent to an additional seven laboratories for further testing. The final selection of the sera was made by a neutral office (NIBSC). The samples included four individual sera from women with natural infection (two with HPV 16, one with HPV 18, and one with HPV 6/11), two pooled sera from naturally infected women reactive against HPV 6+11+16+18, five sera from vaccinated women and one negative serum from a virginal woman. Virus-like particles (VLPs) were identified as another component potentially requiring standardization in immunoassays. If the VLPs are not of good quality and intact then this will impact on the quality of the assay.

There are a number of serological assays for the detection of HPV antibodies, and these were outlined by Dr K. Jansen (*Merck, USA*). Enzyme-linked immunosorbent assays are the most common assays and are highly dependent on the quality of VLPs used in the assay (Carter et al., 1996; Kirnbauer et al., 1992). Competitive radioimmunoassays are also sensitive, type-specific assays as they are based on competition with an HPV type-specific and neutralizing monoclonal antibody (Palker et al., 2001). A competitive multiplex assay (Luminex) has been recently developed which is sensitive and can detect antibodies against multiple HPV types in the same tube (Opalka et al., 2003). Briefly, phycoerythrin-targeted

HPV type-specific neutralizing antibodies compete with a test serum for limited binding sites on VLP-coated beads. Neutralization assays are more difficult to perform but are more type specific. As it is not possible to obtain infectious HPV most neutralization assays are based on pseudovirions.

Three reference laboratories performed a series of titration tests on 16 candidate sera samples, labelled NIB-HPV-01 through NIB-HPV-16, provided by the WHO international laboratory for biological standards (NIBSC). Among reference laboratories some discordance on results was evident (Table 3) and may reflect differences in the sensitivity and specificity of assays used.

