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Results of the First World Health Organization International Collaborative Study of Detection of Human Papillomavirus DNA†

Wim G. V. Quint,[1] Sonia R. Pagliusi,[2] Nico Lelie,[3] Ethel-Michele de Villiers,[4] Cosette M. Wheeler,[5]* and the World Health Organization Human Papillomavirus DNA International Collaborative Study Group

Delft Diagnostic Laboratory, Delft, The Netherlands[1]; Initiative for Vaccine Research, Department of Immunization, Vaccines and Biologicals, World Health Organization, Geneva, Switzerland[2]; Viral Quality Control Laboratory, Central Laboratory for Blood Transfusion, Diagnostic Division, Alkmaar, The Netherlands[3]; Reference Center for Papillomaviruses, Division for the Characterization of Tumorviruses, Deutsches Krebsforschungszentrum, Heidelberg, Germany[4]; and Department of Molecular Genetics and Microbiology, School of Medicine, University of New Mexico, Albuquerque, New Mexico[5]

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Twenty-nine laboratories in 12 countries participated in a study to assess the performance of various human papillomavirus (HPV) detection assays through the use of a recombinant HPV DNA standard reagent panel. The panel was designed by a group of HPV experts, and samples were prepared and distributed by the World Health Organization International Laboratory for Standards and Biologicals in The Netherlands. Each panel consisted of 24 coded samples including a dilution series for HPV types 16 and 18, alone or in combination with five other high-risk (HR) HPV types including HPV types 31, 33, 35, 45, and 52, the low-risk HPV type 6, and a negative control. Qualitative assays were generally consistent across laboratories, and most invalid results reflected a lack of HPV test sensitivity. The combined data sets had a proficiency for HPV 16 of 62.5% (15/24) and for HPV 18 of 73.9% (17/23). HPV 31 was the least accurately detected by participating laboratories. Approximately half of participating laboratories failed to detect high concentrations of HPV 31 and, to a lesser extent, to detect HPV types 35, 52, and 6. The panel sample materials offer a source of renewable and reproducible material that could be used in the future development of international standard reagents for calibration of HPV DNA assays and kits.

Human papillomavirus (HPV) infection has been established as the major cause of cervical cancer in women (40, 46). Among the nearly 100 types of papillomaviruses molecularly identified, about 30 different HPV types are found in cervical carcinomas (18). Epidemiological studies on the global prevalence of HPV types have shown that about 70% of cervical cancer cases are related to infections with two high-risk (HR) HPV types, namely HPV 16 and HPV 18. About 15% are related to HR HPV types 31, 33, 35, 45, 52, and 58, collectively, and approximately 15% are related to other less common types, with some geographical variations (2). Thus, to prevent cervical cancer, several candidate vaccines against HPV have been developed and are currently in clinical testing (5). Prophylactic HPV vaccine candidates are based on recombinant virus capsid proteins, so-called virus-like particles, and are designed to prevent infections by HPV types 16 and 18, the most common oncogenic types, as well as against the common types HPV 6 and HPV 11 that cause genital warts (1, 8, 16).

It was recognized by a group of HPV experts that harmonization of HPV laboratory assays was required at the outset of the development and implementation of new HPV vaccines (41). 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. Hence, an international collaborative study was conducted to consider candidate reference reagents for type-specific HPV DNA assays (42, 43).

To assess the relative value of molecular detection methods, international proficiency panels are already widely used for several microorganisms including hepatitis A, B, and C and human immunodeficiency virus (HIV) (23, 24, 26, 36). Unfortunately, there is no natural source of biological material to generate type-specific HPV standard reagents such as naturally infected or spiked serum or plasma pools used for hepatitis and HIV standards. Cervical scrapes or small genital biopsy specimens obtained for diagnosis of HPV-infected individuals often harbor multiple HPV types and provide only low numbers of HPV genomes. In addition, no adequate culture models or human cell lines containing episomal HPV genomes are readily available to generate reproducible epithelial cell-based HPV standards.

The present international collaborative study was initiated to assess the performance of various HPV DNA detection assays and to examine the feasibility of generating HPV DNA standard reagents consisting of recombinant HPV DNA plasmids placed into a background of HPV-negative human cervical cells. Cloned HPV DNA standards such as those described here allow assessment of the analytic HPV assay sensitivity and

* Corresponding author. Mailing address: Department of Molecular Genetics and Microbiology, School of Medicine, University of New Mexico, Albuquerque, NM 87131-5276. Phone: (505) 272-5785. Fax: (505) 277-0265. E-mail: cwheeler@@salud.unm.edu.

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TABLE 1. HPV DNA standard panel composition

