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Comparison of four different human papillomavirus genotyping methods in cervical samples: Addressing method-specific advantages and limitations

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ABSTRACT

Since human papillomavirus (HPV) is recognized as the causative agent of cervical cancer and associated with anogenital non-cervical and oropharyngeal cancers, the characterization of the HPV types circulating in different geographic regions is an important tool in screening and prevention. In this context, this study compared four methodologies for HPV detection and genotyping: real-time PCR (Cobas® HPV test), nested PCR followed by conventional Sanger sequencing, reverse hybridization (High + Low PapillomaStrip® kit) and next-generation sequencing (NGS) at an Illumina HiSeq2500 platform. Cervical samples from patients followed at the Family Health Strategy from Juiz de Fora, Minas Gerais, Brazil, were collected and subjected to the real-time PCR. Of those, 114 were included in this study according to the results obtained with the real-time PCR, considered herein as the gold standard method. For the 110 samples tested by at least one methodology in addition to real-time PCR, NGS showed the lowest concordance rates of HPV and high-risk HPV identification compared to the other three methods (67–75 %). Real-time PCR and Sanger sequencing showed the highest rates of concordance (97–100 %). All methods differed in their sensitivity and specificity. HPV genotyping contributes to individual risk stratification, therapeutic decisions, epidemiological studies and vaccine development, supporting approaches in prevention, healthcare and management of HPV infection.

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1. Introduction

Human papillomavirus (HPV) is one of the most common sexually transmitted infections worldwide and represents a global public health problem due to its high prevalence and transmissibility [1]. Based on an estimate from 2010, 11.7 % of women with normal cytology are infected by HPV worldwide [2].

HPV infection is usually asymptomatic and resolve spontaneously within a few years, which contributes to the fact that most HPV-positive individuals do not know their status [3]. However, the persistence of high-risk strains of HPV (hr-HPV) can cause intra-epithelial lesions that can develop into cancer if untreated. High-risk HPV infection is a well-established cause of cervical cancer, and more recently has been associated to other anogenital cancers (anus, vulva, vagina and penis) and oropharyngeal cancers [revised by 4]. HPV DNA is found in 99.7 % of cervical cancer cases, 90 % of anal cancers and 20–60 % of other genital and oropharyngeal carcinomas [4,5].

Among the HPV-associated cancers, cervical cancer is the most relevant since it represents the fourth most frequent cancer among women worldwide [6]. In 2020, approximately 600 000 new cases were estimated and 341 000 women died from cervical cancer; more than 90 % of these deaths occurred in low- and middle-income countries [7,8]. Around 16 000 new cases are expected in Brazil for 2023, representing the third most frequent cancer in the female population and the fourth leading cause of cancer death in Brazil (6000 new cases) [9].

There are more than 200 different HPV types currently identified, according to the Papilloma Virus Genome Database (PaVE, https://pave.niaid.nih.gov). Over 40 distinct HPV types infect the genital tract and at least 14 of those types have been implicated in precancerous lesions and cancer of the cervix [10]. In cervical cancer, the most frequent HPV types observed are HPV16 and 18, which are responsible for approximately 70 % of cases globally [11,12]. Although HPV16 is the most common hr-HPV type in all regions, HPV18, 31, 39, 51, 52, 56, 58, and 59 had similar prevalence in the general female population worldwide [2]. Besides the manifestations associated with hr-HPV persistence, infection by low-risk strains of HPV (lr-HPV) can cause benign lesions such as anogenital warts [13]. The lr-HPV types 6 and 11 are the most representative, since they are responsible for approximately 90 % of genital warts [14,15].

In the context of remarkable variability around the world, knowledge of the HPV type distribution is critical to the effectiveness of diagnostic and preventive approaches. Most of the commercial methods available for HPV genotyping are based on PCR, hybridization or restriction fragment length polymorphism analysis [16,17]. The first commercial HPV DNA test for genotyping approved by the US FDA was Hybrid Capture II (Qiagen Corporation, Gaithersburg, MD, USA), based on liquid-phase hybridization and able to identify 13 hr- and five lr-HPV types [18,19]. Other assays such as HPV-HR + HPV16/18 test on Cobas® 4800 HPV System (Roche Molecular Diagnostics, Pleasanton, CA, USA), a real-time PCR-based DNA amplification, have also been approved by FDA for cervical cancer screening. This test simultaneously identifies a pooled result for 12 hr-HPV types and individual results for HPV 16 and HPV 18 [20]. The main disadvantage of commercially available kits is the bias in the detection of rare or unknown genotypes [21]. Recently, next-generation sequencing (NGS) has emerged in HPV genotyping with several advantages, such as analyzing many samples per time, no restriction imposed by specific probes and the high accuracy to detect multiple infections, nucleotide variants and novel types [22–26].

Since HPV genotypes have different distribution around the world and distinct roles in carcinogenesis, genotyping is important for epidemiological studies, improvement of cervical cancer screening and development and use of more effective HPV vaccines. Thus, this study aims to compare four methodologies for HPV detection and genotyping: real-time PCR (Cobas® HPV test; Roche Molecular Diagnostics, Pleasanton, CA, USA), reverse hybridization (High + Low PapillomaStrip® kit; OPERON S.A. Immunodiagnostics, Cuarte de Huerva, Spain), nested-PCR followed by conventional Sanger sequencing and NGS in an Illumina HiSeq2500 platform.

