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Link to publication

The 2010 global proficiency study of Human Papillomavirus genotyping in vaccinology

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Running title: Proficiency study of Human Papillomavirus genotyping for vaccinology

ABSTRACT

Accurate and internationally comparable Human Papillomavirus (HPV) DNA genotyping is essential both for evaluation of HPV vaccines and for effective monitoring and implementation of vaccination programs. World Health Organisation (WHO) HPV Laboratory Network (LabNet) regularly issues international proficiency studies. The 2010 HPV genotyping proficiency panel for HPV vaccinology contained 43 coded samples composed of purified plasmids of sixteen HPV types (HPV 6, 11, 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68a and 68b) and 3 coded extraction controls. Proficient typing was defined as detection in both single and multiple infections of 50 International Units (IU) of HPV 16 and HPV 18 DNA and 500 genome equivalents (GE) for the other 14 HPV types. Ninety-eight laboratories worldwide submitted a total of 132 datasets. Twenty-four different HPV genotyping assay methods were used, with Linear Array being most commonly used. Other major assays used were Lineblot/Inno-LiPa, CLART, type-specific real-time PCR, PCR-Luminex and different microarray assays. Altogether 72 data sets were proficient for detection of more than one type, only 26 data sets proficiently detected all sixteen HPV types. The major oncogenic HPV types, 16 and 18, were proficiently detected in 95.0% (114/120) and 87.0% (94/108) of datasets, respectively. Forty-six datasets reported multiple false positive results and were considered non-proficient. A trend towards increased sensitivity of assays was seen for the 41 laboratories that participated in both 2008 and 2010. In conclusion, continued global proficiency studies will be required for establishing comparable and reliable HPV genotyping services for vaccinology worldwide.

INTRODUCTION

Cervical cancer is the second most common type of cancer among women worldwide, with HPV infection linked to more than 99 % of cervical cancers (3, 37). The most important high risk types HPV 16 and HPV 18 account for about 70 % of all invasive cervical cancers worldwide (27). Accurate and internationally comparable HPV DNA detection and genotyping methodology is an essential component both in the evaluation of HPV vaccines and in the effective implementation and monitoring of HPV vaccination programs. Genotyping assays used today differ in their analytical performance with regard to type-specific sensitivity and specificity (15). Several studies have compared different HPV typing assays using various clinical samples to assess their performance (7, 17, 24). However, in addition evaluation of assay performance needs to be performed in a standardised manner, where different assay performance can be evaluated and results can be compared against a known and accepted standard over time (15).

In 2005 the World Health Organization (WHO) initiated the establishment of a global HPV laboratory network (HPV LabNet) with the objective to facilitate the development and implementation of HPV vaccines by improving and standardising the quality of HPV laboratory services used for HPV surveillance and HPV vaccination impact monitoring. The main activities within the HPV LabNet include harmonisation and standardisation of laboratory procedures by the development of internationally comparable quality assurance methods, international standards and reference reagents and standard operating procedures (SOPs) for vaccinology (8, 9, 38). Regularly issued global proficiency studies are essential for establishing comparable and reliable laboratory services. A number of international proficiency panels for quality assurance of laboratory testing are being conducted widely for a number of infectious agents. E.g., the WHO measles and rubella laboratory networks have been distributing proficiency panels worldwide yearly since 2001, with the purpose to monitor the performance of laboratories and assay methodology over time (31). As there is no natural source of biological material that could be used to generate type-specific HPV international standards (ISs), recombinant HPV DNA plasmids were used to establish ISs of HPV 16 and HPV 18 DNA in 2008 with an assigned potency in International Units (IU) (39). In 2008, the WHO HPV LabNet conducted a proficiency study open for participation to laboratories worldwide based on HPV DNA plasmids containing the genomes from 14 oncogenic HPV types and 2 benign HPV types (6). That study demonstrated that it is possible to perform global proficiency studies with unitage traceable to IS based on plasmid DNA and that such studies can provide an overview of the status of the HPV detection and typing methodology worldwide.

The international WHO proficiency study described herein concerns vaccinology. It should be realized that the proficiency reported cannot be translated into proficiency for cervical screening as the latter demands HPV testing to be informative about the presence of cervical (pre)cancerous lesions and as such has different (analytical) requirements. This report was based on a proficiency panel composed of the same HPV DNA plasmid material as 2008, with the amount of DNA titrated in amounts traceable to the IS. The use of the same panel material allowed a reproducible, standardised evaluation of assay sensitivity over time. Specificity was defined as absence of incorrect typing. The sample pre-processing was evaluated with extraction controls of cervical cancer cell lines. The panel was distributed to 105 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 the HPV types, grouped by the methods used as well as to assess the analytical sensitivity for the detection of the HPV types included in the study, and report on the test results on the comparison of the panel for the years 2008 and 2010.

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, and 66), Dr Ola Forslund (HPV68a L1), Dr Elisabeth Schwarz (HPV 68b), 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 HPV genomes are cloned into different cloning vectors: HPV 6 in pBR322 at position 4724 in the HPV genome, HPV 11 in pGEM4Z at position 4781, HPV 16 in pBR322 at position 6152, HPV 18 in pBR322 at position 2440, HPV 31 in pT713 at position 3362, HPV 33 in pBR322 at position 2797, HPV 35 are cloned in two fragments 5012-956 and 956-5012 in pT713, HPV 39 in pGEM4z at position 1714, HPV 45 in pGEM4Z at position 75, HPV 51 in pGEM4z at position 4511, HPV 52 in pUC19 at position 7559, HPV 56 in pT713 at position 5521, HPV 58 in pGEM4Z at position 1158, HPV 59 in pUC9 at position 69, HPV 66 in pBR322 at position 7484, HPV 68a L1 from a clinical sample cloned in pCR-Script, and HPV68b (ME180) of about 7 kb containing L1, L2, E1, E2, E4, E5, E6, E7 with an incomplete E2 gene in pCR4-TOPO.