Panel ID no.	HPV type	Plasmid dilution of primary HPV/HR HPV pool	HPV genome equivalents per milliliter								
			6	16	18	31	33	35	45	52	C33A
24	HPV 16	10^{-4}		10^{7}							10^{6}
8	HPV 16	10^{-5}		10^{6}							10^{6}
21	HPV 16	10^{-6}		10^{5}							10^{6}
16	HPV 16	10^{-7}		10^{4}							10^{6}
19	HPV 16	10^{-8}		10^{3}							10^{6}
23	HPV 16	10^{-9}		10^{2}							10^{6}
9	HPV 16	10^{-10}		10^{1}							10^{6}
5	HPV 16	10^{-11}		10^{0}							10^{6}
13	HPV 16 low/HR	$10^{-7}/10^{-5}$		10^{4}		10^{6}	10^{6}	10^{6}	10^{6}	10^{6}	10^{6}
10	HPV 16 high/HR	$10^{-5}/10^{-5}$		10^{6}		10^{6}	10^{6}	10^{6}	10^{6}	10^{6}	10^{6}
2	HR type pool	10^{-5}				10^{6}	10^{6}	10^{6}	10^{6}	10^{6}	10^{6}
7	HPV 6	10^{-7}	10^{4}								10^{6}
3	HPV 6/HR	10^{-5}	10^{6}			10^{6}	10^{6}	10^{6}	10^{6}	10^{6}	10^{6}
14	C33A DNA										10^{6}
22	HPV 18	10^{-4}			10^{7}						10^{6}
1	HPV 18	10^{-5}			10^{6}						10^{6}
20	HPV 18	10^{-6}			10^{5}						10^{6}
11	HPV 18	10^{-7}			10^{4}						10^{6}
15	HPV 18	10^{-8}			10^{3}						10^{6}
18	HPV 18	10^{-9}			10^{2}						10^{6}
12	HPV 18	10^{-10}			10^{1}						10^{6}
4	HPV 18	10^{-11}			10^{0}						10^{6}
6	HPV 18 low/HR	$10^{-7}/10^{-5}$			10^{4}	10^{6}	10^{6}	10^{6}	10^{6}	10^{6}	10^{6}
17	HPV 18 high/HR	$10^{-5}/10^{-5}$			10^{6}	10^{6}	10^{6}	10^{6}	10^{6}	10^{6}	10^{6}

specificity but do not provide standards capable of evaluating biological specimen processing.

This report describes the efforts undertaken between September 2002 and September 2004 to develop a reference reagent panel for HPV viral DNA detection and the subsequent assessments conducted in 29 laboratories using a range of the HPV DNA detection assays in use at the time. Samples, derived from cloned plasmid DNA representing double-stranded full genomic DNA sequences of HPV types 6, 16, 18, 31, 33, 35, 45, and 52, were tested by each laboratory in a blinded manner. The results of the study were analyzed to determine the ability of participating laboratories to correctly identify HPV types in a background of human DNA and in the presence or absence of other HPV types. In addition, the analytic sensitivity of detecting HPV 16 and HPV 18, the most common oncogenic HPV types, was evaluated using an end-point dilution series.

MATERIALS AND METHODS

Source of panel material. WHO obtained authorization to use the cloned HPV DNA sequences for the purposes of this study. Institutional permissions allowed the University of New Mexico to purify and transfer the cloned HPV plasmid DNAs to the WHO International Laboratory for Biological Standards in Amsterdam, where the samples were prepared and further distributed among participating laboratories worldwide. The agreements allowed this distribution only for the purposes of the WHO studies presented here. Institutions that granted the use of the HPV reference plasmids and their sequences to WHO were the following: Deutsches Krebsforschungszentrum (H. zur Hausen) for HPV types 6, 16, and 18; Digene Corporation (A. Lorincz) for HPV types 31 and 35; Institut Pasteur (G. Orth) for HPV type 33; Johns Hopkins University (K. Shah) for HPV type 45; and Wayne State University (W. Lancaster) for HPV type 52. The nucleic acid sequences for each of the above HPV reference genomes have been reported previously and were available to the WHO HPV DNA International Collaborative Study Group as follows: HPV16R, http://www.stdgen.lanl.gov/stdgen/virus/cgi-bin/hpv_search.cgi?dbname = hpv &locus = HPV16R&mode = extract; HPV18R, http://www.stdgen.lanl.gov /stdgen/virus/cgi-bin/hpv_search.cgi?dbname = hpv&locus = HPV18R&mode = extract; HPV31, http://www.stdgen.lanl.gov/stdgen/virus/cgi-bin/types_lookup.cgi ?dbname = hpv&type = 31&organism = Human%20papillomavirus; HPV33, http: //www.stdgen.lanl.gov/stdgen/virus/cgi-bin/types_lookup.cgi?dbname = hpv&type = 33&organism = Human%20papillomavirus; HPV35, http://www.stdgen.lanl .gov/stdgen/virus/cgi-bin/types_lookup.cgi?dbname = hpv&type = 35&organism = Human%20papillomavirus; HPV45, http://www.stdgen.lanl.gov/stdgen/virus/cgi -bin/types_lookup.cgi?dbname = hpv&type = 45&organism = Human%20papillomavirus; and HPV52, http://www.stdgen.lanl.gov/stdgen/virus/cgi-bin/types_lookup .cgi?dbname = hpv&type = 52&organism = Human%20papillomavirus.

Purification and characterization of individual panel reagents. Each individual HPV full genome was provided as a plasmid DNA clone from the owners listed above. The HPV plasmid DNAs were used to transform bacteria and produce microgram quantities of bulk-purified DNA using QIAGEN Maxi-Prep kits. Optical density determinations were made at 260 and 280 nm, and fluorimetric measurements by picogreen quantitations (PicoGreen dsDNA Quantitation Reagent; Molecular Probes, Inc., Eugene, Oreg.) were determined as well. Serial 10-fold dilutions of purified bulk plasmid stocks were made, and DNA sequencing was performed to confirm the reported reference HPV sequence within the L1, E6, E7, and LCR genome segments. The sequences of the bulk-purified plasmids were as expected except that a T-to-C change was observed at nucleotide position 7592 in the HPV 18 reference plasmid material. To determine absolute purity of bulk HPV plasmid DNAs, PCR amplification of serial 10-fold dilutions from approximately 10^{11} to 10^{2} genome equivalents was conducted by PCR using PGMY primers and reverse line blot HPV detection for typing (7, 21). In addition, real-time quantitative PCR (qPCR) for HPV 16 E6 and HPV 18 E7 was conducted as previously described (32). Approximately 100 ng of each DNA genotype was sent to the WHO International Laboratory for Biological Standards in Amsterdam.

