Meeting Report

WHO workshop and practical course on Human Papillomavirus (HPV) genotyping and HPV16/18 serology

Lausanne, Switzerland

4-8 June 2007
Summary

The first meeting of the global HPV laboratory network (LabNet) was organized as a joint workshop and practical course. The aims of the workshop were to discuss laboratory aspects linked to the introduction, follow-up and surveillance of the HPV prophylactic vaccines and the role of the global HPV LabNet in promoting internationally recognized quality of the laboratory functions needed. The practical course allowed the participants to provide input on both theoretical and practical issues involved in international standardization and quality control of HPV laboratory methodologies. The course provided an opportunity for participants to conduct HPV genotyping on patient samples and a candidate HPV LabNet DNA proficiency panel using consensus primer PCR and reverse hybridization methodology. Participants also performed HPV serology on patient samples and a candidate International Standard (IS) for HPV16 antibody using both a virus-like particle (VLP)-based enzyme-linked immunosorbent assay (ELISA) and a pseudovirion (PsV)-based neutralization assay. The workshop reviewed the tasks of the appointed HPV LabNet members and made a number of practical decision regarding organization and implementation of the specific tasks of the HPV LabNet, such as the organization of the writing of the WHO Laboratory Manual for HPV diagnosis and the design of the HPV LabNet communication strategy including a bi-annual Newsletter. The workshop/practical course was positively evaluated by the participants and can be used as a template for forthcoming workshops and practical courses for newly appointed HPV LabNet members or trainees involved in HPV surveillance.

1. Background

In 2006, WHO launched the global HPV laboratory network (LabNet) (http://www.who.int/vaccine_research/diseases/hpv/labnet_call/en/index.html). The mission of the WHO global HPV LabNet is to contribute to improving quality of laboratory services for effective surveillance and monitoring of HPV vaccination impact, through enhanced, state-of-the-art laboratory support. The HPV LabNet will support introduction of HPV vaccines and surveillance of disease and infection. The terms of reference with WHO further indicate that laboratories should be instrumental in developing and supporting the HPV laboratory work in their respective geographical areas, including participation in scientific and technical advice, quality assurance, training and communication.

The specific tasks of the global reference laboratory, Malmö are:

- To provide technology platforms and tools for surveillance and to define the circulating HPV types and relationship to vaccine design;
- To provide definitive identification and typing of HPVs isolated, using all available technologies, including sequencing, to ascertain the variant of the isolates;
- To contribute to the preparation and distribution of relevant standard reagents and training materials to regional laboratories;
- To contribute to design, preparation, and validation of proficiency panels which would ensure that all HPV testing (virological and/or serological) is performed at acceptable levels of sensitivity, specificity and reproducibility;
- To provide specialized training and trouble-shooting assistance to regional laboratories;
- To provide confirmatory testing on samples as required;
- To participate in developing WHO guidelines and Standard Operation Procedures (SOPs) for regional laboratories;
To participate in on-site visits to other countries/provinces as part of the WHO evaluation team if required;

To participate in research aimed at improving the sensitivity, specificity, applicability and speed of methods and procedures for the detection of HPV DNA and antibodies;

To report works in progress in a timely manner;

To coordinate activities with the WHO HPV laboratory network.

The specific tasks of the regional reference laboratory for Europe in Lausanne are:

- To contribute to development of an HPV proficiency program for the global laboratory network.
- To provide guidance and technical advice for the transfer of laboratory HPV technologies to low resource settings, as requested, and follow up on outcomes.
- To provide training on laboratory methodologies for type-specific DNA and antibody detection to at least five leading professionals from low resource settings, particularly from countries with high incidence rates of HPV-diseases.
- To provide continuous technical guidance and support for laboratory technology transfer whenever it is required by HPV LabNet members.
- To collaborate or provide technical services on prevalence surveys on populations, or cohorts or groups of people, or monitoring of vaccination, particularly in countries with high disease burden.
- To support HPV laboratory work through capacity building, training and technology transfer, as required for research and implementation activities in “early introducer countries” selected by PATH, namely Peru, Uganda, India and Vietnam.

In order to fulfill the specific tasks of the global and regional reference laboratories, the aims of the workshop were to review and promote progress of the HPV LabNet and provide a practical and theoretical training to personnel of regional reference laboratories or other public health laboratories.

2. Participants

Representatives from the appointed WHO HPV regional reference laboratories were invited to this workshop and a public invitation was also posted on the web site of the regional reference laboratory in Lausanne. Sixteen members of the six appointed HPV LabNet laboratories, including the organizers, heads or their delegates and staff participated in the meeting. In addition one representative of WHO and the National Institute for Biological Standards and Control (NIBSC) each were present as well as representatives of the public health laboratories performing HPV surveillance in Italy and India (see ANNEX 2).

3. Workshop/practical course

The program included consecutive sessions of theoretical presentations and discussions with intermittent practical laboratory work (in groups of two persons) as elaborated in ANNEX 1. In general, two major types of HPV laboratory assays were performed:

- An in house HPV DNA genotyping technique with patient cervical cytology samples and the candidate global HPV LabNet DNA proficiency panel
- VLP-based ELISA and neutralization assay for HPV16 and 18 antibodies with patient serum and the candidate IS sample for HPV16 antibodies.
Detailed assay protocols used in the global reference laboratory, Malmö, and the regional reference laboratory for Europe in Lausanne were provided to the participants.

