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A global proficiency study of Human Papillomavirus genotyping

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ABSTRACT

Internationally comparable quality assurance of Human Papillomavirus (HPV) DNA detection and typing methods is essential for evaluation of HPV vaccines and effective monitoring and implementation of HPV vaccination programs. Therefore, the World Health Organisation (WHO) HPV Laboratory Network (LabNet) designed an international proficiency study. Following announcement at the WHO website, responding laboratories performed HPV typing using one or more of their usual assays on 43 coded samples composed of titration series of purified plasmids of sixteen HPV types (HPV 6, 11, 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66 and 68). A detection of at least 50 International Units (IU) of HPV16 or HPV18 DNA and of 500 genome equivalents (GE) of the other 14 HPV types (in samples with single and multiple HPV types) was considered proficient. Fifty-four laboratories worldwide submitted a total of 84 data sets. There were more than 21 HPV genotyping assays used. Commonly used methods were Linear Array, Lineblot, Inno-LiPa, Clinical-Array, type-specific real-time PCR, PCR-Luminex and microarray assays. The major oncogenic HPV types (HPV16 and 18) were detected in 89.7% (70/78) and 92.2% (71/77) of data sets, respectively. HPV types 56, 59 and 68 were the least commonly detected types (in less than 80 % of data sets). Twenty-eight data sets reported multiple false positive results and were considered non-proficient. In conclusion, we found that international proficiency studies, traceable to International Standards, allow a standardised quality assurance of different HPV typing assays and enables a comparison of data generated from different laboratories worldwide.

INTRODUCTION

Human Papillomavirus (HPV) infection is established as the major cause of cervical cancer (2). Epidemiological studies have classified genital HPV types in high and low-risk HPV types reflecting their association with invasive cancer (19). The most important high risk types HPV 16 and HPV 18 account for about 70 % of all invasive cervical cancers worldwide. The next most common HPV types on all continents are HPV 31, 33, 35, 45, 52 and 58 found in approximately 20 % of cervical cancers (19).

Accurate and internationally comparable HPV DNA detection and typing methodology is an essential component in the evaluation of HPV vaccines and in effective implementation and monitoring of HPV vaccination programs. Genotyping assays used today differ in their performance with regard to type-specific detection rates (10). As the methodology for quality assurance and evaluation of assay performance is not standardised, comparisons between different studies that use different assays is particularly difficult (10).

The World Health Organization (WHO) establishes international biological standard materials and reference reagents for substances of biological origin used in prophylaxis and in therapy or diagnosis of human diseases (*http://www.who.int/biologicals/reference_preparations/en/*). At the WHO meeting held in Geneva, 15-17 August 2005, an expert group recommended the establishment of a global HPV laboratory network (HPV LabNet), to contribute to improving the quality of laboratory services for effective surveillance and HPV vaccination impact monitoring. Major activities within the HPV LabNet include the development of international standard reagents and standard operating procedures (SOPs), and the development of internationally comparable quality assurance methods (5, 26).

International proficiency panels are already widely used for several microorganisms including hepatitis A, B and C, herpes simplex virus (HSV) and human immunodeficiency virus (HIV) (15, 18, 24). As there is no natural source of biological material that could be used to generate type-specific HPV international standards (ISs), the first WHO international collaborative study of detection of HPV DNA examined the feasibility of using recombinant HPV DNA plasmids as standards, focusing on HPV 16 and HPV 18 (13). ISs of HPV16 and HPV18 DNA were established for detection and quantification of HPV 16 and HPV 18 DNA by the WHO Expert Committee on Biological Standardization in 2008 with assigned potency in International Units (IU).

The international WHO proficiency study described in this report was based on a proficiency panel composed of purified plasmids containing the genomes of 14 oncogenic HPV types and 2 benign

HPV types. As the amount of plasmid DNA was titrated in amounts traceable to the IS, the proficiency panel allowed an internationally standardised definition of assay sensitivity.. Specificity was defined as absence of incorrect typing. We also evaluated sample pre-processing with extraction controls of cervical cancer cell lines. The panel was distributed to 61 laboratories worldwide and analyzed using a range of HPV DNA typing assays in a blinded manner. We report the results in terms of the ability of participating laboratories to correctly identify HPV types, grouped by methods performed as well as the analytical sensitivity of detecting the HPV types included.

MATERIALS AND METHODS

Source of panel material. Complete genomes of HPV cloned into plasmid vectors had been provided to the Lund University by the respective proprietors with a written approval to be used in this proficiency panel: Dr Ethel-Michele de Villiers (HPV types 6, 11, 16, 18 and 45), Dr Gérard Orth, (HPV types 33, 39, 66 and 68), Dr Saul Silverstein (HPV type 51), Dr Attila Lörincz (HPV types 31, 35 and 56), Dr Wayne Lancaster (HPV type 52) and Dr Toshihiko Matsukura (HPV types 58 and 59). The agreements allowed distribution of the plasmids only for the performance of this WHO proficiency study.

The nucleic acid sequences for each of these HPV genomes have been reported previously and are available in Gene Bank with the following accession numbers; HPV 6 nr X00203; HPV 11 nr M14119; HPV 16 nr K02718; HPV 18 nr X05015; HPV 31 nr J04353; HPV 33 nr M12732; HPV 35 nr M74117; HPV 39 nr M62849; HPV 45 nr X74479; HPV 51 nr M62877; HPV 52 nr X74481; HPV 56 nr X74483; HPV 58 nr D90400; HPV 59 nr X77858; HPV 66 nr U31794 and HPV 68 nr X67161.