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, HPV 68a nr X67161 and HPV 68b nr FR751039.

Panel composition and production. The plasmids were prepared and characterised as previously described (36), with the following changes: HPV 39 was originally cloned into the L1 gene at the binding site for one of the PGMY primers and was therefore re-cloned so that the vector (pGEM4z) is now positioned in the E1 gene (position 1714). A plasmid for HPV 68b (ME180) with a size of about 7 kb, containing L1, L2, E1, E2, E4, E5, E6, E7 with an incomplete E2 gene was added to the panel. Purified plasmids containing cloned genomic DNAs for HPV types 6, 11, 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68a and 68b were prepared to make up the 43 different panel samples by diluting the HPV recombinant DNA plasmid stock solution in TE buffer in the background of human placental DNA, as previously described (36). 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 (28). 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 2010 and were randomly assigned numbers from 1 through 43. The panels were stored at -20°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 were purchased from the American Type Culture Collection and cultured in Dulbecco's modified Eagle medium (Gibco 11960). The cells were diluted in PreserveCyt™ (Cytyc 0234004) to a concentration of 5 and 500 SiHa cells/µl in a background of 5000 C33A cells/µl, one sample contained only the background C33A cells. 200 µl of each preparation was dispensed in 1.5 ml vials and labelled as WHO HPV DNA A, B and C.

Before distribution of the WHO HPV DNA proficiency panel, the samples were tested (blinded) by the WHO HPV LabNet Global Reference Laboratory (GRL) in Sweden and one other laboratory, namely the German Cancer Research Center (DKFZ) in Heidelberg, Germany (Dr. Michael Pawlita).

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 (29). Ten µl PCR products were analysed by multiplex genotyping using a Luminex based assay that can distinguish HPV types 6, 11, 16, 18, 26, 30, 31, 33, 35, 39, 40, 42, 43, 45, 51, 52, 53, 54, 56, 58, 59, 61, 62, 66, 67, 68a, 68b, 69, 70, 73, 81, 82, 86, 89, 90, 91 and 114 as described earlier (25, 26).
 Appropriate negative and positive controls were used to monitor the performance of the method. DNA from the extraction control A, B and C were extracted using QIAamp DNA Mini and Blood kit (Qiagen) according to the manufacturer's instructions.

(ii) DKFZ. A 10 µl DNA sample was tested by the BSGP5+/ 6+-PCR/MGP assay, as previously described (26). The PCR products were analysed using bead based multiplex genotyping as described (25). 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, 69, 70, 73 and 82 can be distinguished by this method. All samples were

tested for human DNA with PCR primers targeting a part of the β-globin gene and a bead coupled β-globin specific probe used in the genotyping assay.

Organization of the study. A call for participation in the study was advertised on the WHO public website. Laboratories that are or will be involved in HPV surveillance and/or vaccine development were particularly welcome. The panels were prepared by the WHO HPV LabNet GRL in Sweden and were distributed by EQUALIS, the quality assurance company in Sweden at ambient temperature to 105 laboratories worldwide. The number of participating laboratories according to WHO regions are: America Region, 23 laboratories; Africa Region, 1 laboratory; Eastern Mediterranean Region, 5 laboratories; European Region, 49 laboratories; South East Asia Region, 9 laboratories and Western Pacific Region, 18 laboratories. The package also included a letter of instruction 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 EQUALIS online within 4 weeks of receipt of the specimens. In registering to the proficiency study the participating laboratory agree to assign the right to publish the data to the WHO, it was also agreed that only coded results from all participating laboratories will be presented, grouped by methods performed.

All results submitted to EQUALIS were coded and analyzed anonymously by the GRL in Sweden. Datasets generated were designated numerically from 1 through 132. 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, 2, 4, 5, 8, 10-14, 16, 20-22, 25, 26, 29, 30, 32-35) are summarized in Table 2.

Data analysis. Criteria used for considering a dataset as proficient for HPV vaccinology 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 (9) and was based on a consideration of which performance requirements are required and are realistic. A higher requirement for HPV16 and 18 was considered essential, because of the pivotal role of these HPV types in causing cervical cancer.

Four datasets reporting results only as "high" or "low risk" HPV were not included in the overall performance analyses (one such dataset was generated using Hybrid Capture 2 (Qiagen), one dataset was generated using an in-house assay, and two datasets were generated using the Cobas 4800 (Roche)).

RESULTS

Validation of the HPV proficiency panel. The results from the initial panel validation at the GRL Sweden and at DKFZ included qualitative characterization of HPV and human genomic DNAs. Both these laboratories used Luminex based assays with modified GP 5+/ 6+ primers. No false positive HPV type was detected in the samples in any of the reference laboratories. All HPV types were detected by both laboratories in the lowest concentration included in the panel except HPV 31, 33, 39 and 58, when present together with other plasmids that could only be detected by one of these laboratories. Both 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. Ninety-eight of 105 participating laboratories, including the two laboratories that conduct the panel validation, submitted 132 datasets according to the timeline (Table 2). Four datasets 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 73 datasets, the results had been obtained using commercially available tests. The most commonly used assay was Linear Array (Roche) that was used to generate 17 datasets. Other widely used assays include Inno-LiPA (Innogenetics), CLART HPV 2 (Genomica), PGMY-CHUV and other in-house Lineblot, in-house type-specific PCR, Luminex and Microarray based assays (Table 2). Participating laboratories included public health laboratories, research laboratories, diagnostic kit manufacturers and vaccine companies. According to the survey, the annual number of samples analysed for HPV typing per laboratory varied from 60 to 100,000 per year with approximately 52% of the laboratories performing less than 2,000 HPV typing tests per year, around 35 % between 2,000 and 10,000 assays per year and 12 % of the laboratories performed more than 10,000 HPV genotyping assays yearly.