4. Presentations

J. Dillner

The mission of the HPV LabNet was described and elaborated on, in particular the different tasks and their priorities and the different role of global, regional and national reference laboratories. The HPV surveillance system being built in Sweden was described in detail, as an example. The system has been designed by the Swedish Institute for Infectious Disease Control (SIIDC) in collaboration with the WHO global reference laboratory in Sweden. There are currently no laws or regulations about HPV surveillance, so the system is designed as a research project based on ethical review board permission and informed consent by the “opt-out” principle (Newspaper advertisements informing about the possibility to report to the SIIDC that information or samples from yourself should not be used in the HPV surveillance). The major components are: 1) HPV typing of all new cases of HPV-related cancer (cervical, vulvar, vaginal, penile anal and oropharyngeal cancer); 2) HPV typing of an annual subsample of 1000 CIN grade 2 and 3 samples; 3) Sentinel venereology clinics that report cases of condyloma acuminata and submit samples for typing; and 4) Sentinel sampling of sexually active teenagers.

The HPV vaccination registry, which is based on active individual informed consent, also asks all vaccinees for permission to link with health data registries and biobank registries. This will allow careful monitoring of the total effect of the vaccination programs and is expected to be helpful for locating specimens that will enable testing and typing of the HPV type behind the diseases to elucidate if remaining disease is attributable to non-vaccine types or not. Locating serum specimens bio-banked in the health care system is expected to be particularly helpful in investigating possible reasons for vaccination breakthrough and determining immunological correlates of protection.

Some preliminary data was presented. The proportion of condyloma acuminata attributable to vaccine HPV types (6/11/16/18) in Sweden has so far been about 82%. The usefulness of condyloma surveillance as a very early clinical marker of effectiveness was pointed out. Most estimates of incubation time from exposure to disease range from 1 to 9 months and strong decline of condylomas in STD clinics is to be expected shortly after introduction of HPV vaccination programs that effectively break the HPV transmission in the population. The fact that most countries do not have health care clinics specifically focussing on teenage health as in Sweden was discussed and the possibility to instead use the youngest age cohorts entering the cervical screening program (typically at age 23) to monitor if the spread of oncogenic HPV changes subsequent to vaccination was pointed out.

The use of HPV serology in HPV vaccination programs was discussed. Serology is useful to study HPV epidemiology as a measure of cumulative exposure, e.g. in studies of the age at which children start to become exposed to HPV in any given population, as HPV DNA-based studies on cervical samples are not possible in children. Knowing the age at which infection accumulates is of course essential for deciding the target age of programs. It is furthermore essential to monitor if HPV antibody levels wane in vaccinated populations as an early warning of whether effectiveness will be waning and boosters required. Finally, it is of course essential to verify that the antibody levels are satisfactory (e.g. in quality control or research using new vaccine batches, new vaccines, new administration methods et c). The proportion
of the population that is immune can also be assessed by serosurveys, which is expected to be particularly helpful in countries that do not implement a vaccination registry.

The methodological aspects and quality control was reviewed. The main conclusion of previous studies is that serological data is highly reproducible provided that the results are related to an international standard serum that has been assigned a defined unitage. Quality control of VLPs was also discussed, emphasizing the need to measure the antigenic units of neutralizing epitopes on the preparations.

The various HPV DNA detection and typing methods in use were reviewed. Previous studies have found that there is substantial variability in sensitivity and specificity between various methods, emphasizing the urgent need for preparing WHO HPV LabNet proficiency panels for ensuring quality of the tests.

D. Nardelli (Trainer for VLP-based ELISA and neutralization assay)

PsV-based neutralization assay was presented and explained. This assay is performed with PsVs that consist of type-specific HPV capsids that have encapsidated a reporter DNA that encode for secreted alkaline phosphatase (SEAP). Neutralizing antibodies are tittered according to their ability to prevent infection by PsVs of special target cells that stably express SV40 large tumour antigen (293TT cells). These cells allow replication of the infecting encapsidated reporter DNA and thus amplification of SEAP signal upon successful infection. The safety issue of handling PsVs that may have encapsidated, cellular DNA, possibly including SV40 large Tumor antigen or other unknown oncogenes was presented.

Production of PsVs and VLPs necessary for neutralization assays and ELISA, respectively, were explained. PsVs are produced by transfection of 293TT cells with HPV-type-specific plasmids that are available from Dr John Schiller (http://home.ccr.cancer.gov/lco/protocols.asp) who originally developed this method. OptiPrep purified PsV lots should be tested and tittered for SEAP production. Storage problems were discussed. VLPs can be prepared in insect cells using HPV-type-specific recombinant baculoviruses, however this technology has a highly variable yield and is now being replaced by preparation of synthetic mammalian VLP or PsV, which consists of HPV-type-specific PsV that have encapsidated L1-L2-encoding self replicating DNA. Stock of self-propagating VLP/PsV is obtained by transfection of 293 TT cells with L1-L2 producing and replicating plasmids, which can be obtained from Dr. J. Schiller. Such stocks are then used to generate VLP/PsV by infection of 293 TT cells. Quality of OptiPrep purified VLP/PsV lots, similarly to baculovirus-derived VLP lots, need to be assessed by Western blot and/or ELISA analysis.

Comparison of PsV-based neutralization and VLP-based ELISA showed a good correlation using both serum and cervical samples. The results obtained by all groups during the practical workshop were discussed. All groups were able to titrate HPV16 and 18 specific serum antibodies by ELISA. Neutralization assays appeared more difficult to perform, although most groups were successful.