Preparation and characterisation of individual panel reagents. HPV 11 and HPV 58 were originally cloned in the L1 gene and were therefore re-cloned so that the vector (pGEM4z) is positioned in the L2 (position 4781) and the E1 (position 1158) gene respectively. For HPV 35 two clones were included: HPV 35-S contains the entire genes from L1 through E7 including nucleotides 5012-956, and HPV 35-L including nucleotides 956-5012. The plasmid used for HPV 68 contained only the L1 gene. DNA of each individual HPV genome was generated by the use of overnight culture of transformed E. coli and plasmid purification using Qiagen Midi-prep kit. Optical density determinations were made at 260 nm and 280 nm to estimate purity of the preparation. Size and purity of the plasmids were analysed using agarose gel electrophoresis. The double stranded DNA concentration was established using fluorimetric measurements by picogreen quantitations (PicoGreen dsDNA Quantitation Reagent; Molecular Probes, Inc, Eugene, Oreg). The purified plasmid bulk of HPV 16 and HPV 18 were tested in ten-fold serial dilutions in parallel with International Standards for HPV 16 (06/202) and HPV 18 (06/206) distributed by NIBSC (Hertfordshire, UK) using a PCR Luminex assay to establish the amounts in terms of International Units, by traceability of the amount of plasmids in the panel to the IS (16).

Panel composition and production. Purified plasmids containing cloned genomic DNAs for HPV types 6, 11, 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66 and 68 were diluted to a stock

concentration of 10^8 genome equivalents/µl in TE buffer (10mM Tris, 1 mM EDTA pH 8.0) to be used for preparation of 43 samples. Human placenta DNA (Sigma-Aldrich 7011) at a concentration of 10 ng/µl was added to the TE buffer to mimic a molecular matrix background that would typically be present in biological samples. Table 1 summarizes the composition of the panel. The different amounts of plasmid (5-500 GE or IU) were chosen to reflect the lower spectrum of amount of virus that would typically be present in clinical samples. E.g., a study of virus quantities present in cervical samples from healthy HPV-positive women found an average of 18000 GE of HPV16/100ng input DNA (range <300 – 14000000 GE/100ng input DNA) (20). The 43 different panel samples were prepared by dilution of HPV recombinant DNA plasmid stock solution in TE buffer in the background of human placental DNA. Briefly, the HPV DNA plasmids were diluted 100-fold in TE-placenta buffer to 10^4 genome equivalents (GE)/µl, further 10 fold dilutions were made to a final concentration of 1 IU/µl of HPV 16 and HPV 18, for the other HPV types included 10 GE/µl was the final dilution. To ensure high quality of the panel two HPV types were diluted each day with an interval of at least 4 hours in between. The samples containing multiple types were prepared from dilutions of 10^3 genome equivalents/µl. After production of each of the 43 reference samples, the preparation was dispensed in 100 µl volumes in 1.5 ml siliconized vials. The vials were labelled as WHO HPV DNA 2008 and randomly assigned numbers from 1 through 43. The panels were stored at +4^{o}C before shipment to participating laboratories. Participants were instructed to perform HPV typing according to their standard methods using their standard sample input volume.

Two different cell lines were used as controls of the extraction process in participating laboratories. The HPV-negative epithelial cell line C33A derived from human cervical carcinoma and the HPV16-positive epithelial cell line SiHa, derived from a squamous cell carcinoma was purchased from the American Type Culture Collection and cultured in Dulbecco's modified Eagle medium (Gibco 11960). The cells were diluted in PreserveCyt^{TM} (Cytyc 0234004) to a concentration of 400 cells/µl, 100 µl of each preparation was dispensed in 1.5 ml vials and labelled WHO HPV DNA A and B.

Before distribution of the WHO HPV DNA proficiency panel, the samples were tested (blinded) at the WHO HPV LabNet Global Reference Laboratory (GRL) in Sweden and two other laboratories, namely the German Cancer Research Center (DKFZ) in Heidelberg, Germany (Dr. Michael Pawlita) and the WHO HPV LabNet GRL at Centers for Disease Control and Prevention (CDC) in the United States (Dr. Elizabeth Unger).

Technologies used for initial characterization of the panel. (i) GRL Sweden. Three independent experiments testing each sample in duplicate were performed. Five microliters of panel sample DNAs was used for MGP PCR as previously described (21). Ten μl PCR products were analysed by multiplex genotyping using a Luminex based assay as described earlier (16, 17). HPV types 6, 11, 16, 18, 26, 30, 31, 33, 35, 39, 40, 42, 43, 45, 51, 52, 53, 54, 56, 58, 59, 66, 67, 68a, 69, 70, 73, 74, 82, 86, 89, 90 and 91 were distinguished. Appropriate negative and positive controls were used to monitor the performance of the method. DNA from the extraction control A and B was extracted using QIAamp DNA Mini and Blood kit (Qiagen) according to the manufacturer's instructions.

(ii) DKFZ. A 10 μl DNA sample was amplified by the broad-spectrum GP5+ / 6+ primers as previously described (17). The PCR products were analysed using bead based multiplex genotyping as described (16). HPV types 6, 11, 16, 18, 26, 30, 31, 33, 35, 39, 42, 43, 44, 45, 51, 52, 53, 56, 58, 59, 66, 67, 68a, 68b (Me 180), 69, 70, 73 and 82 were distinguished. All samples were tested for human DNA with PCR primers amplifying part of the β-globin gene and a bead coupled β-globin specific probe used in the genotyping assay.