Panel composition and production. Purified plasmids containing cloned genomic DNAs for HPV types 6, 16, 18, 31, 33, 35, 45, and 52 and calibrated at an approximate concentration of 10^{11} HPV genomes/ml were provided to the WHO laboratory to prepare a panel of 24 samples. Table 1 summarizes the composition of panel samples. Given the lack of available biological source materials from human genital samples or from cell-based models, human genomic DNA was included in each sample to minimally mimic a molecular matrix background that would be present in biological samples. For this purpose, the epithelial C33A cell line derived from human cervical carcinoma, which is HPV negative, and purchased from the American Type Culture Collection was cultured in minimal essential medium, and the concentration of cells was determined by a Coulter counter (Beckman). C33A genomic DNA was isolated and purified using a QIAGEN blood and cell culture kit and was provided in TE

buffer (10 mM Tris, 1 mM EDTA, pH 7.5) at a concentration of 2×10^7 genomes/ml. The 24 different reference samples were prepared by gravimetrically recorded dilution of HPV recombinant DNA plasmid standards in the C33A genomic DNA background. The traceability of dilutions from original stocks to final reference samples was guaranteed by the quality system of the WHO Amsterdam laboratory. Briefly, the recombinant HPV DNA plasmids were diluted 1,000-fold in TE buffer. From the 10^{-3} dilution, the subsequent plasmid dilutions (beginning at 10^{-4}) were prepared to a final concentration of approximately 10^7 genomes/ml in TE buffer containing 10^6 genomes/ml of C33A-derived human DNA. This concentration of human genomic DNA is similar to the amount of cellular DNA that is generally found in cervical scrape specimens. Thus, the 10^{-5} dilution of the HPV plasmid DNAs represented an estimated 10^6 HPV genomes/ml or 10^4 genomes/10 μl and the 10^{-7} dilution of the HPV plasmid cDNAs represented an estimated 10^4 genomes/ml or 10^2 genomes/10 μl. After production of the 24 reference samples, each preparation was dispensed in 115-μl volumes using 0.5-ml PCR vials. The vials were labeled as HPV DNA, given a batch identification number, and randomly assigned a number from 1 through 24. The panel sample numbering was different from the original ordering of the prepared dilutions. The PCR vials were snap frozen in liquid nitrogen and kept at −30°C before shipment on dry ice to the collaborative study participants. Participants were instructed to use 10 μl of each panel vial for assessment in their individual HPV DNA assays.

Two samples containing the C33A genomic DNA alone or C33A genomic DNA and a mixture of HR HPV types including HPV types 31, 33, 35, 45, and 52 served as "negative" and "positive" control samples, respectively. After preparation of the samples, a β-globin qPCR was performed in order to verify that the cellular C33A genomic DNA matrix was equally distributed in all samples throughout the panel. Before distribution of the WHO HPV DNA reference panel, the samples were tested at two appointed reference laboratories, namely, the Delft Diagnostic Laboratory in The Netherlands and the laboratory of Molecular Genetics and Microbiology at the University of New Mexico in the United States, as agreed by the group.

Characterization of the panel. Sample DNA of two randomly chosen panels was analyzed. Reference laboratory 1 (Ref 1) performed the PGMY line blot assay (6, 7, 21), and reference laboratory 2 (Ref 2) performed the other panel using the SPF10-LiPA assay (14). The qPCR for HPV 16, HPV 18, and β-globin DNA was performed at the Delft Diagnostic Laboratory (32). Once the reference laboratories (Ref 1 and Ref 2) confirmed the reliability of the samples, the panels were further distributed to participating laboratories.

Technologies used for initial characterizations of the panel. (i) Ref 1. Ten microliters of panel sample DNAs was used for PGMY PCR. The PGMY PCR primer set was used as previously described (6, 7, 21), but 5 pmol of each of the β-globin primers GH20 and PC04 was used instead of 2.5 pmol. PCR products were analyzed using the reverse line blot assay as described earlier (6, 21). HPV types 6, 11, 16, 18, 26, 31, 33, 34, 35, 39, 40, 42, 45, 51, 52, 53, 55, 56, 57, 58, 59, 61, 62, 66, 67, 68, 69, 70, 71, 72, 73, 81, 82, 82v, 83, 84, and 89 were distinguished. Part of the human β-globin gene was amplified in each sample as a control of specimen adequacy. Appropriate negative and positive controls were used to monitor the performance of the method, and appropriate cell-based controls were used to monitor potential contamination and assay performance.

(ii) Ref 2. A 10-μl DNA sample was amplified by the broad-spectrum SPF10 primers. These primers amplify a short fragment of 65 bp. The PCR products were analyzed by HPV DNA enzyme immunoassay, a microtiter plate-based hybridization assay using universal HPV probes as previously described (13). Biotinylated PCR products were captured onto streptavidin-coated microtiter plates and denatured. After denaturation, digoxigenin-labeled universal HPV-specific probes were hybridized to the captured DNA strand under stringent conditions. Hybrids were detected using anti-digoxigenin-horseradish peroxidase conjugate and tetramethylbenzidine substrate.