R. Sahli (Trainer for HPV DNA genotyping)

In-house HPV typing technique based on the PGMY procedure modified to allow custom membrane design and reuse was demonstrated.
Briefly, cervical samples are processed by MagNaPure or Qiagen DNA extraction, followed by PCR using biotinylated PGMY primers and assessment of positives by gel electrophoresis and Gel-Red staining. The types of HPV are determined by reverse blotting hybridization of PCR-positives in a mini-blotter (45 parallel lines) against a panel of 31 HPV probes including all known high-risk HPV types. For hybridization, the probes are covalently attached to a negatively-charged nylon membrane as a parallel array in the mini blotter. Hybrids are detected by chemiluminescence using a streptavidin-peroxydase conjugate. Concentration of primers and MgCl$_2$ in the PCR reaction have been optimized to ensure good analytical sensitivity of the assay (at least 30 copies HPV-16 or HPV-18 per reaction in a background of 5'000 human cell DNA equivalent). The probes have been designed to ensure high specificity and robustness towards hybridization conditions. The PCR reagents and the membranes are subject to rigorous quality controls with known amounts of HPV-16 and HPV-18 positive controls in a background of human DNA, as well as with a panel of HPV-type specific plasmids targeting each probe to ensure reproducibility of HPV detection and typing. The membrane can be reused after washing at least 8-10 times, thus contributing to the low cost of typing by this method.

Results with samples from 3246 patients distributed in 4674 PCR (year 1999 to 2006) have shown that 37.7% were negative, 43.9% corresponded to single infections and 18.8% to multiple infections. Percentage of HPV positives increased with the severity of lesions as expected: normal (36.9%), low grade squamous epithelial lesions (LSIL) 83.8% and high grade squamous epithelial lesions (HSIL) 92.7%. 44.0% samples with undetermined squamous cells of undetermined significance (ASC-US) were positive. The most frequently detected types were HPV-16, HPV-53 and HPV-51, accounting for more than 53% of all types. HPV-18 ranked 7th among the high risk types. Sequencing of PCR-positive and hybridization-negative samples revealed most often types or subtypes that were not included in the probe panel, confirming the specificity of reverse blotting hybridization.

PGMY PCR and typing including DNA extraction with a Qiagen procedure was performed by the trainees during the practical work on 15 positive and 3 negative cervical cell samples selected from our collection. The first panel of standard DNA (14 positive, one negative) from the global reference lab were also analyzed without prior extraction. Concordant results for high risk types were obtained for all cervical samples tested (HPV-16, 18, 31, 33, 35, 51 etc ) but one false-positive for HPV-16 in one of the duplicate reactions (suspected contamination). Quality controls from the global reference laboratory confirmed the analytical sensitivity and specificity of this technique. All participants could perform key steps of the process, except covalent binding of the probes on the membrane. This part was explained in details in a workshop session together with the very important description of the in-house quality controls for validation of the entire procedure according to ISO certification.

HPV-16 real-time PCR assay targeting the E6/E7 region of the viral genome was discussed. Analytical sensitivity was near 1 copy of HPV-16 DNA per reaction (stochastic detection) in a background of 5'000 human cell DNA equivalent. Real-time PCR detected 13 additional positives compared to PGMY PCR with an agreement of both techniques of 91% (kappa 0.78) on a total of 207 samples. If patient's type history was considered, an agreement of 98% was observed (kappa 0.93). No cross reactivity with HPV-18 was detected.
D. Marais  
The HPV VLP–based ELISA methods used at the University of Cape Town, South Africa,  
Division of Medical Virology, Dept Clinical Laboratory Science and Institute of Infectious  
Disease and Molecular Medicine were discussed.

VLP used as antigen for the assays are produced in the laboratory. HPV-16 and HPV-11  
VLPs are prepared using recombinant baculoviruses expressing either HPV-11 or HPV-16 L1  
protein to infect monolayer cultures of Sf21 insect cells in tissue culture. The cell lysate is  
subjected to isopiestic gradient ultracentrifugation, the VLP preparations are dialysed  
extensively against dialysis buffer (PBS plus 400mM NaCl) before being frozen in aliquots at  
-80 °C until use. Quality control for VLPs consists of testing by Western blot, protein  
estimation, electron microscopy and comparison with VLP and serum standards to determine  
ELISA plate coating concentration. VLPs are also run in ELISA against monoclonal  
antibodies to conformational VLP epitopes and epitopes to disrupted VLPs to test for  
conformational quality.

ELISA protocols is a modification of that described by Studentsov et al.,2002 (1) using  
polymer solutions (Sigma) for blocking polyvinyl alcohol (PVA) and secondary antibody  
enhancement polyvinylpyrrolidone (PVP). Polymers significantly lowered non-specific  
background binding in ELISA. For testing antibodies in serum we use a direct ELISA. Plates  
are coated with 100µl VLP at a concentration of 0.5-1.0µg/ml in PBS overnight. For oral and  
cervical antibody assessment, we use a capture ELISA. Monoclonal antibodies H16.V5  
directed against the major conformational, neutralizing epitope on HPV-16, H11 B3 against  
HPV-11 and H18 R5 against HPV-18 (kindly supplied by Neil Christensen) are used as  
capture antibodies. The enhancement of the capture ELISA by polymer enabled the use of  
oral and cervical fluid at a 1:5 dilution instead of a previous 1:1 dilution and serum at a1:100  
dilution in direct ELISA. Serum plate controls (with low, medium and high optical density  
values) are used to measure plate to plate variability. A cut-off point for antibody positivityin  
serum and oral fluid samples is estimated using the ELISA mean plus 3 or 4 standard  
deviations (SD) of children’s sera (aged 2-10 years) after elimination of outliers. For cervical  
mucous/lavage samples, antibody responses are first standardised for total IgA or IgG to  
account for fluctuations during stages of the menstrual cycle. Cut points for cervical samples  
are determined by viewing scatterplot analysis of the absorbance values obtained for both  
standardized cervical IgA and IgG values.