(iii) GRL CDC. Ten microliters of sample DNAs was used in the 100 μl PCR otherwise following the manufacturers protocol for Roche Linear Array which is designed to detect 37 individual HPV types, 6, 11, 16, 18, 26, 31, 33, 35, 39, 40, 42, 45, 51, 52, 53, 54, 55, 56, 58, 59, 61, 62, 64, 66, 67, 68, 69, 70, 71, 72, 73, 81, 82, 83, 84, 89 and IS39. As the probe for detecting HPV 52 cross-hybridizes to types 33, 35 and 58, the presence of HPV 52 in samples with one or more of these three other types was tested with an HPV 52-specific real-time PCR assay.

Organization of the study. Participants to the study were recruited by advertisement at the WHO website. The panels were distributed from the WHO HPV LabNet GRL in Sweden at ambient temperature to 61 laboratories worldwide, by WHO region: America Region16 laboratories, Africa Region 1 laboratory, Eastern Mediterranean Region 1 laboratory, European Region 28 laboratories, South East Asia Region 2 laboratories and Western Pacific Region 13 laboratories. The package also included a letter of instruction as well as a form for reporting the results of the testing of the panel as well as technical information on the procedures to be performed. Laboratories were asked to submit the results of the tests performed to the WHO GRL in Sweden within 4 weeks of receipt of specimens. The agreement included assigning the right to publish the data to the WHO, but it was agreed that only coded results from all laboratories will be presented, grouped by methods performed.

All results submitted to the WHO HPV LabNet GRL Sweden were coded and analyzed anonymously. Data sets generated were designated numerically from 1 through 84. Individual results of the proficiency study were disclosed only to the participating laboratory that generated the data.

HPV technologies used by study participants. The different HPV typing methods that were used to generate results for the WHO LabNet proficiency study to detect HPV DNA (1, 3, 6-9, 17, 21, 22, 25) are summarized in Table 2.

Data analysis. Criteria used for considering a data set as proficient were the following: (i) detection of at least 50 IU per 5 μl of HPV 16 and HPV 18, both in single and multiple HPV infection; (ii) detection of at least 500 GE per 5 μl of the other HPV types included, both in single and multiple infection; (iii) at most one false positive result. These criteria were arrived at by a consensus opinion of international experts participating in an international WHO workshop in Geneva, 2008 (5) and was based on a consideration of which performance requirements were required and realistic. A higher requirement for HPV16 and 18 was considered essential, because of the pivotal role of these HPV types in causing cervical cervical cancer.

Four data sets reporting results only as “high” or “low risk” HPV were not included in the overall performance analyses (one data set that used the Roche Amplicor assay, one data set that used the Seeplex HPV 4 ACE assay and two data sets used in-house PCR with agarose gel analyses).

RESULTS

Validation of the HPV proficiency panel. The results from the initial panel validation at the 2 GRLs and at DKFZ included qualitative characterization of HPV and human genomic DNAs. Two of these laboratories used Luminex based assays with modified GP 5+ / 6+ primers, the third laboratory used Linear Array, which is based on PGMY primers for the analyses. No false positive HPV type was detected in the samples in any of the reference laboratories. HPV 31 was not detected in the lowest concentration, when present together with other plasmids, in both laboratories that used GP-based assays. HPV 18 was not detected in the lowest concentration in the laboratory using Liner Array. All other HPV types were detected at the lowest concentration included in the panel, except HPV 39 and HPV 68 that could not be detected using Linear Array. The HPV 39 plasmid used in the panel cannot be detected by systems using PGMY primers as it was cloned into the vector at the binding site of one of the PGMY primers. Linear Array and other PGMY based assays are designed to detect HPV68 subtype B and can not detect the HPV68 prototype virus because of several mismatches.

All 3 reference laboratories detected HPV 16 DNA in the DNA extraction control containing SiHa cells and had negative results in the negative control for DNA extraction (C33A cells).

The results from the reference laboratory evaluation advised that the panel performed as expected and the panel was then distributed to participating laboratories worldwide.

Panel distribution and response. Fifty-four of 61 participating laboratories, including the three laboratories who did the panel validation, submitted 84 data sets according to the timeline (Table 2). Two laboratories that responded after the deadline are not included in this report. Four data sets were generated using assays that did not discriminate specific HPV types and were therefore not included in the overall type-specific analyses presented here.

Some participating laboratories did not perform tests for typing of all HPV types included in the proficiency panel. Therefore, the denominator for the number of test results included in the analyses varies for the different HPV types. In 37 data sets, the results had been obtained using commercially available tests. The most commonly used assay was Liner Array (Roche) that was used to generate 15 data sets. Other widely used assays were CLART HPV 2 (Genomica), Inno-LiPA (Innogenetics), PGMY-LINEBLOT, in-house type specific PCR, Luminex and Microarray based assays (Table 2). Participating laboratories included both public health laboratories, research laboratories, diagnostic kit manufacturers and vaccine companies. The annual number of samples

analysed for HPV per laboratory varied from 100 to 100 000 per year with approximately 40 % of the laboratories performing <2000 HPV typing tests per year and around 40 % between 2000 and 10 000 HPV typings per year.