Samples scored positive for HPV DNA were subsequently genotyped by the INNO-LiPA HPV genotyping assay, SPF10 system version 1 (Innogenetics, Ghent, Belgium, manufactured by Labo Bio-Medical Products, Rijswijk, The Netherlands), as described previously (14). In this assay, genotype-specific probes for HPV genotypes 6, 11, 16, 18, 31, 33, 34, 35, 39, 40, 42, 43, 44, 45, 51, 52, 53, 54, 56, 58, 59, 66, 68, 70, and 74 are immobilized in parallel lines on a nitrocellulose membrane strip. Briefly, 10 μl of amplimer, containing 5′ end biotinylated primers, was denatured by adding 10 μl of NaOH solution. After hybridization of the amplimer to the probes on the strip under stringent conditions and stringent washing conditions, hybrids were detected by alkaline phosphatase-streptavidin conjugate and substrate (5-bromo-4-chloro-3-indolylphosphate and nitroblue tetrazolium) treatment, resulting in a purple precipitate at the positive probe lines. After drying, the strips were interpreted visually.

TABLE 2. Different HPV DNA typing methods used to generate results

Method	No. of data sets	Detection primers
PGMY line blot	8	PGMY
SPF10-LiPA	5	SPF-10
Digene Hybrid Capture	3	Not applicable
qPCR HPV 16 and 18	3	(32)
Multiple Well Plate assay	1	PVUP/PVDP
Deg GP5+/6+ reverse line blot[a]	1	GP5+/6+
Biomed DNA chip	1	GP5+/6+ degenerated nested
Other PCR methods	11	In-house type-specific and universal PCR

[a] Deg, degenerate.

The panel was also analyzed by qPCR directed to the HPV 16 E6 region, the HPV 18 E7 region, and part of the β-globin gene as described previously (32). Panel sample DNA was analyzed in three independent assays. Briefly, the different qPCRs were performed in a final reaction volume of 50 μl containing 10 μl of sample DNA, 1× AmpliTaqGold PCR buffer II, 3.3 mM $MgCl_2$, a 200 μM concentration of each deoxynucleoside triphosphate, 100 to 400 nM concentrations of each primer, 100 nM TaqMan probe, and 3 U of AmpliTaqGold. The PCR conditions were as follows: AmpliTaqGold was activated for 12 min at 95°C denaturation, and target DNA was amplified by 50 cycles of 15 s at 95°C denaturation and 30 s at 55°C annealing and by extension using the I-cycler (Bio-Rad, Veenendaal, The Netherlands).

Organization of the study. The panels were distributed from the production laboratory in Amsterdam (WHO International Laboratory for Biological Standards) on dry ice to 29 laboratories in Australia, Belgium, Brazil, France, Germany, India, Italy, The Netherlands, Republic of South Korea, South Africa, Spain, Sweden, Switzerland, the United Kingdom, and the United States. The package also included an information sheet with technical data. A questionnaire was included in order to obtain technical information on the procedures employed by individual participants. Laboratories were asked to complete the panel assessments at their convenience and to return the results to the WHO International Laboratory for Biological Standards within 6 months of receipt.

All results were submitted to the WHO International Laboratory in Amsterdam, which acted as a neutral office. The neutral office coded the results that were then analyzed anonymously by both reference laboratories 1 and 2, the neutral office, and the WHO secretariat. Although individual results of the study were returned to each participating laboratory, it was agreed prior to conducting the study that specific laboratories and HPV assays would not be linkable in the study results to individual institutions. Thus, individual HPV assays are designated numerically from 1 through 19 and individual laboratories are designated alphabetically from A through U (see Fig. 2).

HPV technologies used by study participants. Different HPV typing methods were used to generate results for the first WHO international collaborative study to detect HPV DNA (4, 6, 7, 9, 10, 11, 12, 14, 15, 17, 27, 33, 34, 35, 37), and these are summarized in Table 2.

Data analysis. Criteria used for analyzing proficiency of HPV 16 and 18 detection were the following: (i) correct identification of the negative sample containing only C33A-derived cellular DNA as HPV negative (sample 14); (ii) a logical order in the detection of HPV 16 and 18 DNA-positive samples in both dilution series (more than one negative sample result between positives was considered not logical); (iii) positive detection of HPV types 16 and 18 at a dilution of 10^{-5} or approximately 10^4 genome equivalents per 10 μl assayed in a background of other HR HPV DNA (each HR HPV type [samples 10 and 17, respectively] was present at approximately 10^4 genome equivalents per 10 μl assayed) (Table 1); (iv) no false-positive detection of HPV types 6, 16, 18, 31, 33, 35, 45, and 52; and (v) no false-negative detection of the 10^{-5} dilution or approximately 10^4 genome equivalents per 10 μl assayed of HPV types 6, 16, 18, 31, 33, 35, 45, and 52.

Thus, criteria for proficiency of HPV 6, 31, 33, 35, 45, and 52 detection (10^{-5} dilution or approximately 10^4 genome equivalents per 10 μl assayed) included detection of these HPV types in the relevant samples (i.e., codes 2, 3, 6, 10, 13, and 17) and no false-positive results.

Data sets from the two reference laboratories and four data sets without specific, single HPV type results were not included in the overall performance