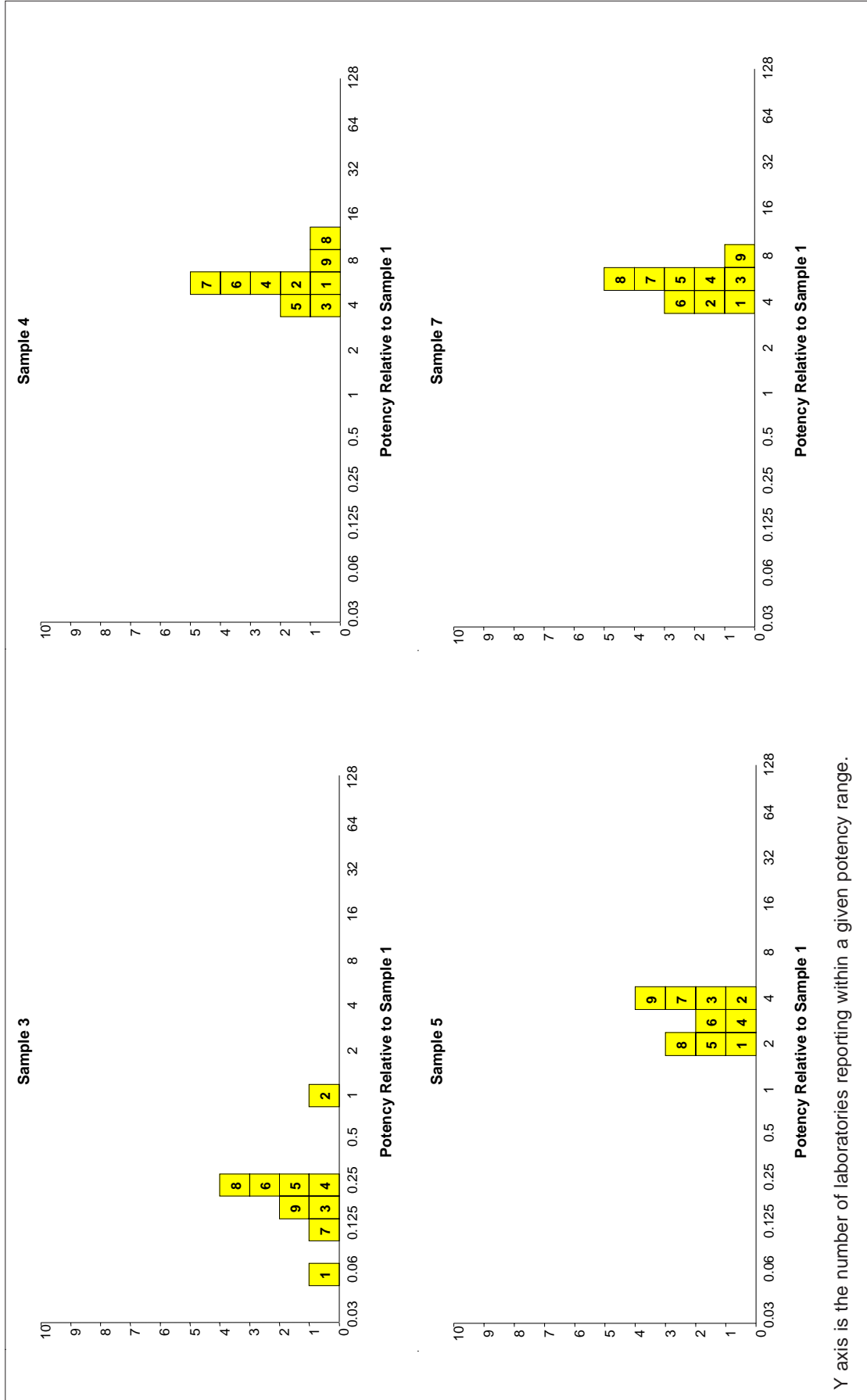
Analysis of the data from the laboratories was presented by Dr M. Ferguson (*NIBSC, United Kingdom*). There was variation in the sensitivity and specificity of the laboratories on the various immunoassays. However, when the HPV 16 serology results were presented by potency of sera relative to a positive HPV 16 arbitrarily defined as potency “one”, then performance was similar in all the laboratories as exemplified in Figure 3 (NIBSC report). It was concluded that the expression of antibody concentration in each sample relative to NIB-01 improves the reproducibility between laboratories, and this indicated the value of having an international standard serum. It was agreed that these results should be published.

Dr J. Schiller (*National Institute of Health, USA*) presented a novel method of pseudovirions production and a novel neutralization assay. The capacity of antisera to neutralize viral infection in vitro is well accepted as the “gold standard” assay to test protective immunity conferred by vaccination. However, as no efficient system for replicating HPV in laboratory conditions exists to date, the evaluation of HPV neutralization relies mostly on pseudo-neutralizing assays. Those are xenograph or organotypic graft assay, intracellular or extracellular assembly of pseudovirions based on reporter genes (Kreider et al., 1987; Roden et al., 1996; White et al., 1998; Yeager et al., 2000). To overcome sensitivity and feasibility problems, a high sensitivity papilloma pseudovirus neutralization assay has been developed based on the expression of L1/L2 genes in transfected 293 TT cells under the control of the SV40 promoter and carrying a reporter gene. Following an efficient lysis and extraction procedure to separate pseudovirions from cellular debris, within three days billions of pseudovirions can be produced in this way in relatively small T 150 flasks. Supernatants of transfected cells, with or without neutralizing sera, can be assayed for the reporter gene activity, secreted alkaline phosphatase using chemiluminescent substrates that amplify the sensitivity of the assay. It is a high throughput assay because it is processed in multiple well plates. The specificity of this assay warrants further investigation for applicability in vaccine research.