Performance of HPV assays and participating laboratories. Participating laboratories were requested to perform testing using their standard protocols. Accordingly, the input volume of the DNA panel varied between 2 µl and 50 µl between laboratories. Data is presented by lowest category of concentration (5, 50 or 500 GE or IU) proven to be detectable. E.g., a lab using a 2 ul input instead of 5ul input that does detect 2 GE is considered to be able to detect 5 GE. The sample containing 100 IU HPV 16/µl was the sample that most datasets, 94.9 %, identified correctly (Table 1). Single HPV types in 100 GE/µl were correctly identified, without false positive types detected, in an average of 84 % of the data sets. HPV 56 and 59 were correctly identified by less than 80 % of the datasets HPV 68 was correctly identified only by 37.9 % of laboratories. In the samples containing multiple HPV types, between 50 % and 73 % of the datasets could correctly identify the types. The negative control sample containing only human genomic DNA was correctly identified as negative by 74 of 80 datasets.

The proficiency of detecting HPV types (restricted to data sets testing for more than 12 HPV types) is shown in Table 2. Nineteen data sets were 100 % proficient (detecting at least 50 IU of HPV 16 and HPV 18 in 5µl and 500 GE in 5µl of the other HPV types tested for (also when present together with other HPV types), without having more than one false positive result. As the Linear Array assays used a large (50 µl) input volume, the Linear Array system did not test for presence of amounts below 50 IU of HPV 16 and HPV 18 in 5µl and 500 genome equivalents in 5µl of the other HPV types. Two different Microarray assays were the commercial tests that had the highest number of proficient results (100%). Several in-house assays based on type-specific PCR and on general-primer PCR-Luminex were also 100 % proficient.

The non-commercial PGMY-LineBlot assay was transferred to all WHO HPV LabNet members in 2008 as an effort to build up testing capacity and evaluate the ease of technology transfer of this assay. The PGMY-LineBlot assay was used by seven members of the WHO HPV LabNet but with 100 % proficiency in only one laboratory. Only one laboratory (the originator) had been routinely using this assay before and the other laboratories had recently set up the assay according to instructions. Indeed, when a subsequent, similar proficiency panel was sent to the WHO HPV LabNet members, two additional laboratories using PGMY-LineBlot were 100% proficient and one additional lab was 88% proficient (data not shown).

To be considered as proficient in this study no more than one false positive sample per data set was acceptable. The number of false positive HPV types detected per data set is shown in Table 3. Thirty-four of the 80 data sets did not have any false positive results, whereas 12 data sets reported more than 3 false positive results. Among these, 3 datasets reported false positive HPV types in more than 15 samples. Data sets generated by the commercial tests CLART and InnoLiPA reported more than one false positive sample in 4 out of 6 datasets. Several in-house assays as well as some commercial assays that were performed by only one or only a few laboratories reported no false positive results at all.

The lowest genome equivalent or IU of each HPV type included in the panel that was detected in both single and multiple infections by different assays are shown in Table 4. HPV 16 and HPV 18 were the types detected at lowest concentration in most data sets. Only 1 and 3 datasets, respectively, could not detect the highest concentration of HPV16 and 18. By contrast, for HPV 52, HPV 59 and HPV 56 there were 25, 19 and 17 data sets, respectively, that could not detect these viruses in the highest concentration (Table 4).

DISCUSSION

We report on the development of an internationally comparable quality assurance methodology that traceable to ISs. Accurate and internationally comparable HPV DNA detection and typing methodology is an essential component in the evaluation of HPV vaccines and in effective implementation and monitoring of HPV vaccination programmes. Standardised methodology for evaluation of laboratory performance is a fundamental basis to enable any comparison of the methodologies used in laboratories worldwide. The major tools for achieving progress towards this goal are developing international biological standards and preparing and validating proficiency panels to qualify methods. The current study has established that such international proficiency panels with unitage traceable to ISs can be used in global studies. We have also demonstrated that such studies provide a unique overview of the status of the HPV detection and typing methodology that are being used globally and how well they perform in different laboratories.

Overall, it can be said that a majority of laboratories in this study had a good performance of their HPV DNA typing tests. However, some limitations were revealed.

There was a clear tendency towards systematically different limits of sensitivity for different HPV types. E.g., HPV 16 and HPV 18 were the types detected at the lowest amount in most data sets (only 1 and 3 datasets, respectively, could not detect 500 IU / 5 μl) whereas HPV 52, HPV 59 and HPV 56 could not be detected in the 500 GE / 5 μl amount by 25, 19 and 18 data sets respectively. Thus, many surveys of circulating HPV types might systematically underestimate the prevalence of HPV 52, 56 and 59 compared to HPV 16 and 18.

There was also a tendency for lower sensitivity of tests when multiple HPV types were present. In the samples containing multiple HPV types, between 50 % and 73 % of the data sets could correctly identify the types present, but in samples with only 1 HPV type present an average of 84 % of HPV types could be identified without false positive results. This tendency will cause a systematic underestimation of the prevalence of multiple infections and will introduce a systematic detection bias in epidemiological studies with detectability being dependent on determinants of HPV acquisition (e.g., a given HPV type will be more difficult to detect in high risk groups, because of higher likelihood of other HPV infections).

There was a surprisingly high amount of false positive results reported, with only 34/80 datasets being 100% specific. The proficiency panel contained only 1 entirely HPV-negative sample. The present study was designed to primarily evaluate HPV typing (rather than mere HPV detection) and we considered that specificity should in this context be measured primarily as absence of

detection of a specific HPV type also when other HPV types are present. Thus, for each HPV type evaluated there are at least 39 negative samples included in the panel and 1 false positive result thus equals >97% specificity. There was only 1 indication of a systematic mistyping (some Linear Array-based data sets reporting HPV56-containing samples as positive for HPV66), but otherwise there was no single sample that had systematic false positivity for the same type in several laboratories. These very common false positives are therefore neither associated with the panel nor with the assays used, but rather appear to result from laboratory environment and performance. Considering the deleterious consequences that a false positive result may have, it appears that a substantial effort towards increased specificity of testing is warranted.