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 1 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. HPV 16 and 18 were included as single plasmids at the highest concentration of 10 IU/μl, which could be correctly detected in 95% and 87% of the datasets respectively. The samples containing single plasmids at a concentration of 100 GE/μl of HPV 6, 11, 33, 58 and 66 were correctly identified, without false positive types detected in more than 95% of the datasets (Table 1). HPV 39 and 68b were correctly identified by less than 80% of the datasets. HPV 68a cannot be detected by Linear Array and other PGMY-based assays because of several primer mismatches. HPV 68a was correctly identified only by 36.8% of the laboratories. In the samples containing multiple HPV types, between 44% and 78% of the datasets could correctly identify the types. The negative control sample containing only human genomic DNA was correctly identified as negative by 128 of 132 datasets.

The proficiency of detecting HPV types by assay (restricted to datasets testing for more than 2 HPV types) is shown in Table 2. Twenty-six datasets 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 in some laboratories, these Linear Array datasets 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. For the commercial assay Papillocheck, the panel did not evaluate the ability to detect HPV18 since the HPV 18 plasmid included in the panel is cloned in the region targeted by this assay (E1).

Two different Microarray assays, Papillocheck (Greiner Bio-one) and EASYCip (King Car) were the commercial tests that had the highest number of proficient results (100%). About half of the datasets generated by Linear Array were 100 % proficient. Several in-house assays based on general-primer PCR followed by hybridisation (PGMY-CHUV) or Luminex were also 100% proficient.

To be considered as proficient in this study, no more than one false positive sample per dataset was acceptable. The number of false positive HPV types detected per dataset is shown in Table 3. Seventy-one of the 132 datasets did not have any false positive results, whereas 26 datasets reported more than 3 false positive results. Among these, 4 datasets reported false positive HPV types in more than 25 samples. Datasets generated by the commercial tests InnoLiPA, CLART and

Linear Array reported more than one false positive sample in 75 %, 50% and 35 % of the datasets respectively. Several in-house and commercial assays that were performed by only a few laboratories reported no false positive results at all.

The lowest GE 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 11, 16, 18, 33, 52 and 66 were the types detected at lowest concentration in most datasets. Only 3 datasets could not detect the highest concentration of HPV16. By contrast, for HPV 39, HPV 59 and HPV 56 there were 41, 37 and 32 datasets, respectively, that could not detect these viruses in the highest concentration (Table 4).

Three additional samples (A, B, C) were used to evaluate the DNA extraction step before the HPV testing and typing. Two of the samples contained cells from the cervical cancer cell line SiHa mixed with the HPV negative cancer cell line C33A in different amounts and one sample with only C33A cells was the negative control. We did not observe any obvious difference in performance between different extraction methods (data not shown). In the sample containing 2500 cells /5 μl of the cervical cancer cell line SiHa, HPV 16 was correctly identified by 83% of the datasets. Four datasets reported false positive HPV types in this sample. The negative control containing only C33A cells was correctly reported as negative by only 83% of the laboratories (Table 1).

Comparison of results for laboratories that participated in the proficiency study both 2008 and 2010. Forty-one laboratories analysed the proficiency panels in both 2008 and 2010. Some of the laboratories used the same tests in both years whereas some laboratories had changed at least one of the tests used. Percent proficiency, for both years and in comparison with the results from all datasets submitted in 2010 is shown in Table 5. Laboratories that used the same assay both years were 27 % proficient in 2008 compared to 30% in 2010. There was a definite trend towards increased sensitivity of assays, e.g. 50 IU of HPV 16 could be detected by all (100%) laboratories this year compared to 86% in 2008 (data not shown). However, the increase in sensitivity is for several laboratories accompanied by an increased amount of false positive results, resulting in non-proficiency (Table 6).

DISCUSSION

We report on a reproducible, internationally comparable quality assurance methodology traceable to ISs. The methodology for evaluation of laboratory performance needs to be standardised, in order to enable accurate comparison of the methodologies used in laboratories worldwide. The current study has established that repeated issuing of international proficiency panels containing known amounts of virus plasmids with unitage traceable to ISs can be used to follow the development of the HPV typing methodologies for vaccinology that are being used globally and how robust they are when performed in different laboratories.

Overall, a majority of HPV DNA typing methodologies used by laboratories participating in this study had a proficient performance according to the established criteria. However, some limitations were revealed.

The 2008 study findings that there were systematic differences in the sensitivity to detect different HPV types remained in 2010. E.g., HPV 16, HPV 11 and HPV 18 were still the types detected at the lowest amount in most datasets (only 3, 9 and 11 datasets, respectively, could not detect 500 IU / 5 μl) whereas HPV 39, HPV 59 and HPV 56 could not be detected in the 500 GE / 5 μl amount by 41, 37 and 32 datasets respectively. This suggests that many surveys of circulating HPV types systematically underestimate the prevalence of HPV 39, 56 and 59 compared to HPV 16 and 18. As also found in 2008, HPV52, 56 and 59, were the types most difficult to detect.

Correct typing of samples containing multiple HPV types was reported in 44 % to 78% of the datasets, compared to an average of 86% when only 1 HPV type was present in the sample. A lower sensitivity in samples with multiple types was seen also in the 2008 study. The underestimation of the prevalence of multiple infections will introduce a systematic detection bias in epidemiological studies, with detectability being dependent on determinants of HPV acquisition. Some high risk HPV types will thus be more difficult to detect in patients in high risk groups, because of higher likelihood of multiple HPV infections.

There was a rather high amount of false positive results reported, with only 71/132 (54%) of datasets being 100% specific. This is a small, but noteworthy, improvement compared to 2008, when only 42 % (34 of 80) datasets were 100% specific.