In a study of seroresponses to HPV-16 in >900 control women and >400 women with cervical  
cancer, the HPV-16 IgG seroprevalence was 44.4% and IgA 28.7% in control women and  
significantly higher (61.8% and 52.7%, respectively) in women with cervical cancer (OR 2.1  
and OR 2.8, respectively). Multivariate analysis showed cervical HPV infection to be  
associated with HSV-2 infection and multiple sexual partners. HPV-16 IgG antibodies were  
association with younger age and multiple sexual partners and HPV-16 IgA antibodies with  
HSV-2 infection and less than 10 years education. The prevalence of cervical HPV infection  
and HPV-16 IgG decreased while HPV-16 IgA increased with the increasing age of control  
women. There were indications of high exposure to HPV-16 infection in this population  
(submitted for publication).

Assessing the significance of cervical HPV-16 antibodies in women with cervical  
 intraepithelial neoplasia (CIN), both the frequency and magnitude of HPV-16-specific  
cervical IgA was significantly elevated in women with CIN 2/3 compared with women with  
CIN 1 (p=0.0073 frequency; p=0.0045 magnitude). Women with cervical HPV-16 infection
had significantly higher magnitude and frequency of cervical HPV-16 IgA responses than women without cervical HPV-16 DNA (p=0.0002 frequency; p=0.0052 magnitude).

In conclusion, it seems that the assays in the lab have been verified as the results compare favourably with those found for similar studies(2, 3). A problem identified with the HPV ELISAs is with the determination of cut point for positivity which can be too low so the number of positives might be overestimated at times. A set of positive and negative serum standards run on ELISA plates would significantly assist in accurate cut point determination.

S. Sukvirach

Cervical cancer is the top leading cancer in Thai female with overall age-standardized incidence rate of 24.7 per 100,000 population. Although HPV type 16 and HPV 18 are the two most common HPV types found in cervical cancer, there are slightly different in the distribution of HPV types in different geographic areas.

In order to implement HPV DNA testing in clinical use for women with ambiguous cytological results at National Cancer Institute, Thailand, a project was set up. HPV DNA test for free of charge was offered to the patients with abnormal cytology results during October 2002 to September 2004. This project was reviewed by the National Research Council and National Cancer Institute’s Research and Ethical Committee. Financial support was from Department of Medical Service, Ministry of Public Health. Exfoliated cell samples were collected by gynecologic oncologists under permission of patients with abnormal cytology results who were referred to gynecologic oncology section. The cells were extracted for DNA using freeze-thaw-boiling method. HPV DNA was detected by PCR-EIA using GP5+/6+ primers and 36 HPV probes. In total, 713 samples were screened for HPV DNA. They were diagnosed as ASCUS, ASC-H, AGUS, LSIL, HSIL, invasive cervical cancer and other abnormalities with the number of 36 (5%), 37 (5%), 9 (1%), 42 (6%), 182 (26%), 400 (56%) and 7(1%) accordingly. HPV DNA were detected in 521 cases (73%), within these positive 56 cases (7.8%) were multiple infection. Twenty three HPV types were detected. They were HPV -6,-11,-16,-18,-26,-31, -33,-35,-39,-42,-45, -51, -52,-53, -56, -58,-59,-66,-68,-70,-72,-73, and -81. Five most common HPV types found in invasive cervical cancer in descending order frequency were HPV -16, -18, -58, -33, and -52. There were 8 unidentified HPV types.

In conclusion, this data supports that HPV- 58 and HPV- 52 may be two candidates for future vaccine development especially for Asian population.

D. Wilkinson

NIBSC-coordinated projects towards International Standardization of HPV DNA and antibody measurements were presented. In close collaboration with international experts on HPV, the WHO is supporting the preparation and characterization of International Standards (ISs) for HPV antibody and DNA analysis. As a WHO Collaborating Center for Biological Standards, NIBSC has initiated two international collaborative studies to characterize a candidate IS for antibody to HPV16 L1 capsid VLP for use in serological assays and candidate ISs for HPV16 and HPV18 DNA for use in nucleic acid amplification technology (NAT) assays.

The candidate IS for HPV16 antibody (05/134) is derived from serum collected over 18 months from three women naturally infected with HPV 16 only. The donated serum was tested and found negative for HBsAg, antibodies to HIV 1/2 and HCV RNA by NAT assay. The serum was pooled at NIBSC and freeze-dried in 0.5mL aliquots in ampoules.
The aims of the WHO collaborative study in the evaluation of candidate standard 05/134 include: i) the assessment of the suitability of the freeze-dried serum to serve as the IS for antibodies to HPV16 with an assigned unitage in International Units per ampoule for use in neutralization assays and enzyme immunoassays; ii) the characterization of candidate 05/134 in terms of reactivity and specificity; iii) the determination of the reactivity of the candidate 05/134 in a range of typical serological assays performed in different laboratories; iv) the assessment of the commutability of the candidate IS. In other words, to establish the extent to which candidate 05/134 is suitable to serve as a standard for the variety of different samples being assayed.

Eleven laboratories from nine countries participated in the study which was initiated in early 2006. In addition to candidate 05/134, coded samples were distributed to study participants. These coded samples included a freeze-dried serum negative for HPV antibody, a blind duplicate of 05/134; three serum samples from vaccinated individuals; an anti-HPV16 serum, an anti-HPV18 serum, and a HPV antibody-negative serum. Participants were asked to assay dilution series of study samples on three separate occasions for antibodies using the method(s) in routine use in their laboratories. The serological assays used by the participants in the collaborative study included those that tested for neutralizing antibody against in vitro infection of cells by HPV16 pseudoviron carrying a reporter gene (5 participants); and enzyme immunoassays using VLPs as substrate (11 participants). VLPs were considered a major source of variation in the study in that they were obtained by the participants from different sources and produced by different means. Furthermore, the VLPs were utilized in various assay formats. For example, some formats utilized VLPs that were coated directly onto plates while others utilized VLPs that were indirectly captured onto plates or attached to microspheres.