On the other hand, there were some needs for improvement of the proficiency panel itself that were identified by this study. The HPV 39 plasmid used in the panel was cloned into the vector at the binding site of one of the most commonly used PCR primers (PGMY). All assays using the PGMY primer system, including Linear Array and CLART, could not detect the HPV 39 plasmid in the panel. As this was because of the way the plasmid was constructed, all these data sets were considered as not having been evaluated for HPV39 in this study.

The plasmid used to test for HPV 68a was not full-length, but contained only the L1 gene. We noted that Linear Array and all other PGMY-based assays that are indeed directed against L1 could not detect the HPV68a plasmid. Comparison of the sequences of HPV68a and HPV68b (ME180 isolate) showed significant differences in the sequence corresponding to the PGMY primer binding site. As the sequence of HPV68b was published before the sequence of HPV68a, it appears that these systems are designed to only detect HPV68b (11, 14). All data sets reporting usage of primers directed to genes other than L1 or that used the PGMY primers were considered as not testing for HPV 68 in this study. Accordingly only 29 data sets could be analysed for detection of HPV 68a and only 11 of the 29 laboratories (38 %) could detect HPV 68a. For the next WHO HPV LabNet proficiency panel, HPV39 will be recloned to change the cloning site and full-length genomes of both HPV68a and HPV68b will also be included.

The Linear Array can not exclude HPV 52 when the sample is positive for HPV 33, HPV 35 or HPV 58. Some laboratories have developed a type-specific PCR for HPV 52 to test HPV 33, 35 and 58-positive samples, whereas some laboratories (4/15) scored all sample with multiple infections containing HPV 52 as negative for HPV 52 (4, 23). This resulted in that they are regarded as not proficient for HPV 52 in this study. Four data sets generated using Linear Array were considered as not proficient since they reported 2 or even 3 false positive results. HPV 66 was

detected as false positive in 7 of in total 15 false positive results submitted in the 15 data sets using Linear Array, 6 of these samples contained 500 GE of HPV 56 that was correctly identified. The detection of HPV 66 in these samples was not reported by any other assay, indicating that the false detection of HPV 66 in HPV 56-positive samples is a problem that is commonly seen with the Linear Array assay.

For two commercial tests (InnoLiPA and CLART), 4 out of 6 data sets were not proficient because of too many false positives. InnoLiPa could not identify HPV 52 in 5 of 6 data sets. On the other hand, HPV 52 was reported in 9 samples where it was not present. The number of false positive samples reported by InnoLiPA was between 3 and 5 for the 4 laboratories that were not proficient. Three laboratories using CLART reported 7, 17 and 21 false positive results respectively, some with more than 3 false positives in each sample. Four laboratories using CLART could not detect HPV 56 and 45 in samples with multiple types. There was no consistent false positivity for any specific sample for these two assays. The false positivities for these assays appeared to be randomly distributed among the samples and were always different for the different laboratories, indicating that the problem is not related to the assay kit itself. Indeed, there were examples of several laboratories that had completely proficient results using these assays.

A major conclusion of the present study is that differences in performance were much larger between laboratories than between different types of assays. Proficiency panel testing is particularly useful to stimulate a learning process of improved performance in laboratories. Once regular feed-back on proficiency testing results is implemented, improvement of performance usually follows rapidly. An example of this was the results of the PGMY-LineBLOT assay that was recently set up in the HPV LabNet. Several laboratories who were using this assay for the first time had suboptimal results, but became proficient in a subsequent proficiency testing performed when there had been more time for practise.

The 2 samples that evaluated the DNA extraction step before the HPV testing and typing had a surprisingly low proportion of correct results. The sample containing 2000 cells of the cervical cancer cell line SiHa with about 1 copy of HPV16 per cell (i.e. total 2000 IU of HPV16/5ul) was detected only in about a third of the datasets. Also, a large number of datasets (six) reported false positive results in the sample containing an HPV-negative human cell line. This indicates that low yield in the DNA extraction step, potentially reducing sensitivity, as well as contamination in the DNA extraction step may be significant problems in the field of HPV DNA testing. Future

proficiency panels will contain a larger set of samples designed to specifically evaluate the DNA extraction step before the actual HPV testing and typing.

There are additional steps in the laboratory detection process that are not evaluated by the present strategy, notably sampling technique, handling and storage, natural variability of circulating virus strains, PCR inhibiting substances and naturally occurring genome modifications (e.g. integration and rearrangement). The HPV LabNet has chosen to perform quality control for these aspects of testing by launching a confirmatory testing scheme, where part of the clinical samples being tested are annually submitted for retesting to a higher level reference laboratory (5). The alternative strategy to include clinical samples in proficiency testing schemes was not chosen, because of the need to have exactly reproducible panels with defined content that can be used by hundreds of laboratories over many years and since confirmatory testing schemes were considered to better reflect the actual testing being done.