The proficiency panel contained only 2 entirely HPV-negative samples. The study was designed to evaluate HPV typing 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 38 negative samples included in the panel and 1 false positive result thus equals >97% specificity.

We searched the datasets for patterns of consistent false positivity for any specific sample in the panel. The false positivities appeared to be essentially randomly distributed among the samples, indicating that the problem with false positives is usually not related to a property of the assays itself (e.g. cross-reactivity), but rather with the laboratory conditions of use (e.g. contamination). A systematic false positivity was found in the samples that contain the HPV58 plasmid, where 15 datasets also detected HPV52 in at least one of the HPV58-containing samples. This could be related to the fact that both the Linear Array and InnoLiPA assays state that these tests cannot exclude HPV52 in samples that contain HPV58. Most of the HPV52 detections in the HPV58-positive samples were generated using the SPF10 primers used in InnoLiPA, but there were also other assays, including HPV52 type-specific PCRs. As HPV52 and HPV58 are closely related viruses, it is conceivable that several assays could have problems to distinguish these HPV types. However, it should also be considered whether the proficiency panel itself could have been contaminated in these samples. There were no less than 94 datasets from laboratories proficient to detect HPV52 in the lowest dilution that did not report this false HPV52 positivity in these samples - several of them using the same assays as those reporting the false HPV52 positivity - suggesting that a general proficiency panel contamination is unlikely as explanation.

There were some needs for improvement of the proficiency panel itself that were identified by this study. The commercial test Papillocheck, used by 4 laboratories, is using primers directed to the E1 gene. Since the plasmid used for HPV 18 is cloned at one of the primer binding sites in E1, this assay cannot detect the HPV 18 plasmid and is considered as not testing for HPV 18 in the study. The plasmid used to test for HPV 68a was not full-length, but contained only the L1 gene. We noted in 2008 that Linear Array and all other PGMY-based assays that are indeed directed against L1 could not detect the HPV68a plasmid. In this new panel a plasmid containing HPV 68b was included in addition to HPV 68a (18, 23). All datasets reporting usage of primers directed to genes other than L1 or that used the PGMY primers were considered as not testing for HPV 68a in this study. Accordingly, only 61 datasets could be analysed for detection of HPV 68a. Still, only 17 of these laboratories (28 %) could detect HPV 68a. In order to allow detection systems with targets outside L1, full-length genomes of HPV68a will be included in the next panel

The most commonly used commercial assay, Linear Array, used to generate 17 datasets cannot exclude HPV 52 when the sample is positive for HPV 33, HPV 35 or HPV 58. In the 2008 study,

4/15 laboratories scored all samples with multiple infections containing HPV 52 as negative for HPV 52. This year no laboratory scored HPV 52 as negative in multiple infections and all laboratories using Linear Array were proficient in detecting HPV 52. Six datasets generated using Linear Array reported between 2 and 10 false positive results and were considered as not proficient. HPV 66 was detected as false positive in 11 of in total 31 false positive results submitted in the 17 datasets using Linear Array. Ten of these 11 false positives were detected in samples that contained HPV 56. This confirms the observation made already in 2008 that the Linear Array assay is prone to false detections of HPV 66 in HPV 56-positive samples.

For the commercial test InnoLiPA, 9 out of 12 datasets reported between 2 and 8 false positive results. Fifteen out of the 42 false positive results reported were HPV 52 detected in HPV 58 plasmids, as described above, and four datasets detected HPV 52 in samples that contain HPV 68b. The other false positivities appeared to be randomly distributed among the samples and were always different for the different laboratories.

Four of eight laboratories using the assay CLART submitted datasets with between 2 and 4 false positive. This is a major improvement compared to the study results in 2008 when 3 laboratories using this assay reported 7, 17 and 21 false positive results respectively, some with more than 3 false positives in each sample. This indicates that the previous problem with low specificity is not related to the assay kit itself and there are also examples of several laboratories that had completely proficient results using this assay.

The Line-Blot assay PGMY-CHUV is described in the WHO HPV Laboratory Manual (36). The assay was developed within the WHO HPV LabNet (9) in order to provide an inexpensive assay that would be independent of any specific commercial vendor. The 6 different laboratories in X different continents that had used this assay had generally good results, with no false positive results and 4/6 laboratories being fully proficient, supporting that this assay is suitable for standardisation and technology transfer.

As also found in our previous study (6), differences in performance were much larger between laboratories than between different types of assays. Proficiency panel testing is thus particularly useful to stimulate a learning process of improved performance in laboratories.

Three samples were included in the panel to evaluate the DNA extraction step before the HPV testing and typing. These contained cells from the cervical cancer cell line SiHa in a background of the HPV negative cell line C33A to mimic a clinical sample. SiHa cells has about 1 copy of HPV 16 per cell and 2500 cells /5ul was correctly identified in 83 % of the datasets. This is a major improvement compared to 2008 when only one third of the datasets could detect 2000IU of HPV

16/5ul. In the sample containing only the HPV negative cell line, 12 datasets reported false positive results and in total 21 false positive results was reported in the 3 extraction samples. This suggests that, for a noteworthy minority of laboratories, contamination in the DNA extraction step is an issue.

The HPV LabNet has chosen to perform proficiency testing using a panel of HPV plasmids since this material can be used to generate exactly reproducible panels with defined content in quantities that can be distributed to hundreds of laboratories over many years. The use of clinical samples in proficiency panels does not allow the same reproducibility over time. To assess the additional steps in the laboratory detection process that are not evaluated by the current proficiency panel, e.g. the sampling technique, handling and storage, PCR inhibiting substances, the HPV LabNet instead performs quality control by a confirmatory testing scheme. Participating laboratories submit a part of their clinical samples tested annually for retesting to a higher level reference laboratory (5).