Participants returned raw data to NIBSC, indicating whether a sample was positive or negative and providing the cutoff value used to determine this. This data was submitted for statistical analysis at NIBSC in January 2007. The assessment of the candidate standard also included an accelerated degradation study conducted at NIBSC. After incorporation of participants’ suggestions, NIBSC will submit a final report on the candidate HPV16 antibody standard to WHO in October, 2007. It is anticipated that the candidate standard will be established by the WHO Expert Committee on Biological Standardization (ECBS) as the IS for antibodies to HPV type 16, human serum. In order to facilitate the standardization of serological assays performed by HPV LabNet members, NIBSC will enquire with ECBS as to whether it will be possible to distribute candidate 05/134 to HPV LabNet members prior to its approval for use as an IS.

An ongoing project is the development of HPV types 16 and 18 DNA standards. The candidate ISs for HPV16 DNA (06/202) and HPV18 DNA (06/206) were formulated at NIBSC using plasmid preparations of HPV16 or HPV18 whole genome DNA cloned into pBR322 and supplied by Professor Cosette Wheeler’s laboratory. Each candidate HPV DNA standard was formulated to contain 1 x 10^7 genomes/mL of HPV 16 DNA or HPV 18 DNA; C33a human cellular DNA at a final concentration of 1 x 10^6 genomes/mL and 10mM Tris buffer (pH7.4) containing 1mM EDTA and 5 mg/mL trehalose. The HPV DNA preparations were then dispensed into ampoules in 0.5 mL aliquots and freeze-dried.

19 laboratories, including two reference laboratories, from 13 countries accepted WHO’s invitation to participate in the collaborative study to evaluate the candidate ISs for HPV16 DNA and HPV18 DNA for use in NAT assays. In addition to the candidate standards, aliquots of the liquid bulk HPV DNA preparations were included in the collaborative study to ascertain differences between the HPV DNA concentration in the bulk and in the freeze dried samples. Sample diluent containing C33a human cellular DNA, at final concentration of 1 x
$10^6$ genomes/mL dH$_2$O, was also dispatched with the study samples. In order to approximately mimic a DNA background that would be present in biological samples, participants were asked to make all sample dilutions using the supplied C33a DNA diluent.

Participants were requested to assay the study samples on four separate occasions for HPV DNA using the method(s) in routine use in their laboratories. The NAT assays used by the participants in the collaborative study included both qualitative and quantitative HPV DNA determinations as well as HPV genotyping.

September 2007 is the deadline for participants to return data to NIBSC with statistical analysis of results and accelerated degradation studies of the candidate standards scheduled for completion in October 2007. An important consideration in drawing up the report for submission to ECBS in November 2007 is whether the candidate HPV DNA standards should be assigned an arbitrary International Unitage (IU) or copy number. In order to facilitate the standardization of NAT assays performed by HPV LabNet members, NIBSC will enquire with ECBS as to whether it will be possible to distribute candidates 06/202 and 06/206 to HPV LabNet members prior to their approval for use as ISs.

Additional NIBSC-coordinated projects for developing international reference materials and reagents towards international standardization of HPV DNA and antibody measurements include: i) the development of an IS for HPV 18 antibody; ii) the development of HPV DNA ISs of additional genotypes; iii) freeze-drying of SiHa (HPV16) or HeLa (HPV 18) whole cells for use as a process control to monitor centrifugation and extraction steps for NAT assays; iv) the provision of C33a DNA diluent; v) sourcing of high-quality VLPs and/or pseudoviron for use in serological assays; vi) the development of HPV working standards or proficiency panels.

When established by the WHO ECBS, these International reference materials and reagents will play essential roles in harmonizing the detection of HPV worldwide thus facilitating in the development and implementation of HPV vaccines and related in vitro diagnostic devices.

**B. Allan**

Mr Allan gave a presentation about "HPV Diagnosis in South Africa: A Third World Perspective" which highlighted the serious nature of cervical HPV infection and associated cervical cancer, both worldwide and in South Africa. The history of laboratory methods used to detect the presence of cervical HPV infection locally was sketched. What was of note was that the increasing sophistication, standardization and cost of HPV detection methods was linked to the development and use of commercially developed assays. One detection method is the Digene hybrid capture test which is being used by the laboratory for clinical and research purposes to test for oncogenic HPV types. A major advantage of its use in Third World situations is the robustness of the collection technique. Unfortunately, the present cost of the test excludes its large scale use in the public sector in South Africa. Its application in a large study of the role played by injectable contraceptives in cervical cancer (4) provided useful epidemiological information as regards the prevalence of oncogenic HPV in the Greater Cape Town area. A complimentary commercial test based on the use of the polymerase chain reaction, the Roche Linear Array, is also being used to provide data of specific HPV types present in cervical infections in local women. An examination of study findings showed for instance that HPV types 83, 53, 52 and 16 were the most prevalent types in Cape Town women graded Papanicolaou smear negative. Cost considerations do, however, limit the use of this test.
The severe impact of human immunodeficiency virus (HIV) on regional cervical HPV infection and on cervical cancer prevalence was presented (5). Features of this impact mimic international findings and include persistent infection by a greater number of HPV types with high viral loads as well as a high risk of cervical lesions (6) compared to findings in HIV negative women. Another local study presented (7) showed the importance of the use of innovative techniques in Third World settings: the efficacy of self-collection devices for obtaining cervical samples for HPV testing was demonstrated in a comparison with cervical samples drawn clinically.