It should be emphasised that the current proficiency panel study was designed to evaluate the performance of HPV testing and typing tests used in HPV vaccinology and HPV surveillance, but not for evaluation of HPV tests used in cervical cancer screening (12). The demands on performance of HPV typing assays vary depending on the purpose of the testing. In vaccinology, a high sensitivity is needed for clinical vaccine trials as failure to detect prevalent infections at entry may result in apparent vaccine failures. By contrast, the clinical HPV-associated diseases, such as high grade CIN, typically contain larger amounts of virus and cervical screening programs using HPV testing do not have as high demands on sensitivity (12). Guidelines for evaluations of such tests have recently been published (12).

In conclusion, we find that global HPV DNA proficiency studies are both feasible and informative. The launch of an internationally standardised methodology to analyse the specificity and sensitivity for different HPV typing assays (as well as the performance of participating laboratories) to correctly identify the 16 HPV types which are the most important in HPV surveillance and vaccinology is likely to greatly enhance quality and comparability of studies in these fields.

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TABLE 1. HPV DNA proficiency panel composition and HPV testing results

HPV types	HPV genome equivalents per 5 µl	Percent correct data sets* (N)
16	500	**94,9 (74 / 78)**
16	50	89,7 (70 / 78)
16	5	67,9 (53 / 78)
18	500	**92,2 (71 / 77)**
18	50	92,2 (71 / 77)
18	5	59,7 (46 / 77)
6	500	**91,3 (63 / 69)**
6	50	81,1 (56 / 69)
11	500	**88,4 (61 / 69)**
11	50	94,2 (65 / 69)
31	500	**86,4 (64 / 74)**
31	50	67,6 (50 / 74)
33	500	**90,5 (67 / 74)**
33	50	86,5 (64 / 74)
35	500	**86,5 (64 / 74)**
35	50	78,4 (58 / 74)
39	500	**90,5 (38 / 42)**
39	50	69,0 (29 / 42)
45	500	**89,0 (65 / 73)**
45	50	80,8 (59 / 73)
51	500	**88,9 (64 / 72)**
51	50	75 (54 / 72)
52	500	**85,1 (63 / 74)**
52	50	78,4 (58 / 74)
56	500	**75,3 (55 / 73)**
56	50	68,5 (50 / 73)
58	500	**90,5 (67 / 74)**
58	50	75,7 (56 / 74)
59	500	**72,6 (53 / 73)**
59	50	65,7 (48 / 73)
66	500	**84,6 (55 / 65)**
66	50	77,3 (51 / 65)
68	500	**37,9 (11 / 29)**
68	50	34,4 (10 / 29)
16, 45, 52, 33	500	**58,9 (46 / 78)** [a]
16, 45, 52, 33	50	47,4 (37 / 78) [a]
11, 18, 31, 51	500	**72,7 (56 / 77)** [a]
11, 18, 31, 51	50	59,7 (46 / 77) [a]
35, 39, 59, 66	500	**59,7 (44 / 74)** [b]
35, 39, 59, 66	50	50 (37 / 74) [b]
6, 56, 58, 68	500	**50,0 (37 / 74)** [b]
6, 56, 58, 68	50	41,9 (31 / 74)[b]
None	0	**92,5 (74 / 80)**
HPV 16 Cervical cancer cells	2000 cells	34,3 (23 / 67) (3 false positives)
HPV-negative cells	0	65,7 (44 /67) (6 false positives, 17 invalid)

* Data sets detecting correct type as claimed, no false positive type detected.

[a] Including data sets generated by type specific HPV 16 / HPV 18 PCR.

[b] Data sets known not to detect HPV 39 or HPV 68 are considered as correct when the other HPV types in the sample are detected.

c) The plasmid concentration that is equivalent to 50 genome copies (IU) varied from 0,53fg to 0,67fg/5 µl because of small variation in the length of the HPV genome and use of different cloning vectors. HPV68 had only an L1 plasmid and the plasmid concentration equivalent to 50 genome copies was therefore 0,23fg/5µl. The background concentration of human DNA was in all samples 50ng/5 µl.

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563 **TABLE 2. Proficiency of detecting HPV types tested for, by laboratory**

HPV assay type	Number of data sets	HPV region targeted (primers)	No. of proficient data sets				
			100% proficient	99-90 % proficient	89-80 % proficient	<80 % proficient	Not proficient
All assays	73	L1/E1/E6/E7	19	10	5	11	28
Linear Array (Roche)	15	L1 (PGMY)	6	5	0	0	4
CLART HPV 2 (Genomica)	6	L1 (PGMY)	0	0	2	0	4
InnoLiPA (Innogenetics)	6	L1 (SPF10)	0	1	1	0	4
PGMY-LINEBLOT	6	L1 (PGMY)	1	1	0	2	2
In-house Type-specific PCR	6	L1 / E6 / E7	2	0	0	1	3
DNA chip (Biocore)	4	L1	0	0	0	3	1
In-house Lineblot (Snijders)	4	L1 (GP)	0	1	0	2	1
In house PCR Luminex	4	L1 (GP)	3	0	0	0	1
In house PCR Luminex	4	E6 / E7	2	0	0	0	2
In-house Microarray	3	L1 / E7	1	0	0	0	2
PCR-RFLP	3	L1	0	0	0	2	1
Microarray (Genetel)	2	L1	2	0	0	0	0
DEIA LiPA assays (DDL)	2	L1 (SPF 10)	0	0	0	0	2
In house PCR EIA	2	L1	0	0	1	0	1
Papillocheck Microarray	1	E1	1	0	0	0	0
Type specific PCR (GenoID)	1	L1	0	1	0	0	0
In-house PCR Luminex	1	L1 (PGMY-GP)	0	0	0	1	0
PCR Luminex (Multimetrix)	1	L1 (GP)	0	0	1	0	0
PCR EIA (GenoID)	1	L1	0	1	0	0	0
In-house PCR sequencing	1	L1 (PGMY-GP)	1	0	0	0	0