This was the second HPV DNA proficiency panel issued by HPV LabNet that was open for worldwide participation. The number of participating laboratories almost doubled from 54 laboratories in 2008 to 98 laboratories in 2010. This increased participation in the study shows that many laboratories are interested in quality assurance of their assay methodologies and laboratory performance. Comparing the results of the laboratories that tested both the 2008 and 2010 WHO HPV DNA proficiency panel, we observe only marginal overall improvements. Among laboratories that used the same assay both years, 27 % were proficient in 2008 as compared to 30% in 2010. However, there are several noteworthy examples of laboratories that have achieved major improvements. We also see a strong trend towards increased sensitivity of assays. E.g., among the laboratories using the same assay in 2008 and 2010, 50 IU of HPV 16 could be detected by all (100%) laboratories this year compared to 86% in 2008. However, for several laboratories the increased sensitivity was accompanied by an increased amount of false positive results, resulting in non-proficiency. We suggest that recommendations for HPV laboratory testing should include an increased emphasis on the use of negative controls in the assays. Furthermore, we suggest that the requirements for proficiency in future proficiency panels should at the outset announce that proficiency will require that there are no false positives at all.

The demands on sensitivity of HPV typing assays vary depending on the purpose of the testing. The WHO HPV LabNet proficiency panels are designed to evaluate the performance of HPV typing tests used in HPV vaccinology and HPV surveillance. In vaccinology, a high analytical

sensitivity is needed as failure to detect prevalent infections at trial entry may result in false vaccine failures in vaccination trials. It should be noted that HPV tests used in cervical cancer screening programs have different requirements for evaluation, since for that purpose only HPV infections associated with high-grade cervical intraepithelial neoplasia or cancer are relevant and not the transient infections that do not give rise to clinically meaningful disease. Since the latter are characterized by low viral loads, HPV screening assays do not have as high demands on analytical sensitivity (19).

In conclusion, we find that the use of global HPV DNA typing proficiency panels for validating different HPV DNA tests and laboratories promotes the comparability of data generated from different laboratories worldwide. Regularly issued global HPV DNA typing proficiency panels that allow comparison of global results over time will be required for the continuing work towards international standardisation and quality improvement of HPV DNA typing results worldwide.

Conflict of interest

Joakim Dillner has acted as a consultant for and received research grants from Merck/SPMSD, a manufacturer of HPV vaccines.

Carina Eklund, Keng-Ling Wallin, Ola Forslund and Tiequn Zhou have disclosed no potential conflicts of interests.

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

HPV types	HPV IU/genome equivalents per 5 μl	Percent correct datasets* (N)
16	50	**95.0 (114 / 120)**
16	5	77.5 (93 / 120)
18	50	**87.0 (94 / 108)**
18	5	71.9 (82 / 114)
6	500	**96.1 (99 / 103)**
6	50	69.5 (73 / 105)
11	500	**98.1 (102 / 104)**
11	50	77.1 (81 / 105)
31	500	**87.4 (97 / 111)**
31	50	59.3 (67 / 113)
33	500	**97.2 (104 / 107)**
33	50	74.5 (82 / 110)
35	500	**89.1 (98 / 110)**
35	50	67.2 (76 / 113)
39	500	**76.2 (80 / 105)**
39	50	53.7 (58 / 108)
45	500	**85.4 (94 / 110)**
45	50	67.6 (75 / 111)
51	500	**85.7 (96 / 112)**
51	50	66.7 (74 / 111)
52	500	**87.4 (97 / 111)**
52	50	73.4 (83 / 113)
56	500	**87.2 (95 / 109)**
56	50	61.8 (68 / 110)
58	500	**95.4 (105 / 110)**
58	50	66.7 (70 / 105)
59	500	**85.7 (96 / 112)**
59	50	56.5 (61 / 108)
66	500	**95.3 (102 / 107)**
66	50	78.1 (82 / 105)
68a	500	**36.8 (21 / 57)**
68a	50	21.8 (12 / 55)
68b	500	**75.0 (78 / 104)**
68b	50	57.1 (64 / 112)
6, 16, 18, 51	500	**78.3 (101 / 129)** [a]
6, 16, 18, 51	50	69.0 (89 / 129) [a]
11, 16, 31, 33, 58	500	**57.9 (73 / 126)** [a]
11, 16, 31, 33, 58	50	46.1 (59 / 128) [a]
39, 45, 52, 56, 68a	500	**44.5 (53 / 119)** [b]
39, 45, 52, 56, 68a	50	35.0 (41 / 117) [b]
35, 59, 66, 68b	500	**50.0 (59 / 118)**
35, 59, 66, 68b	50	32.2 (38 / 118)
None	0	**97.0 (128 / 132)**
HPV 16 pos cells	25	61.1 (77 / 126) (5 false positives)
HPV-negative cells	0	83.3 (105 /126) (12 false positives)
HPV 16 pos cells	2500	83.3 (105 / 126) (4 false positives)

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

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

Datasets known not to detect the HPV 68a plasmids in this panel 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.

TABLE 2. Proficiency of detecting HPV types tested for, by assay. Table restricted to assays

testing for more than 2 types.