The presentation ended with the fact that HPV testing in South Africa was geared to extending knowledge about regional cervical HPV infection and cervical cancer and that a role for HPV diagnostic testing was being sought in public sector screening programs.

M. Bharadwaj
Cervical cancer is the second-most common cancer in women worldwide. But it is the most common cancer and is a leading cause of cancer related death in women in India. It has been estimated that more than 1,20,000 women develop this cancer every year with about 75,000 annual death, constituting about 16% of the world’s annual incidence. The primary causative agent of cervical cancer is infection by certain high-risk types of HPV (HR-HPV), while low-risk types are associated with benign cervical lesions and genital warts. In India, almost all cases of cervical cancer show presence of HR-HPV infection and HPV type 16 alone present in more than 85% of cervical cancer.

Although incidence of cervical cancer is preventable by organized cytological screening and early detection of HR-HPV infection to a great extent recent development of two prophylactic vaccines against most prevalent HR-HPV types have made prevention of cervical cancer most promising. Recently US-FDA has approved the VLP-based quadrivalent HPV vaccine “Gardasil” (HPV 6/11/16/18). The bivalent HPV vaccine “Cervarix” (HPV 16/18) is also likely to be licensed soon. These vaccines are recommended for vaccinating young adolescent girls at or before onset of puberty.

Indian Council of Medical Research (ICMR), Government of India has signed a Memorandum of Understanding (MOU) with Merck & Co. to initiate clinical trial of Gardasil in India. Institute of Cytology and Preventive Oncology (ICPO) is supposed to coordinate this trial. Therefore, before introduction of HPV vaccines in India it is important to know the prevalence of HPV infection among pre-adolescent children as well as other age-group women, as no data is available till date. A study to determine the prevalence of HPV infection among healthy school children of ages 8-15 years has been initiated using non-invasive urine sampling. This approach can bypass the many social issues in Asian region for successful implementation and monitoring of HPV vaccine programme.

F. Carozzi
The Italian randomized trial (NTCC trial), observed a prevalence of HR-HPV of 13-14% in the age group of 25-39 years old Italian women; prevalence decreased in older age groups to 5%. The overall observed prevalence was 8.8%. Because of the relative paucity of appropriate data on HPV type distribution in Italy, the Ministry of Health financed studies to investigate the HPV type prevalence including a pilot study on impact of HPV vaccination on women aged 18-26 and a study to evaluate a statistical model of HPV vaccine impact on the screening program. To describe the overall and age-stratified prevalence of HPV infection on a population level in women before the introduction of vaccination, three different studies have
been planned: 1) in women aged 25-60 participating in a screening program for cervical cancer, including women enrolled in the NTCC, 2) in young women aged 18-24, and 3) typing 1000 CIN2+ lesions on tissue samples obtained in Italy in the past 10 years. These studies will be also the basis to evaluate future potential non-vaccine type replacement in the post-vaccination era. In these studies, HC2 will be performed in each site and positive samples will be genotyped in three laboratories in Italy using the same methodology based on GP5+/GP6+. The molecular laboratory in CSPO (Centro per lo Studio e la Prevenzione Oncologica) is the coordinator of HPV test and typing and furthermore is responsible for setting protocol and internal/external quality control of molecular analysis.

One of the most neglected aspects of the ongoing debate on the potential impact of HPV vaccination is the need to examine existing screening practices to permit synergy between primary and secondary prevention efforts. It is sensible to consider that incorporation of HPV vaccination cannot be cost-effective without substantial changes to existing screening policies. The best protocols to be applied in the new situation are to be determined. HPV types 16 and 18 are account, worldwide, for about 70% of cervical cancers, therefore screening needs to be continued also for vaccinated women in order to prevent cancers arising from oncogenic types other than 16 and 18. Data from efficacy studies provided little information about the actual impact of vaccination on routine screening, as study women were at younger age and were screened much more intensively than in regular programmes. To study the impact of HPV vaccination on screening for cervical cancer, a pragmatic effectiveness trial based on an active offer of the vaccine to women at the age of starting screening, or just below, will be conducted. Vaccination is expected to slightly reduce costs of screening, particularly because of a reduced referral to colposcopy. Reduced CIN prevalence will result in reduced Positive Predictive Value. In a situation with a low HPV prevalence testing could be the best method to screen and possibly longer screening intervals could be applied. It will allow understanding the impact of vaccination on regular screening activity, therefore allowing to decide the best screening protocols in the vaccination era. A secondary objective will be to implement an electronic data collection system that creates a data transmission flow between the vaccination centres and the local health care units where cervical cancer screening is performed. A computerized medical database will be created for all participating vaccination centres, local health units and the laboratory in charge of HPV genotyping.

5. Evaluation of the course

A questionnaire for self-assessment of the course by the trainees was filled by 9 participants. Overall the content of the course was highly appreciated and was felt to be adequate to the needs of the participants. The time for the practical part was considered sufficient for the participants who were already familiar with the techniques used, but additional time would have been appreciated by some other participants.

6. Recommendations and conclusions

Some specific actions were decided at the meeting, including:

6.1 The WHO HPV Laboratory Manual will contain a chapter on the role of the HPV LabNet and importance of HPV surveillance as part of HPV vaccination programs. The Manual will also contain examples of SOPs for methods that have been demonstrated to work. The methods covered by the SOPs should be as simple as possible, so that they can be
implemented globally in a variety of laboratory settings. A WHO consultation with external experts examining each section of the manual will be held in January 2008.

6.2 Essential items of laboratory-based HPV surveillance were discussed.

6.2.1 Sampling strategies and populations studied include i) population-based samples for HPV DNA prevalence and type distribution among normal women; ii) HPV type distribution among cases of cervical cancer and women with cervical intraepithelial neoplasia grade II or III (CIN2/3). In some settings, surveillance of HPV type distribution among patients with low grade cervical lesions may also be of value.