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565 Table restricted to assays testing for more than 12 types.
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TABLE 3 False positive HPV types detected, by assay

HPV assay type	Number of data sets	HPV region targeted (primers)	No. of false positive samples per data set				
			0 samples	1 sample	2 samples	3 samples	> 3 samples
All assays	80	L1/E1/E6/E7	34	16	9	9	12
Linear Array (Roche)	15	L1 (PGMY)	6	5	2	2	0
CLART HPV 2 (Genomica)	6	L1 (PGMY)	1	1	1	0	3
InnoLiPA (Innogenetics)	6	L1 (SPF10)	1	1	0	2	2
PGMY-LINEBLOT	6	L1 (PGMY)	3	0	0	3	0
In-house Type-specific PCR	7	L1 / E6 / E7	1	3	1	0	2
In-house 16 /18 specific PCR	6	E6 / E7	5	0	1	0	0
DNA chip (Biocore)	4	L1	1	2	0	1	0
In-house Lineblot (Snijders)	4	L1 (GP)	2	1	0	0	1
In house PCR Luminex	4	L1 (GP)	3	0	0	0	1
In house PCR Luminex	4	E6 / E7	2	0	1	0	1
In-house Microarray	3	L1 / E7	0	1	1	0	1
PCR-RFLP	3	L1	1	1	1	0	0
Microarray (Genetel)	2	L1	2	0	0	0	0
DEIA LiPA assays (DDL)	2	L1 (SPF 10)	0	0	1	1	0
In house PCR EIA	2	L1	0	1	0	0	1
Papillocheck Microarray	1	E1	1	0	0	0	0
Type specific PCR (GenoID)	1	L1	1	0	0	0	0
In-house PCR Luminex	1	L1 (PGMY-GP)	1	0	0	0	0
PCR Luminex (Multimetrix)	1	L1 (GP)	1	0	0	0	0
PCR EIA (GenoID)	1	L1	1	0	0	0	0
In-house PCR sequencing	1	L1 (PGMY-GP)	1	0	0	0	0

[a]Data including the 2 extraction control samples, that were not included in the proficiency evaluation

585 **Table 4a**: HPV IU/GE detected per 5 µl in both single and multiple infections (commercial assays)

HPV type	HPV IU /GE	All Assay (%)	Linear Array* (Roche)	CLART HPV 2 (Genomica)	InnoLiPA (Innogentics)	DNA chip (biocore)	Microarray (Genetel)	Papillocheck Microarray	Luminex (Multimetrix)	PCR-EIA (GenoID)	Type specific PCR (GenoID)
16	5	50 / 79 (63)	7 / 15	4/6	2 / 6	4 / 4	2 / 2				1 / 1
16	50	69 / 79 (87)	15 / 15	5/6	3 / 6			1 / 1	1 / 1		
16	500	78 / 79 (99)			6 / 6					1 / 1	
18	5	41 / 78 (53)	4 / 15	1/6	5 / 6	4 / 4	2 / 2		1 / 1		1 / 1
18	50	69 / 78 (88)	14 / 15	6/6						1 / 1	
18	500	75 / 78 (96)	15 / 15					nt			
6	50	48 / 70 (69)	6 / 15	6 / 6	6 / 6	4 / 4	2 / 2	1 / 1			1 / 1
6	500	62 / 70 (88)	15 / 15							1 / 1	
11	50	56 / 70 (80)	6 / 15	6 / 6	5 / 6	4 / 4	2 / 2	1 / 1	1 / 1	1 / 1	1 / 1
11	500	67 / 70 (96)	14 / 15								
31	50	36 / 75 (48)	6 / 15	3 / 6	5 / 6		2 / 2	1 / 1		1 / 1	1 / 1
31	500	61 / 75 (81)	15 / 15	4 / 6							
33	50	55 / 75 (73)	7 / 15	5 / 6	6 / 6	4 / 4	2 / 2		1 / 1	1 / 1	1 / 1
33	500	70 / 75 (93)	15 / 15					1 / 1			
35	50	50 / 75 (67)	7 / 15	4 / 6	5 / 6	4 / 4	1 / 2		1 / 1	1 / 1	1 / 1
35	500	65 / 75 (87)	14 / 15		6 / 6		2 / 2	1 / 1			
39	50	25 / 42 (60)			5 / 6	1 / 4	1 / 2	1 / 1	1 / 1		1 / 1
39	500	38 / 42 (90)	Nt	nt	6 / 6	3 / 4	2 / 2			1 / 1	
45	50	48 / 74 (65)	7 / 15	1 / 6	2 / 6	4 / 4		1 / 1	1 / 1	1 / 1	1 / 1
45	500	63 / 74 (85)	15 / 15	2 / 6	5 / 6		2 / 2				
51	50	49 / 73 (67)	7 / 15	6 / 6	5 / 6	2 / 4	2 / 2	1 / 1	1 / 1	1 / 1	1 / 1
51	500	64 / 73 (88)	15 / 15								
52	50	40 / 75 (53)	3 / 15	4 / 6	1 / 6	2 / 4	2 / 2		1 / 1		1 / 1
52	500	50 / 75 (67)	9 / 15			3 / 4		1 / 1		1 / 1	
56	50	41 / 74 (55)	4 / 15	1 / 6	6 / 6		2 / 2	1 / 1	1 / 1	1 / 1	1 / 1
56	500	56 / 74 (76)	14 / 15	2 / 6							
58	50	48 / 75 (64)	7 / 15	5 / 6	1 / 6	3 / 4	2 / 2			1 / 1	1 / 1
58	500	68 / 75 (91)	15 / 15	6 / 6	4 / 6	4 / 4		1 / 1	1 / 1		
59	50	42 / 74 (57)	7 / 15	4 / 6	1 / 6		2 / 2	1 / 1		1 / 1	1 / 1
59	500	55 / 74 (74)	15 / 15						1 / 1		
66	50	44 / 66 (67)	6 / 15	6 / 6	6 / 6		1 / 2	1 / 1	1 / 1	1 / 1	1 / 1
66	500	58 / 66 (88)	14 / 15				2 / 2				
68	50	7 / 29 (24)					1 / 2				
68	500	10 / 29 (34)	Nt	nt	1 / 5		2 / 2	nt			