HPV assay type	Number of data sets	HPV region targeted (primers)	No. of proficient datasets				
			100% proficient	99-90 % proficient	89-80 % proficient	<80 % proficient	Not proficient
All assays	118	L1/L2/E1/ E2/E4/E6/E7	26	8	15	23	46
Linear Array (Roche)	17	L1 (PGMY)	8	1	1	1	6
InnoLiPA (Innogenetics)	12	L1 (SPF10)	0	1	1	1	9
In-house Lineblot	10	L1 (GP /PGMY)	1	0	1	4	4
CLART HPV 2 / 3 (Genomica)	8	L1 (PGMY)	0	0	2	2	4
In-house Type-specific PCR	6	L1/E6/E7	0	0	1	1	4
In-house realtime PCR	5	L1/E1/E4/E6/E7	0	1	0	1	3
In-house PCR-RFLP	7	L1/E6/E7	0	0	1	4	2
In- house PCR Luminex	7	L1 (GP/MGP/ BSGP/PGMY)	3	0	1	1	2
In-house PGMY-CHUV	6	L1 (PGMY)	4	1	0	1	0
In-house PCR sequencing	6	L1 / E6	0	0	0	5	1
Papillocheck (Greiner bio-one)	4	E1	4	0	0	0	0
PCR Luminex (Multimetrix)	3	L1(GP)	0	0	3	0	0
Digene HPV genotyping LQ test (QIAGEN)	3	L1 (GP)	0	0	0	2	1
Digene HPV genotyping RH test (QIAGEN)	2	L1 (GP)	0	1	1	0	0

Hybribio microarray	2	11	0	0	0	0	2
DEIA LiPA assays (Lab.Bio)	2	L1(SPF 10)	0	0	0	0	2
In- house Dot-Blot	2	L1	1	0	0	0	1
LCD array (Chipron)	2	L1 (PGMY)	0	0	2	0	0
EASYChip (King Car)	2	L1	2	0	0	0	0
Other Commercial assays [a]	9	L1	1	3	1	0	4
Other In-house assays [b]	3	L1/L2/E1/E2/E6	2	0	0	0	1

a) Other commercial assays include one laboratory using each of; Medical Device Microarray, PnE HPV genotyping Luminex, Ampliquality reverse hybridization, Panagene microarray, PANArray TM HPV, GenoFlow HPV array kit, MolGENTIX multiplex-fluorescent PCR, Hybridcapture 2, High risk screen TM Sacace, GENOSERCH HPV 31 Luminex

b) Other In-house assays include one laboratory using each of; In-house PCR EIA, In-house multiplex PCR gel-analysis, In-house mass-spectrometry

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 dataset				
			0 samples	1 sample	2 samples	3 samples	> 3 samples
All assays	132	L1/L2/E1/ E2/E4/E6/E7	71	12	19	4	26
Linear Array (Roche)	17	L1 (PGMY)	10	1	2	1	3
InnoLiPA (Innogenetics)	12	L1 (SPF10)	2	1	5	0	4
In-house Lineblot	10	L1 (GP /PGMY)	4	2	1	1	2
CLART (Genomica)	8	L1 (PGMY)	3	1	2	1	1
In-house RFLP	7	L1/E6/E7	4	1	0	1	1
In house PCR Luminex	7	L1 (GP/MGP/ BSGP/PGMY)	5	0	1	0	1
In-house realtime PCR	6	L1/E1/E4/E6/E7	2	1	1	0	2
PGMY-CHUV	6	L1 (PGMY)	6	0	0	0	0
In-house type-specific PCR	6	L1/E6/E7	2	0	2	0	2
In-house PCR sequencing	6	L1 / E6	3	2	0	0	1
In-house 16 /18 specific PCR	6	E6/E7	4	1	1	0	0
16 /18 specific PCR (TS Lab.Bio)	4	L1/E6/E7	4	0	0	0	0
Papillocheck (Greiner bio-one)	4	E1	4	0	0	0	0
PCR Luminex (Multimetrix)	3	L1 (GP)	3	0	0	0	0
Digene HPV genotyping LQ test (QIAGEN)	3	L1 (GP)	2	0	1	0	0
Digene HPV genotyping RH test (QIAGEN)	2	L1 (GP)	2	0	0	0	0
Hybribio Microarray	2	L1	0	0	1	0	1
DEIA LiPA assays (Lab.Bio)	2	L1 (SPF10)	0	0	0	0	2
In house Dot-blot	2	L1	0	1	0	0	1
LCD array (Chipron)	2	L1 (PGMY)	2	0	0	0	0
EASYChip (King Car)	2	L1	2	0	0	0	0
Cobas 4800 (Roche)	2	L1	0	0	1	0	1
Other Commercial assays [a]	10	L1	5	1	1	0	3
Other In-house assays [b]	3	L1/L2/E1/E2/E6	2	0	0	0	1

a) Other commercial assays include one laboratory using each of; Medical Device Microarray, PnE HPV

genotyping Luminex, Ampliquality reverse hybridization, Panagene microarray, PANArray TM HPV,

GenoFlow HPV array kit, MolGENTIX multiplex-fluorescent PCR, Hybridcapture 2, High risk screen TM

Sacace, GENOSERCH HPV 31 Luminex

b) Other In-house assays include one laboratory using each of; In-house PCR-EIA, In-house multiplex PCR gel-