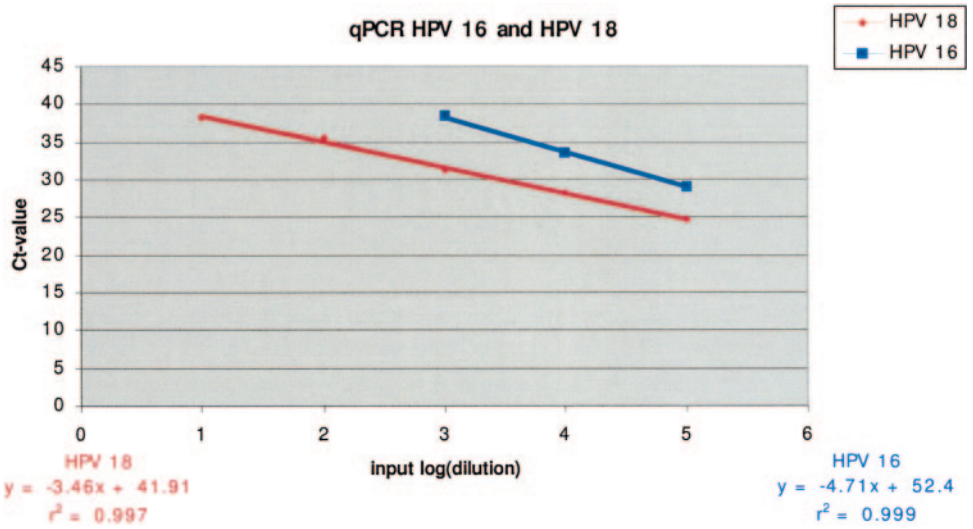

FIG. 1. Linearity of HPV 16 and HPV 18 plasmid DNA dilution series. C_T, cycle threshold; y, slope. The theoretical ideal slope is expressed as $y = -3.34x$. Values for the input log (dilution) are as follows: 0, 10^{-9}; 1, 10^{-8}; 2, 10^{-7}; 3, 10^{-6}; 4, 10^{-5}; and 5, 10^{-4}.

analysis; three data sets used the Digene Hybrid Capture 2 (HC2), assay and one data set used the Roche Multiple Well Plate (MWP) assay for HPV DNA detection, both providing non-type-specific results.

RESULTS

Validation of the HPV panel reagents by reference laboratories. The results from Ref 1 and Ref 2 included quantitative and qualitative characterizations of human and HPV DNAs. For TaqMan qPCR assays, targets included HPV 16 E6, HPV 18 E7, and human β-globin DNA (32). Linearity of the HPV 16 and 18 DNA dilution series within the panel was confirmed by qPCR analysis, and correlation coefficients of 0.999 and 0.997 were obtained for HPV 16 and 18, respectively, as shown in Fig. 1. qPCR amplification efficacy was 4.71 and 3.46 for HPV types 16 and 18, respectively. (These values represent the slopes, not the efficiency. The efficiency calculation is $E = [10^{1/(-\text{slope})}] - 1$. Therefore, E is 63% and 95% for the HPV 16 and 18 qPCR assays, respectively.) These values indicate that HPV 16 is less efficiently amplified than HPV 18 by this qPCR assay (Fig. 1).

Endpoint detection in blinded panel samples was observed by qPCR at dilutions of 10^{-6} for HPV 16 and 10^{-8} for HPV 18. In both dilution series the next dilution (i.e., 10^{-7} and 10^{-9}, respectively) was positive in one out of three tests, indicating that the dilution had reached Poisson distribution.

Equivalence of distribution of C33A-derived DNA matrix was characterized by β-globin DNA qPCR in a randomly selected panel set of 24 samples. A mean cycle threshold value of 27.8 with a range of 26.48 to 29.23 was observed, indicating equal distribution of C33A genomic DNA background throughout the panel (data not shown).

HPV 16 was detected in both reference laboratories at a dilution of 10^{-7} using qualitative assays. For HPV 18, Ref 1 detected up to 10^{-7} and Ref 2 detected up to 10^{-8}. When HPV types 16 and 18 were at dilutions of 10^{-7} and 10^{-5} with other HPV types present at a dilution of 10^{-5}, both reference laboratories detected HPV 18 down to 10^{-7}; for HPV 16, both laboratories detected only the 10^{-5} dilution. (Fig. 2).

Panel distribution and response. Twenty-four of 29 participating laboratories, including the two reference laboratories submitted 33 data sets as summarized in Table 2. The three qPCR data sets for HPV 16 and 18 DNA were subsequently combined into a single data set representing the mean result obtained for each panel specimen (Fig. 2, Ref2*). Of the remaining 31 data sets, 4 data sets were generated using assays that did not discriminate specific HPV types, and, therefore, they were not included in the overall type-specific analyses presented here. Despite their good performance, the non-type-specific data sets that were excluded used the Digene HC2 and the Roche MWP assays. The HC2 HPV DNA assay demonstrated an approximate detection limit of 10^5 and 10^4 genome equivalents per assay for HPV 16 and 18, respectively. The MWP demonstrated a detection limit of 10^2 and 1 genome equivalent(s) per assay for HPV 16 and 18, respectively (data not shown). The remaining 27 data sets are shown in Fig. 2. Further, the data sets from reference laboratories were not included in the overall performance analysis.

Performance of the participating laboratories. (i) Detection of HPV 16. Overall, the level of HPV 16 detection varied 1,000-fold among the participating laboratories, as shown in Fig. 2. HPV 16 was detected in 100% of the data sets at a dilution of 10^{-4}; 19 of 24 (79.2%) data sets from participating laboratories detected a dilution of 10^{-5}, 14 of 24 (58.3%) detected a dilution of 10^{-6}, and 7 of 24 (29.2%) detected a dilution of 10^{-7}. However, positive results from two data sets from one laboratory for the 10^{-7} dilution (Fig. 2, compare F10 and F14) were not detected in a logical order (see Materials and Methods).