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588 **Table 4b**: HPV IU or GE detected per 5 µl in, both single and multiple infections (in-house
589 assays).

HPV type	HPV IU /GE	PGMY - CHUV	Type specific PCR	Lineblot	Luminex (GP)	Luminex (E6/E7)	Microarray	PCR-RFLP	DEIA LIPA	PCR-EIA	Luminex (PGMY-GP)	PCR sequencing
16	5	4 / 7	6 / 7	2 / 4	3 / 4	3 / 4	2 / 3	1 / 3	2 / 2	1 / 2	1 / 1	1 / 1
16	50	6 / 7		3 / 4	4 / 4		3 / 3	2 / 3		2 / 2		
16	500	7 / 7	7 / 7	4 / 4		4 / 4		3 / 3				
18	5	2 / 7	5 / 7	1 / 4	4 / 4	2 / 4	2 / 3		2 / 2		1 / 1	1 / 1
18	50	6 / 7		4 / 4		4 / 4	3 / 3			1 / 2		
18	500		7 / 7					2 / 3		2 / 2		
6	50	4 / 7	3 / 5	1 / 4	4 / 4	1 / 2	1 / 2	3 / 3	2 / 2	1 / 2	1 / 1	1 / 1
6	500	5 / 7	5 / 5			2 / 2						
11	50	5 / 7	4 / 5	4 / 4	4 / 4	1 / 2	2 / 2	3 / 3	2 / 2	2 / 2	1 / 1	1 / 1
11	500	7 / 7	5 / 5									
31	50		5 / 7	2 / 4	1 / 4	2 / 4	1 / 3	1 / 3	2 / 2	2 / 2		1 / 1
31	500	5 / 7	6 / 7	4 / 4	4 / 4	3 / 4	3 / 3	2 / 3				
33	50	2 / 7	7 / 7	3 / 4	4 / 4	3 / 4	2 / 3	2 / 3	2 / 2	2 / 2		1 / 1
33	500	5 / 7		4 / 4			3 / 3	3 / 3				
35	50	2 / 7	6 / 7	4 / 4	4 / 4	2 / 4	2 / 3		2 / 2	2 / 2	1 / 1	1 / 1
35	500	5 / 7				4 / 4						
39	50		4 / 6		4 / 4	3 / 4	2 / 3		2 / 2			
39	500	nt	6 / 6	3 / 4		4 / 4				1 / 1	nt	nt
45	50	6 / 7	5 / 7	4 / 4	4 / 4	4 / 4	2 / 3		2 / 2	1 / 1	1 / 1	1 / 1
45	500						3 / 3					
51	50	5 / 7	5 / 6	1 / 4	4 / 4	2 / 4	1 / 3	1 / 3	2 / 2		1 / 1	1 / 1
51	500	7 / 7	6 / 6	3 / 4		3 / 4				1 / 1		
52	50	5 / 7	7 / 7		4 / 4	4 / 4	3 / 3		2 / 2			1 / 1
52	500			1 / 4								
56	50	1 / 7	5 / 6	4 / 4	4 / 4	4 / 4	1 / 3		2 / 2	1 / 2	1 / 1	1 / 1
56	500	4 / 7								2 / 2		
58	50	5 / 7	7 / 7	3 / 4	3 / 4	2 / 4	2 / 3	2 / 3	2 / 2		1 / 1	1 / 1
58	500			4 / 4	4 / 4	3 / 4				2 / 2		
59	50	6 / 7	5 / 7	3 / 4	3 / 4	2 / 4	2 / 3		1 / 2	1 / 1	1 / 1	1 / 1
59	500	7 / 7			4 / 4	3 / 4			2 / 2			
66	50	5 / 7		2 / 4	4 / 4	2 / 3	2 / 3	1 / 3	2 / 2	1 / 1	1 / 1	1 / 1
66	500	6 / 7	nt	4 / 4		3 / 3	3 / 3					
68	50				4 / 4		1 / 2		1 / 2			
68	500					nt		nt	2 / 2		nt	nt

590 Includes laboratories with multiple false positives. Detection with input volume 50 µl classified as
591 data for the next 10-fold dilution compared to input with 5 µl. Input with 10 or 15 µl classified as
592 same dilution compared to input with 5 µl.
593 * 8 laboratories used 50 µl input volume in Linear Array.
594 One InnoLiPA assay does not detect HPV 68.
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