Table 3: Results of serological testing

Assigned code	Neutral. HPV serotype	Lab A HPV16	Lab B HPV 16	Lab C HPV 16	Lab A HPV 16	Lab B HPV 18	Lab C HPV 18	Lab A HPV 18	Lab B HPV 6	Lab A HPV 6	Lab B HPV 11	HPV 11
NIB-HPV-01-02	nat. infection (16)	(+)	(+)	(+)	(+)	(-)	(-)	(-)	(-)	(+)	(+)	(-)
NIB-HPV-02-02	nat. infection (16)	(+)	(+)	(+)	(-)	(-)	(-)	(-)	(-)	(+)	(-)	(-)
NIB-HPV-03-02	Pool nat. infection (6,11,16,18)	(+)	(+)	(+)	(+)	(-)	(+)	(+)	(-)	(+)	(-)	(-)
NIB-HPV-04-02	vaccine	(++)	(++)	(++)	(++)	(+)	(+)	(-)	(-)	(+)	(+)	(-)
NIB-HPV-05-02	vaccine	(+)	(++)	(++)	(+)	(+)	(+)	(-)	(+)	(+)	(+)	(+)
NIB-HPV-06-02	vaccine	(++)	(+)	(++)	(+)	(+)	(-)	(-)	(-)	(-)	(+)	(-)
NIB-HPV-07-02	vaccine	(++)	(++)	(++)	(++)	(+)	(-)	(-)	(-)	(-)	(-)	(-)
NIB-HPV-08-02	nat. infection (16)	(+)	(-)	(+)	(+)	(-)	(-)	(-)	(-)	(-)	(-)	(-)
NIB-HPV-09-02	nat. infection (6,11,16,18)	(+)	(-)	(+)	(+)	(+)	(+)	(+)	(-)	(+)	(-)	(+)
NIB-HPV-10-02	vaccine	(+++)	(+++)	(+++)	(+++)	(-)	(+)	(-)	(-)	(-)	(-)	(+)
NIB-HPV-11-02	vaccine	(+)	(++)	(++)	(+)	(+)	(+)	(-)	(-)	(+)	(+)	(-)
NIB-HPV-12-02	negative	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)
NIB-HPV-13-02	nat. infection (18)	(-)	(-)	(-)	(-)	(+)	(+)	(+)	(-)	(-)	(-)	(-)
NIB-HPV-14-02	vaccine	(+++)	(+++)	(+++)	(+++)	(+++)	(++)	n.a.	(+++)	(++)	(+++)	(+)
NIB-HPV-15-02	nat. infection (18)	(-)	(-)	(-)	(-)	(+)	(++)	(+)	(-)	(-)	(-)	(-)
NIB-HPV-16-02	nat. infection (6,11)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(+)	(-)	(-)

Figure 3: Potency of random samples relative to sample NIB-HPV-01-02



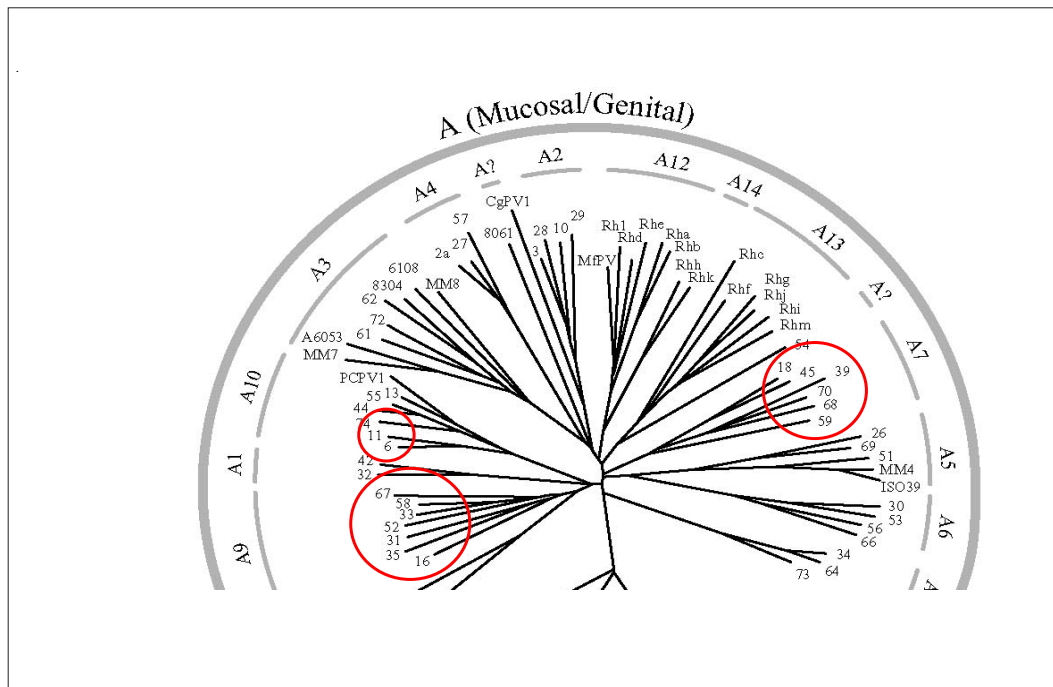
Y axis is the number of laboratories reporting within a given potency range.