analysis, In-house mass-spectrometry

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

HPV type	HPV IU /GE	Linear Array	InnoLiPA	CLART (HPV 2)	Digene genotyping LQ	Papillocheck Microarray	Luminex (Multimetrix)	Digene genotyping RH	Hybribio Microarray	DEIA-LiPA (Lab.Bio)	EASY chip (King Car)	LCD array (chipron)	Other Commercial [a]
16	5	3 / 17	10 / 12	8 / 8	2 / 3	4 / 4	3 / 3	2 / 2		2 / 2	2 / 2	2 / 2	7 / 9
16	50	16 / 17	12 / 12		3 / 3								7 / 9
16	500	17 / 17							1 / 2				9 / 9
18	5	3 / 17	9 / 12	5 / 8	3 / 3	nt[b]	3 / 3	2 / 2		2 / 2	2 / 2	2 / 2	6 / 9
18	50	17 / 17	12 / 12	5 / 8									6 / 9
18	500			8 / 8					1 / 2				9 / 9
6	50	4 / 17	11 / 12	6 / 8		4 / 4	2 / 3		1 / 2	2 / 2	2 / 2	2 / 2	8 / 8
6	500	17 / 17	12 / 12	8 / 8	nt		3 / 3	nt	2 / 2				8 / 8
11	50	4 / 17	12 / 12	8 / 8		4 / 4	3 / 3		2 / 2	2 / 2	2 / 2	2 / 2	6 / 8
11	500	16 / 17			nt			nt					8 / 8
31	50	4 / 17	12 / 12	8 / 8	1 / 3	1 / 4		2 / 2		2 / 2	2 / 2		4 / 9
31	500	15 / 17			3 / 3	4 / 4			1 / 2				
33	50	4 / 17	10 / 12	8 / 8	3 / 3	4 / 4	3 / 3	2 / 2	2 / 2	2 / 2	2 / 2	2 / 2	7 / 8
33	500	16 / 17											8 / 8
35	50	4 / 17	12 / 12	8 / 8	3 / 3		3 / 3	2 / 2		2 / 2	2 / 2	2 / 2	6 / 9
35	500	14 / 17				4 / 4							9 / 9
39	50	4 / 17	9 / 12			4 / 4	3 / 3	1 / 2		2 / 2	2 / 2	2 / 2	5 / 9
39	500	15 / 17	11 / 12	1 / 8									7 / 9
45	50	4 / 17	7 / 12	1 / 8	3 / 3	3 / 4	3 / 3	2 / 2		2 / 2	2 / 2	2 / 2	9 / 9
45	500	17 / 17	12 / 12	4 / 8		4 / 4			1 / 2				
51	50	4 / 17	11 / 12	8 / 8	1 / 3	4 / 4	3 / 3	1 / 2		2 / 2	2 / 2	2 / 2	8 / 9
51	500	15 / 17	12 / 12		3 / 3			2 / 2					
52	50	4 / 17	11 / 12	8 / 8		4 / 4	3 / 3		2 / 2	2 / 2	2 / 2	2 / 2	9 / 9
52	500	17 / 17	12 / 12		1 / 3			2 / 2					
56	50	4 / 17	10 / 12		3 / 3	4 / 4	3 / 3	2 / 2		2 / 2	2 / 2		7 / 9
56	500	15 / 17	12 / 12	3 / 8									
58	50	4 / 17	1 / 12	8 / 8	1 / 3	3 / 4	1 / 3	2 / 2		2 / 2	2 / 2	2 / 2	7 / 9
58	500	17 / 17	5 / 12		3 / 3		2 / 3		1 / 2				9 / 9
59	50	3 / 17	1 / 12	7 / 8	1 / 3	4 / 4	3 / 3	1 / 2		2 / 2	2 / 2	2 / 2	7 / 9
59	500	16 / 17	2 / 12		2 / 3			2 / 2	2 / 2				8 / 9
66	50	4 / 17	11 / 12	8 / 8	3 / 3	3 / 4	3 / 3	2 / 2		2 / 2	2 / 2	2 / 2	6 / 7
66	500	14 / 17				4 / 4			2 / 2				7 / 7
68a	50		1 / 12							2 / 2			2 / 6

68a	500	nt	4 / 12	nt	nt				nt		nt	nt	
68b	50	4 / 17	11 / 12		4 / 4	3 / 3	1 / 2	2 / 2	2 / 2	2 / 2	2 / 2	7 / 9	
68b	500	14 / 17	11 / 12										

a) Other commercial assays include one laboratory using each of; Medical Device Microarray, PnE HPV genotyping Luminex, Ampliquality reverse hybridization, Panagene microarray, PANArray TM HPV, GenoFlow HPV array kit, MolGENTIX multiplex-fluorescent PCR, High risk screen TM Sacace, GENOSERCH HPV 31 Luminex