6.2.2 The HPV DNA surveillance needs to start now, as a baseline before vaccination is needed in order to be able to evaluate vaccination strategies.

6.2.3 The role of HPV serology was discussed. It was envisaged that it will not be used in diagnostic, but rather largely for seroprevalence surveys before vaccination (particularly in young girls, where cervical samples are not available for DNA testing) and in immunity surveys post vaccination.

6.2.4 As part of the effort to standardize and control the laboratory methodologies used by HPV LabNet members, it was suggested that certain test samples should be selected as reference samples to be sent to the global reference laboratory for confirmatory testing. The global reference laboratory is currently using several technologies, which were reviewed (Luminex, Mass Spectrometry, rolling circle amplification, cloning and sequencing). It was decided that the testing of reference samples should be performed in the format of a publishable scientific study, but should also be continuously ongoing and annually reported to WHO.

In principle, it was agreed that reference samples selected by the LabNet members and sent to the global reference laboratory should include:

- 5% of positive samples, but not less than 100 samples. Preferred: from cervical cancers. If not available from CIN2/3.
- All samples classified as “HPVX” (i.e. HPV positive, but with unknown type).
- All samples from histologically confirmed cervical cancers and CIN2/3 that test HPV-negative.
- 50 samples/laboratory classified as “grey zone” samples (questionable positivity).

6.3 The HPV LabNet communication strategy will include:

- A six-monthly newsletter, with emphasis on wide distribution to all stakeholders.
- A website presenting the HPV LabNet should be maintained and continuously updated at the WHO website.
- For internal communication “SharePoint” systems, i.e. password-protected websites, should be used. This is available at the global reference lab for the writing of the WHO Laboratory Manual of HPV Diagnosis and at the regional reference lab for Europe, where all the documents and teaching materials from this Workshop are posted. A share-point should also be set up at the WHO Headquarters.

6.4 Standard operating procedures (SOPs) and quality control:

- SOPs on how to perform quality assurance should be agreed upon and enforced. These SOPs will be written with the intent to be included in the HPV Laboratory Manual and will be tested by the LabNet members (preferably before January 2008).
• HPV DNA proficiency panels will be prepared by the global reference lab. Also the
development of a DNA extraction control was highly recommended. Cervical cancer
cell lines fixed in PreservCyt medium or freeze-dried are possible options.
• Serology: The critical reagents (VLPs and PsVs) are missing, as they are too difficult
to prepare to suggest that every laboratory should produce them in-house. Several
options to obtain these urgently needed reagents should be investigated by WHO and
NIBSC. A request will be made to Dr. Neil Christensen of the University of
Pennsylvania enquiring as to whether he will be able to provide monoclonal antibodies
that are suitable for use as positive controls
• The IS for HPV16 antibodies is absolutely necessary for standardization of serology
results. In order to arrive at a standardized system of reporting of HPV antibody
levels, at least within the HPV LabNet, NIBSC will be asked to provide the candidate
IS for HPV16 to the HPV LabNet prior to its approval by the WHO Expert Committee
on Biological Standardization. ISs for HPV18 and serum proficiency panels are also
needed.

The participants emphasized that the usefulness of laboratory-based HPV surveillance for
effective implementation and surveillance of HPV vaccination policies needs to be elaborated
and made clear to all stakeholders. Because baseline data needs to be collected before
vaccination in order to allow for the international evaluation and comparison of HPV vaccine
programs, it is particularly urgent that surveillance programs for HPV infection and HPV-
associated diseases be implemented. It was recognized by the Global HPV LabNet as
particularly urgent and important that the careful design of surveillance programs is needed to
allow for the international comparison of data. The HPV LabNet needs to support awareness of
the possibilities of HPV laboratory diagnosis in vaccination programs and design and
disseminate the requirements for reliable and internationally comparable methodology.

REFERENCES

immunosorbbent assay for detection of antibodies to virus-like particles of human

and cancer of the cervix, anogenital organs, oral cavity and pharynx, oesophagus and prostate

3. Passmore JA, Marais DJ, Sampson C, et al. Cervicovaginal, oral, and serum IgG and IgA
responses to human papillomavirus type 16 in women with cervical intraepithelial neoplasia. J

in relation to the use of injectable progestogen contraceptives and combined
estrogen/progestogen oral contraceptives (South Africa). Cancer Causes Control. 2003;

5. Denny L, Walraven S, Myer L, Allan B et al. HPV infection in untreated HIV-1 infected
women in Cape Town South Africa. Presented at the International HPV Conference 2005,
Canada.


AUTHORS

Joakim Dillner, Head, Global Reference Laboratory, Malmö, Sweden; Denise Nardelli Haefliger, Head, Regional Reference Laboratory for Europe, Lausanne, Switzerland and Tiequn Zhou, Scientist, QSS/IVB/FCH/WHO, with support of the presenters and participants in the meeting.
# ANNEX 1