When the HPV 16 10^{-5} dilution was combined with a dilution of 10^{-5} for each of the other HR HPV types (types 31, 33, 35, 45, and 52), 22 of 24 (91.7%) data sets detected the HPV 16 DNA. In contrast, only 3 of 24 (12.5%) data sets detected the HPV 16 10^{-7}

HPV16

Detection method			1	2	3	4	5	6	7	8	9	10	11	12	13	14	14	14	14	14	14	15	16	17	17	17	18	19
Code	Sample	Dilution	Ref 1	Ref 2	Ref 2*	A	B	C	A	D	E	F	G	H	I	F	J	K	L	M	N	O	P	Q	R	S	O	T	U
24	HPV 16	10^{-4}																											
8	HPV 16	10^{-5}																											
21	HPV 16	10^{-6}																											
16	HPV 16	10^{-7}																											
19	HPV 16	10^{-8}																											
23	HPV 16	10^{-9}																											
9	HPV 16	10^{-10}																											
5	HPV 16	10^{-11}																											
13	HPV 16 low + HR	10^{-7}																											
10	HPV 16 high + HR	10^{-5}																											
2	HR types	10^{-5}																											
7	HPV 6	10^{-7}																											
3	HPV 6 + HR	10^{-5}																											
14	C33A DNA	N.A.																											
22	HPV 18	10^{-4}																											
1	HPV 18	10^{-5}																											
20	HPV 18	10^{-6}																											
11	HPV 18	10^{-7}																											
15	HPV 18	10^{-8}																											
18	HPV 18	10^{-9}																											
12	HPV 18	10^{-10}																											
4	HPV 18	10^{-11}																											
6	HPV 18 low + HR	10^{-7}																											
17	HPV 18 high + HR	10^{-5}																											

HPV18

Detection method			1	2	3	4	5	6	7	8	9	10	11	12	13	14	14	14	14	14	14	15	16	17	17	17	18	19
Code	Sample	Dilution	Ref 1	Ref 2	Ref 2*	A	B	C	A	D	E	F	G	H	I	F	J	K	L	M	N	O	P	Q	R	S	O	T	U
24	HPV 16	10^{-4}																											
8	HPV 16	10^{-5}																											
21	HPV 16	10^{-6}																											
16	HPV 16	10^{-7}																											
19	HPV 16	10^{-8}																											
23	HPV 16	10^{-9}																											
9	HPV 16	10^{-10}																											
5	HPV 16	10^{-11}																											
13	HPV 16 low + HR	10^{-7}																											
10	HPV 16 high + HR	10^{-5}																											
2	HR types	10^{-5}																											
7	HPV 6	10^{-7}																											
3	HPV 6 + HR	10^{-5}																											
14	C33A DNA	N.A.																											
22	HPV 18	10^{-4}																											
1	HPV 18	10^{-5}																											
20	HPV 18	10^{-6}																											
11	HPV 18	10^{-7}																											
15	HPV 18	10^{-8}																											
18	HPV 18	10^{-9}																											
12	HPV 18	10^{-10}																											
4	HPV 18	10^{-11}																											
6	HPV 18 low + HR	10^{-7}																											
17	HPV 18 high + HR	10^{-5}																											

true positive
false positive
negative
not performed

FIG. 2. Ability to detect HPV DNA 16 and 18. Each reference and each participating laboratory are listed on the top row from 1 through 19. Participating laboratories are identified as letters of the alphabet from A to U. Methods used for HPV DNA detection are identified as numbers from 1 through 19. Gray boxes, correct detection (positive); blank boxes, no detection (negative); dark boxes, incorrect detection (false positive); light gray boxes, not performed. Samples for HPV 16 and HPV 18 are listed to the left as dilution series (in descending order of DNA concentration). Actual numbers of the coded samples are listed to the left, in addition to the HPV types and dilutions.

TABLE 3. Proficiency of detecting HPV types 16, 18, 6, 31, 33, 35, 45, and 52

HPV type data sets	No. of data sets (% of total) by HPV type							
	16[a]	18[a]	6[b]	31[b]	33[b]	35[b]	45[b]	52[b]
Total no. of data sets	24	23	21	21	21	21	21	21
Proficient data sets	15 (62.5)	17 (73.9)	15 (71.4)	9 (42.9)	20 (95.2)	15 (71.4)	20 (95.2)	15 (71.4)
Data sets with false positives	5 (20.8)	6 (26.1)	5 (23.8)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	2 (9.5)
Data sets with false negatives at 10^{-5} dilution	6 (25.0)	0 (0.0)	1 (4.8)	12 (57.1)	1 (4.8)	6 (28.6)	1 (4.8)	4 (19.0)
Data sets with detection out of logical order	5 (20.8)	0 (0.0)	NA[c]	NA	NA	NA	NA	NA

[a] Detection of HPV types 16 or 18 at a dilution of 10^{-5}; no false-positive results and no detection out of logical order.
[b] Detection of HPV types 6, 31, 33, 35, 45 and 52 at a dilution of 10^{-5} and no false-positive results.
[c] NA, not applicable.

dilution when combined with other HR HPV types; the HPV 16 10^{-7} dilution in the absence of other HR types (sample 16) was detected in 5 (20.8%) data sets.

The single negative control sample was detected as negative for HPV by all assays in all laboratories (Fig. 2). However, based on the study proficiency definition, 5 of 24 data sets (20.8%) presented false-positive detection of HPV 16 DNA, including detection of HPV 16 in material containing HPV 6 and 18 and mixtures of other HR HPVs, particularly when in-house HPV 16 type-specific detection systems were used. Only one laboratory had a false-negative result because it was not able to detect HPV 16 at a dilution of 10^{-5} in a background of the other HR HPV types. In total, for HPV 16 identification, 15 (62.5%) data sets met the study criteria for proficient HPV 16 detection.