Discussion

The group agreed that an international standard was needed. The first standard would be ideally antisera against HPV 16 followed by HPV 18. HPV 6 and HPV 11 antiserum standards were not seen as a priority, as these types of HPV are not strongly linked to cancer development. It is desirable to have serum that is monospecific and that does not cross-react with other HPV types, indicative of a single HPV infection. Therefore, the sera should be checked for cross-reactivity against a panel of oncogenic HPV types and particularly clade A9 HPVs (see Figure 4 below). Dr P. Coursaget (Tours University, France) offered to provide such a panel. Dr M. Pawlita (DKFZ, Germany) offered to provide HPV type-specific capsomers. Dr Jansen offered to test for cross-reacting sera using RIA.

In order to have an international standard, 3000–4000 vials of serum at 0.5–1 ml per vial are needed. The reagent should be pooled as undiluted serum that is negative for human pathogens. Standard sera should be from naturally infected women. There was some discussion of the potential sources of the serum. The possibilities include women participating in longitudinal studies or serum from a commercial company. Whatever the source of serum, it should be collected with informed consent and the collection approved by the appropriate ethics committee. There was a requirement for serum to be collected, as heparin in plasma is known to inhibit some of the assays. This serum would provide an international standard that would be used for the calibration of in-house tests.

Figure 4: Phylogenetic tree of mucosal HPV genotypes



Panel recommendations on HPV sera standards

1) Assessment of need

The panel was reminded that for WHO to propose and develop an international standard, a need must be demonstrated. The panel agreed on the need for such a standard as this would improve HPV serology assays, allow valid comparison of results among laboratories, and facilitate vaccine effectiveness monitoring. The first item of this proposal was that monotypic antisera be developed for HPV types 16 and 18. Identified sera would be prepared and distributed via NIBSC to selected laboratories for formal testing. The laboratories of Virologie Moleculaire, Tours University, National Institutes of Health, Bethesda, and Lund University were suggested as laboratories that could help in this characterization. In addition, Dr Schiller suggested that Johns Hopkins, USA, be included in the studies as he has a source of VLPs that can serve as antigens for testing the sera. The selected laboratories would test the antisera for specificity, titre and reactivity with other HPV types. In addition, Dr Schiller's laboratory would test the sera for their neutralization capacity. The testing results would be assembled by NIBSC and a consensus would be reached on the specific sera to be pooled. Most likely, each testing laboratory would individually rank the tested sera. Specific criteria (e.g. cross-reactivity with other HPV serotypes) would still need to be defined.

2) Reference materials or international standards

The intent would be for these antisera to become international standards, agreed by all participants.