**Agenda for WHO workshop/practical course on HPV genotyping and HPV16/18 serology (4-8 June 2007)**

## Monday June 4th 2007

<table>
<thead>
<tr>
<th>Time</th>
<th>Theory/Discussion</th>
<th>Practical course</th>
</tr>
</thead>
<tbody>
<tr>
<td>9h00</td>
<td>Role of the HPV Lab network. J. Dillner</td>
<td></td>
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<tr>
<td>9h30</td>
<td>PsV-based Neutralization assay: D. Nardelli</td>
<td></td>
</tr>
<tr>
<td>10h00</td>
<td>Coffee and Welcome Address from the Institute of Microbiology</td>
<td>11h00 Neutralization assay: cell plating</td>
</tr>
<tr>
<td>12h00</td>
<td>Lunch</td>
<td>12h45 Neutralization assay: mix PsV, VLP and samples</td>
</tr>
<tr>
<td>13h30</td>
<td>PsVs and VLPs production in Lausanne. D. Nardelli</td>
<td></td>
</tr>
<tr>
<td>14h00</td>
<td>PsVs and VLPs production. Round table discussion</td>
<td>14h30 Neutralization assay: infection</td>
</tr>
<tr>
<td>15h00</td>
<td>Coffee</td>
<td></td>
</tr>
<tr>
<td>15h30</td>
<td>Neutralization assays and ELISA methods in Lausanne. D. Nardelli</td>
<td></td>
</tr>
<tr>
<td>16h00</td>
<td>HPV serological assays in use at the regional HPV reference lab in South Africa. Experience of performance and quality control. D. Marais</td>
<td>17h00 ELISA: coating plate</td>
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## Tuesday June 5th 2007

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<tr>
<th>Time</th>
<th>Theory/Discussion</th>
<th>Practical course</th>
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<tbody>
<tr>
<td>8h45</td>
<td>Quality control work/proficiency panels. J. Dillner</td>
<td>8h15 ELISA: blocking plate</td>
</tr>
<tr>
<td>9h30</td>
<td>ELISA: samples</td>
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</tr>
<tr>
<td>10h15</td>
<td>Coffee</td>
<td></td>
</tr>
<tr>
<td>10h45</td>
<td>Ordinary ELISAs, quality control of VLPs and Multiplex serology. J. Dillner</td>
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</tr>
<tr>
<td>11h30</td>
<td>Newsletter and communication in the HPV Lab Network. S. Garland</td>
<td>12h15 ELISA: 2nd antibody.</td>
</tr>
<tr>
<td>12h30</td>
<td>Lunch</td>
<td>13h15 ELISA: detection</td>
</tr>
<tr>
<td>15h00</td>
<td>ELISA Results and discussion. D. Nardelli</td>
<td></td>
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<tr>
<td>15h30</td>
<td>Coffee</td>
<td></td>
</tr>
<tr>
<td>Time</td>
<td>Event</td>
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<tr>
<td>16h00</td>
<td>Overview of HPV DNA detection and typing: methods in use at the global reference lab and internal Quality controls  J. Dillner</td>
<td></td>
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<tr>
<td>16h45</td>
<td>HPV DNA detection and typing: methods in use in the South African reference lab. A. Bruce</td>
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<tr>
<td>17h15</td>
<td>HPV DNA detection and typing: methods in use in the Lausanne reference lab. (PGMY PCR and RLBH, real-time PCR). R. Sahli</td>
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**Wednesday June 6th 2007**

**Theory/Discussion**

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<th>Time</th>
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<tr>
<td>8h30</td>
<td>HPV genotyping: DNA extraction</td>
</tr>
<tr>
<td>10h00</td>
<td>HPV genotyping: PCR</td>
</tr>
<tr>
<td>10h30</td>
<td>Coffee</td>
</tr>
<tr>
<td>11h00</td>
<td>HPV genotyping: production of customized nylon membranes. R.Sahli</td>
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<tr>
<td>11h30</td>
<td>Services offered at the global reference lab. J. Dillner</td>
</tr>
<tr>
<td></td>
<td>Presentations: F Carrozzi and M Bhardwaj</td>
</tr>
<tr>
<td>12h30</td>
<td>lunch</td>
</tr>
<tr>
<td>14h00</td>
<td>HPV genotyping: gel preparation and migration</td>
</tr>
<tr>
<td>15h00</td>
<td>Coffee</td>
</tr>
<tr>
<td>15h45</td>
<td>HPV genotyping discussion of result. R. Sahli</td>
</tr>
<tr>
<td>16h30</td>
<td>HPV types found in women with abnormal cytology at National Cancer Institute Thailand. S. Sukvirach</td>
</tr>
<tr>
<td>17h00</td>
<td>Ongoing NIBSC-coordinated projects towards international standardization of HPV DNA and Antibody measurements. D. Wilkinson</td>
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<table>
<thead>
<tr>
<th>June 7th</th>
<th>Theory/Discussion</th>
<th>Practical course</th>
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<tbody>
<tr>
<td>10h30</td>
<td>coffee</td>
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<tr>
<td>11h00</td>
<td>The WHO HPV Laboratory Manual. What should it contain and how work should be organized. J. Dillner and round table discussion</td>
<td>12h00 HPV genotyping: washing monoclonal antibody + peroxidase</td>
</tr>
<tr>
<td>12h45</td>
<td>lunch</td>
<td>13h30 HPV genotyping: washing and film exposure</td>
</tr>
<tr>
<td>15h15</td>
<td>Coffee</td>
<td>14h00 Neutralization: detection</td>
</tr>
<tr>
<td>15h45</td>
<td>HPV genotyping: results and discussion. R. Sahli</td>
<td></td>
</tr>
<tr>
<td>16h15</td>
<td>Neutralization: results and discussion. D. Nardelli</td>
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</tr>
<tr>
<td>17h00</td>
<td>End of the theory/discussion part</td>
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<table>
<thead>
<tr>
<th>June 8th</th>
<th>Practical course</th>
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<tbody>
<tr>
<td>8h30</td>
<td>Practical aspects at the trainee’s demand</td>
</tr>
<tr>
<td>11h00</td>
<td>End of practical course</td>
</tr>
</tbody>
</table>
ANNEX 2

LIST OF PARTICIPANTS

Organizers
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