b) Nt: Not tested

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

HPV type	HPV IU /GE	All Assays (%)	Lineblot	PCR -RFLP	Luminex	PGMY-CHUV	Type-specific PCR	Realtime PCR	PCR sequencing	Dot-Blot	Other In-house[a]
16	5	85 / 127 (67)	3 / 10	2 / 7	7 / 7	4 / 6	4 / 6	4 / 5	3 / 5	1 / 2	3 / 3
16	50	118 / 127 (93)	9 / 10	5 / 7		5 / 6		5 / 5	4 / 6	2 / 2	
16	500	125 / 127 (98)	10 / 10	7 / 7		6 / 6	5 / 6		5 / 6		
18	5	72 / 119 (60)	2 / 10	1 / 7	5 / 7	4 / 6	3 / 6	3 / 5	3 / 5	2 / 2	2 / 3
18	50	106 / 119 (89)	7 / 10	3 / 7	7 / 7	5 / 6	4 / 6	4 / 5	4 / 6		
18	500	108 / 119 (91)	8 / 10			6 / 6	5 / 6	5 / 5			
6	50	64 / 110 (58)	2 / 10	3 / 7	2 / 6	5 / 6	4 / 5	2 / 4	2 / 6		1 / 3
6	500	97 / 110 (88)	8 / 10	6 / 7	4 / 6	6 / 6	4 / 5	3 / 4	3 / 6	1 / 2	3 / 3
11	50	79 / 109 (72)	6 / 10	4 / 7	6 / 6	5 / 6	4 / 5	2 / 4	1 / 6	2 / 2	3 / 3
11	500	100 / 109 (92)	8 / 10	7 / 7		6 / 6	4 / 5	3 / 4	2 / 6		
31	50	52 / 118 (44)	3 / 10		3 / 7	3 / 6	2 / 6	4 / 5			2 / 3
31	500	84 / 118 (71)	5 / 10	3 / 7	5 / 7	5 / 6	4 / 6		1 / 6	1 / 2	3 / 3
33	50	76 / 117 (65)	4 / 10	2 / 7	4 / 7	5 / 6	3 / 6	5 / 5		2 / 2	3 / 3
33	500	97 / 117 (83)	7 / 10	3 / 7	5 / 7		4 / 6		2 / 6		
35	50	70 / 118 (59)	5 / 10	1 / 7	5 / 7	5 / 6	3 / 6	2 / 5	1 / 6	1 / 2	3 / 3
35	500	98 / 118 (83)	8 / 10	2 / 7	7 / 7		4 / 6	4 / 5	3 / 6	2 / 2	
39	50	45 / 118 (38)	3 / 10		3 / 7	3 / 6	1 / 6	4 / 5		1 / 2	2 / 3
39	500	77 / 118 (65)	6 / 10		5 / 7	6 / 6	3 / 6	5 / 5			3 / 3
45	50	71 / 118 (60)	7 / 10	2 / 7	6 / 7	5 / 6	3 / 6	5 / 5	2 / 6	2 / 2	2 / 3
45	500	98 / 118 (83)	9 / 10	2 / 7	7 / 7				3 / 6		
51	50	69 / 117 (59)	3 / 9	1 / 7	5 / 7	5 / 6	3 / 6	4 / 5			2 / 3
51	500	92 / 117 (79)	5 / 9	2 / 7	6 / 7	6 / 6	4 / 6	5 / 5		1 / 2	3 / 3
52	50	79 / 117 (67)	4 / 9	3 / 7	7 / 7	5 / 6	3 / 6	5 / 5	1 / 6	1 / 2	3 / 3
52	500	98 / 117 (84)	7 /9	3 / 7							
56	50	63 / 118 (53)	5 / 10	1 / 7	6 / 7	3 / 6	3 / 6	4 / 5		2 / 2	3 / 3
56	500	86 / 118 (73)	8 / 10		7 / 7	4 / 6					
58	50	62 / 118 (52)	4 / 10	4 / 7	6 / 7	5 / 6	3 / 6	3 / 5		1 / 2	2 / 3
58	500	91 / 118 (77)	7 / 10	5 / 7	7 / 7			4 / 5			3 / 3
59	50	52 / 117 (44)	3 / 9	1 / 7	5 / 7	4 / 6	2 / 6	1 / 5		1 / 2	1 / 3
59	500	80 / 117 (68)	6 / 9	2 / 7	6 / 7	5 / 6	3 / 6	3 / 5			2 / 3
66	50	75 / 113 (66)	4 / 9	3 / 7	6 / 7	5 / 6	3 / 5		3 / 6	1 / 2	3 / 3
66	500	95 / 113 (84)	7 / 9	6 / 7	7 / 7		3 / 5		4 / 6		
68a	50	10 / 61 (16)		1 / 5	3 / 6					1 / 1	

68a	500	17 / 61 (28)	2 / 8		4 / 6	nt[b]	nt	nt		1 / 1
68b	50	53 / 114 (46)	3 / 10	1 / 7	3 / 7	5 / 6	2 / 5	1 / 3		1 / 3
68b	500	73 / 114 (64)	5 / 10	2 / 7	6 / 7				1 / 2	3 / 3

Includes laboratories with multiple false positives. Detection with input volume 50 µl classified as data for the next 10-fold dilution compared to input with 5 µl. Input with 10 or 15 µl classified as same dilution compared to input with 5 µl.

a) Other In-house assays include one laboratory using each of; In-house PCR EIA, In-house multiplex PCR gel-analysis, In-house Mass-spectrometry

b) Nt: Not tested

Table 5: Proficiency of detecting HPV types by laboratories that participated in both 2008 and 2010 proficiency studies in comparison with all datasets submitted 2010*.

Proficiency	Identical assays used		All tests by laboratories that participated in both 2008 and 2010		All datasets 2010
	2008 (%)	2010 (%)	2008 (%)	2010 (%)	2010 (%)
100 % proficient	10 / 37 (27)	11 / 37 (30)	13 / 60 (22)	13 / 52 (25)	26 / 118 (22)
99-90 % proficient	2 / 37 (5.4)	2 / 37 (5.4)	9 / 60 (15)	4 / 52 (7.7)	8 / 118 (6.8)
89-80 % proficient	6 / 37 (16)	4 / 37 (11)	7 / 60 (12)	7 / 52 (13)	15 / 118 (13)
<80 % proficient	5 / 37 (14)	6 / 37 (16)	11 / 60 (18)	7 / 52 (13)	23 / 118 (19)
Not proficient	14 / 37 (38)	14 / 37 (38)	20 / 60 (33)	21 / 52 (40)	46 / 118 (39)

*Table restricted to assays testing for more than two HPV types.

Table 6: Number of false positive HPV types detected per data set reported by laboratories participating in both 2008 and 2010 years proficiency studies in comparison with all datasets submitted 2010.

No of false positive samples	Identical assays used		All test by laboratories participating both 2008 and 2010		All datasets 2010
	2008 (%)	2010 (%)	2008 (%)	2010 (%)	2010 (%)
0 samples	18 / 37 (49)	20 / 37 (54)	34 / 67 (51)	34 / 59 (58)	69 / 132 (52)
1 sample	5 / 37 (14)	3 / 37 (8.1)	13 / 67 (19)	4 / 59 (6.8)	12 / 132 (9.2)
2 samples	4 / 37 (11)	6 / 37 (16)	7 / 67 (10)	8 / 59 (14)	19 / 132 (14)
3 samples	4 / 37 (11)	3 / 37 (8.1)	5 / 67 (7.5)	3 / 59 (5.1)	4 / 132 (3.0)
>3 samples	6 / 37 (16)	5 / 37 (14)	8 / 67 (12)	10 / 59 (17)	27 / 132 (21)