3) Composition of international standards

- a) **HPV types.** Following an agreement on the first item of the proposal to develop monotypic HPV 16 and HPV 18 international standards, the focus of the discussion turned to the specifics of implementation. It was determined that 2–3 litres of high-titre serum were necessary for each HPV type to be used as an international standard. To obtain this volume, it will be necessary to use multiple donors. There was general agreement on the sources of donors for the identification of the monotypic antisera. Such donors should be less than 25 years of age; the sex of the donor is irrelevant; and details of the donor's history should be optional, not mandatory. However, it is essential that donor sera be screened for all relevant blood-borne pathogens. Donor sera could also be obtained from commercial sources for logistic reasons. However, sera used in the previous collaborative serology study would be re-evaluated in the new study to identify any sera that could be included in the monotypic sera pools. The panel noted that sera should be obtained from reputable sources. In addition, during screening it should be verified that heparin was not used during collection, as this would compromise the use of sera for subsequent neutralization assays. Dr Jansen offered to conduct the initial screening of commercially available blood samples. She stated that she will be able to screen hundreds of sera samples and will obtain approximately 20 of moderately high titre for both HPV type 16 and type 18. Once the initial screening is completed, the sera will be better characterized by individual laboratories.

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- b) **Negative controls.** There was some discussion about whether negative HPV sera should be provided along with the type 16 and 18 sera standards. The group concluded that monotypic type 16 and 18 sera should be developed as international standards and that the negative control used in the initial study would be provided by NIBSC as a running control to help laboratories in the evaluation of their assays. It would be expected that laboratories receiving the standards and the negative control would develop their own set of negative controls for their individual assays in the long term. Lyophilization of sera has been successful with other international standards and it is expected that it will not pose any hurdles in the case of HPV sera.
- c) **Lyophilization.** Based on results of characterization, selected pools will be combined, filled and lyophilized at NIBSC. These vials will constitute the international standard and will be distributed to test laboratories for determination of the potency. The potency for each monotypic antisera pool will be defined with respect to sera characterized in the initial studies.
- d) **Calibration of IS.** It was noted that the actual number assigned is arbitrary and should be given a potency value that is functional with respect to results of the serological tests that the sera will be used for.

4) **Other monotypic antisera**

The panel noted that it was likely that other monotypic antisera (for oncogenic HPV types other than 16 and 18) would become increasingly important in the future and other standards might be needed.

Other comments

Three other related issues were discussed briefly.

- The time line for implementation of this proposal is as soon as possible. This ended the discussion concerning the recommendations for development and implementation of HPV antisera standards.
- Dr Schiller remarked that the previously discussed proposal was not dependent upon standardization of VLP quality. But this was an issue that the group should be thinking about: i.e. how to obtain high quality preparations of VLP. There was a brief discussion that companies involved in vaccine manufacturing might be willing to supply such reagents. The probability of this is unknown at the present time.
- Finally, there was a brief discussion on whether the panel should also consider the need for standards for cell-mediated immunity assays (i.e. particularly for evaluation of therapeutic HPV vaccines). It was concluded that this area should be kept under review as product development advances.

3. Brief summary of recommendations

Recommendations to WHO on developing HPV DNA standards were:

- International standards (IS) for HPV 16 and 18, followed by HPV 31, 33, 35, 45, 52 and 58 should be developed.
- Reagents (i.e. HPV DNA) should be from recombinant plasmids.
- Reagents should be monovalent.
- Human genomic DNA purified from C33a cells should be used as diluent/stabilizer.
- Pilot studies of lyophilization of reagents are required.
- Six to seven WHO contact laboratories will initially test rHPV IS.
- A future collaborative study for standardization is to be planned.

A proposal was presented for the further development of WHO HPV reference antisera into international standards.

- Monotypic reference antisera developed for HPV types 16 and 18 would become international standards.
- Other oncogenic monotypic antisera (for HPV types other than 16 and 18) would become increasingly important in the future and might be needed.
- Selected pools of antisera would be combined, filled and lyophilised, to be distributed to test laboratories for determination of the titres and will constitute the international standard.
- The potency for each monotypic antisera pool would be defined with respect to sera characterized in the initial studies. It was noted that the actual number assigned is arbitrary and should be given a potency value that is functional with respect to results of the serological tests that the sera will be used for.

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