(ii) Detection of HPV 18. Of the 24 data sets from participating laboratories included in the analysis, one method used did not perform assays capable of distinguishing HPV 18, and, therefore, only 23 data sets were analyzed here. The lower limit of detection of HPV 18 between laboratories varied by 100,000-fold (Fig. 2). All data sets detected HPV 18 at a dilution of 10^{-4} and 10^{-5}. Twenty-two of 23 (95.7%) data sets detected the 10^{-6} dilution, 17 of 23 (73.9%) detected the 10^{-7} dilution, 10 of 23 (43.5%) detected the 10^{-8} dilution, 4 of 23 (17.4%) detected the 10^{-9} dilution, and 1 laboratory detected up to the 10^{-10} dilution.

When the 10^{-5} dilution of HPV 18 was combined with a dilution of 10^{-5} for the HR HPV types (types 31, 33, 35, 45, and 52), all laboratories detected the HPV 18 DNA. Only 12 of 23 (52.2%) of the data sets detected the HPV 18 10^{-7} dilution when combined with the HR HPV types. In total, for HPV 18 identification, 17 of 23 (73.9%) data sets met the study criteria for proficient HPV 18 detection. Examples of the false-positive HPV 18 observations included detection of HPV 18 in material containing HPV types 6 and 16 and the mixture of other HR HPV types, similar to HPV 16.

(iii) Detection of other HPV types. Some participating laboratories did not perform tests for typing of HPV types 6, 31, 33, 35, 45, and 52. Thus, the test results of only 21 data sets from participating laboratories were available for analysis (see Fig. SA, SB, and SC in the supplemental material) and are presented in Table 3. The negative control sample was detected as negative for all HPV types (see Fig. SA, SB, and SC in the supplemental material) except for one laboratory, which detected HPV 6 in this sample. Detection of HPV 31 had an overall proficiency below 50% (Table 3). The low proficiency rate for HPV 31 was caused by false-negative results in 12 data sets. In contrast, for HPV 6 the low proficiency was mainly caused by false-positive ($n = 5$) results. All laboratories except one were proficient (95.2%) in the detection of HPV types 33 and 45. HPV types 6, 35, and 52 had intermediate proficiency results (71%).

DISCUSSION

The goal of accurate detection of infectious agents is to provide consistent and meaningful results in the research and clinical setting to help target and focus resources in disease prevention and control. Over the past years, WHO has worked with the scientific community, national regulatory authorities, other standards-setting bodies, and users through a series of consultations to review the scientific basis of biological reference materials. WHO reference reagents, which may serve as interim standards, and the published catalogue of WHO biological reference materials includes over 300 materials (a list of reference materials may be found at www.who.int/biologicals). It is recognized that some international standards may be used for qualitative rather than quantitative purposes. It may also be necessary to establish materials that might act as a reference panel to aid in the evaluation of diagnostic tests. Indeed, a reference panel of 10 individual genotypes of HIV-1 has been previously established to help assess the specificity of nucleic amplification technology-based assays for HIV-1 (44).

In addition to evaluating the performance of various HPV DNA detection methods, the present international collaborative study evaluated the feasibility of generating HPV DNA international standard reagents and the suitability of recombinant plasmids containing full-length HPV-cloned genomes for this purpose. Historically, international standards in the form of nucleic acids have been isolated from pools of virus-infected biological material, such as the hepatitis C RNA standards established in 1997, hepatitis B DNA standards established in 1999, and HIV-1 RNA standards established in 1999 (45). International standards must fulfill several criteria, including the following: demonstrate consistent performance, demonstrate long-term stability under selected storage conditions, contain sequences found often in the real target nucleic acid (i.e., full viral genomes if possible), perform in detection assays

like the naturally occurring target, and be readily available and renewable. Ideally, standards should mimic properties of actual biological samples under measurement and allow evaluation of the full laboratory sample processing procedures. HPV clinical samples are not plasma derived, and the creation of international standards represented by pools of cervicovaginal specimens is not feasible and not as reliable and reproducible as recombinant HPV nucleic acid standards. At a minimum, HPV DNA standards should contain full HPV genomes to allow identification of any genomic region that may be targeted in detection assays and should be presented in a background matrix of human genomic DNA. Indeed, the proposed materials fulfilled these requirements. The studied materials also contained a matrix of epithelial DNA derived from a cervical carcinoma cell line (C33A), which is of human origin and is HPV DNA negative and offers a source of renewable and reproducible matrix material. Studies need to determine if HPV DNA candidate standards can be further developed to assess the full spectrum of sample processing schemes that would mimic diagnostic sample preparation including DNA extraction, precipitation, or centrifugation procedures. This would require the further development of culture models that would harbor authentic episomal HPV genomes. Future panel assessments will need to also include pooled biological specimens to relate standard materials to clinically relevant levels of HPV DNAs.

The overall detection limits observed among participating laboratories, across all HPV detection systems employed, were significantly different between HPV types 16 (dilutions between 10^{-5} and 10^{-7}) and 18 (dilutions between 10^{-6} and 10^{-10}) in this study. These dilutions corresponded to a detection limit ranging for HPV 16 from approximately 10^4 to 10^2 genome equivalents per assay and for HPV 18 from approximately 10^3 to 10^{-1} genome equivalents per assay. Although initial characterizations by both reference laboratories suggested similar levels of HPV types 16 and 18 DNA within the panel dilutions, a subsequent analysis by qPCR revealed a difference between HPV types 16 and 18. In general HPV 18 plasmid material appeared at least 1 order of magnitude more concentrated than the HPV 16 plasmid material. This is reflected in the qPCR results shown in Fig. 1 and in the apparent lower detection limits observed for HPV 18 in the majority of participating laboratories using a variety of HPV DNA assays. In part for this reason, the data obtained in this panel evaluation were displayed as a function of the dilution that the original material was subjected to rather than as an estimate of genome equivalents. Data from this study demonstrate that future evaluations of candidate HPV recombinant DNA standards will require rigorous examination of longitudinal stability. In addition, it may be best to designate HPV international standard reagents using arbitrary international units rather than genome equivalents.

In this panel, the determination of sensitivity for the five HR HPV types (HPV types 31, 33, 35, 45, and 52) and one low-risk HPV type (HPV 6) was not addressed using a dilution series. HPV 6 was included in the panel because it is a component of one vaccine preparation aimed at preventing genital warts and a proportion of low-grade cervical dysplasia. The HR HPV types 31, 33, 35, 45, and 52 were included in this panel to assess potential cross-priming, or hybridization, or competitive amplification within a particular HPV DNA detection system. The results showed that in the model proposed here, HPV 16 and 18 DNA detection was not compromised by the codetection of these additional HR DNAs using two selected concentrations of other HR HPV types. This result is somewhat surprising since HPV types 16, 31, 33, 35, and 52 phylogenetically belong to the same species 9, while HPV 45 is the nearest relative of HPV type 18 (species 7) (3). Results also suggest that HPV types 33 and 45 were equally detected by all HPV DNA tests evaluated in this study. HPV 31 was the least accurately detected by participating laboratories (Table 3). Approximately half of participating laboratories failed to detect high concentrations of HPV 31 and, to a lesser extent, to detect HPV types 35, 52, and 6. The failure to detect HPV types 31, 35, 52, and 6 could reflect inherent assay differences in sensitivity and specificity that have been previously reported (22, 38) (Table 3).

The results of this study support the concept that recombinant HPV DNA constructs can be used to develop international standard reagents. The international collaborative study group recommended that the focus of international standard reagents be first on HR types HPV 16 and 18 and not on low-risk HPV types not related to cancer, and then expand to the most prevalent HR HPV types as follows: HPV types 31, 33, 35, 45, 52, and 58. It should be noted that an initial assessment of HPV type 58 in this panel was not conducted due to the fact that it is cloned within the L1 gene segment and would have required reengineering. This effort will be undertaken for future generation of an HPV 58 international standard.

The use of HPV DNA standards will vary depending on the setting in which they are applied. For example, in clinical vaccine trials, where women are under evaluation for prophylaxis of HPV infections and related disease, highly sensitive HPV DNA assays are desirable (8, 16). In contrast, the management of genital HPV-related clinical disease has demonstrated that less sensitive HPV detection limits may be appropriate (25, 30, 31). For genital HPV infections, the high prevalence of HPV DNA versus clinical disease has demonstrated that overly sensitive HPV detection would result in excessive triage of women for diagnosis and treatment. Establishment of appropriate sensitivity for any HPV assay used in clinical settings requires evaluation in very large, preferably randomized, trials and issues of cost-effectiveness as related to use in public health settings must be considered. With the introduction of highly sensitive technologies to detect HPV, quantitative assays may be useful for establishing clinically relevant sensitivity. An intrinsic part of using such technology should be the use of well-characterized standards or proficiency panels.

For HPV DNA international standards, it is desirable to develop monovalent or individual HPV type standards. This will allow unequivocal calibration of individual HPV DNA material and will allow assessment of potential detection interference when multiple HPV types are present. The HPV DNA international standard unit remains to be established and could be defined using genome equivalents, micrograms, copy numbers, or other units. International standard units for hepatitis B DNA reagents, for example, were arbitrarily assigned a potency of 10^6 international units (IU)/ml, as agreed based on the sensitivity of assays used at the time that the international standard was established (24).

Conclusion. Because cervical cancer prevention is a high priority for public health interventions in many countries, WHO supported the preparation of this panel of candidate HPV reference reagents aimed at facilitating interlaboratory comparisons and detection worldwide. HPV types 16 and 18 were the focus of this panel because they are responsible for the majority of cervical cancer cases worldwide and are the primary targets of current prophylactic HPV vaccines. The results of this pilot study show that the majority of participating laboratories accurately detected HPV types at the highest concentrations represented in the panel. Both the individual laboratory proficiency with a given test and the HPV DNA detection system itself are contributors to the interlaboratory variations observed here. For instance, a single HPV detection method used by seven laboratories demonstrated several orders of magnitude of variation in sensitivity for HPV 16 detection. Similar observations have been reported in proficiency studies of hepatitis B (36), hepatitis C (20), HIV (19, 29), herpes simplex virus (28), and *Chlamydia trachomatis* (39) using nucleic acid detection. These data underscore the need to critically consider information on HPV type-specific prevalence in epidemiology studies and point to the utility of developing HPV DNA standards. In addition, the outcome of this study underlines the need for standard operating procedures, quality control panels, and reference reagents. To address these needs the following should be considered: (i) large batches of analytical reference HPV DNA reagents, similar to those used in the present studies, must be prepared according to international guidelines for worldwide use; (ii) written standard operating procedures that describe all steps in the handling, processing, and storage of the reference reagents must be provided with the reagents; (iii) active quality assurance programs must be promoted that use external quality control panels of known HPV type and concentration; and (iv) designation of regional WHO HPV reference laboratories could serve laboratories and act to facilitate high levels of performance in HPV DNA detection.

The potential benefits of available reference reagents are many. The sensitivity and specificity of HPV DNA assays can be determined, validated, and monitored. In addition, performance of HPV DNA detection methods as related to international standards will facilitate comparisons of data from multiple studies. Thus, the availability of international HPV DNA standards will contribute to the field of HPV prevention, diagnosis, and treatment. In particular, such standards, if available worldwide, will allow for reference calibration of HPV DNA tests, thereby enabling manufacturers to further validate and develop HPV detection reagents and kits, and will allow reliable disease monitoring and improve health care worldwide.

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