2. Materials and methods

2.1. Study population

For the current study, 114 samples were selected from a group of 2076 anonymized cervical swabs collected from women recruited in two units of the Family Health Strategy Program of Juiz de Fora, Minas Gerais, Brazil, between 2010 and 2012. These women were asymptomatic, and enrollment was performed during a routine pap smear screening. All samples were previously tested for HPV using the HPV-HR + HPV16/18 test on Cobas® 4800 HPV System (Roche Molecular Diagnostics, Pleasanton, CA, USA) [27]. This test determines the presence of HPV16 and HPV18 individually and other 12 hr-HPV types collectively (HPV31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66 and 68) and was used herein as the gold standard method. Samples were randomly selected to complete three groups with different HPV results: Co-infection with HPV16 or HPV18 and at least one of the other 12 hr-HPV types (HPV16/18 + HR - seven samples); infection with at least one of the 12 hr-HPV types from the pool and negative for HPV16/18 (HR-HPV - 26 samples); and negative for both HPV16/18 and hr-HPV types pool (HPV-NEG - 81 samples). The study was approved by the Ethics Committee of the Instituto de Medicina Social of Universidade do Estado do Rio de Janeiro (UERJ) (reference protocol 0026.1.259.180-09).

2.2. DNA isolation

Cervical samples stored in PreservCyt® solution (Hologic, Marlborough, MA) were centrifuged for 30 min at 3000 rpm and the supernatant was partially discarded to concentrate the cells. Total DNA was extracted from the concentrated sample using QIAamp DNA mini kit (QIAGEN, Valencia, CA), following the manufacturer's instructions. A PCR of a 412-bp fragment of the *TP53* gene exon 4

was performed under previously described conditions [28] to evaluate the quality of the specimen and DNA isolation. Samples with successful amplification for this endogenous gene were considered satisfactory for the downstream experiments.

2.3. HPV identification by Sanger sequencing

Isolated DNA was submitted to a semi-nested PCR using MY09/MY11 primers in the first round and MY09/GP05 primers in the second round to amplify a final 410-bp fragment of the HPV *L1* gene as previously described [29–31]. Products were purified with the Illustra GFX PCR DNA & Gel Band Purification kit (GE Healthcare, Piscataway, NJ) and sequenced using the BigDye Terminator kit (Life Technologies, Carlsbad, CA) in an automated 3130XL Genetic Analyzer (Life Technologies). Sequences obtained were assembled with SeqMan (DNAStar, Madicon, WI) and classified according to the best hit on a Blast search of the query sequence at the NCBI website.

2.4. HPV identification by reverse hybridization

HPV genotyping using reverse hybridization was performed with the High + Low-Risk PapillomaStrip® kit (OPERON S.A. Immunodiagnostics, Cuarte de Huerva, Spain). The method is based on a PCR amplification of HPV E6/E7 regions, followed by hybridization of the PCR product with HPV type-specific probes spanning 37 different low- and high-risk HPV types (HPV6, 11, 16, 18, 26, 31, 33, 35, 39, 40, 42, 43, 44, 45, 51, 52, 53, 54, 56, 58, 59, 61, 62, 66, 67, 68, 69, 70, 71, 72, 73, 74, 81, 82, 83, 84 and 91). This test was carried out following manufacturer's instructions.

2.5. HPV identification by next-generation sequencing

Rolling circle amplification was performed to enrich the total circular DNA with the Illustra TempliPhi Amplification kit (GE Healthcare Life Sciences, Piscataway, NJ) following manufacturer's instructions. Positive products were purified and used to prepare DNA libraries with the Nextera XT DNA Sample Preparation kit (Illumina Inc., San Diego, CA). During this step, each library was indexed with a different pair of barcodes. Real-time qPCR was performed with the KAPA Library Quantification kit (KAPA Biosystems, Wilmington, MA) to estimate the libraries' concentration. Libraries were pooled and sequenced by synthesis in an Ilumina HiSeq-2500 system using a 200-cycles paired-end run (2 × 100).

Reads obtained from the sequencing were evaluated for quality using FastQC (Babraham Bioinformatics, Cambridge, UK). The bases with quality score lower than 28 Phreds and reads lower than 70 bp were trimmed out with Sickle [32]. Remaining reads were mapped to 167 different reference sequences of HPV types available at the PAVE database (https://pave.niaid.nih.gov/) using BWA [33] and the number of mapped reads were counted to each HPV reference genome used with Samtools [34]. When a given HPV type was identified only by NGS, we defined a number of reads threshold to avoid false positive results due to sample bleeding problem [35]. Two thresholds' values were calculated: the highest number of reads obtained for each HPV type by submitting HPV-negative libraries sequenced at the same HiSeq lane as the samples from this study to the HPV read count analysis described above; and the lowest number of reads obtained from each HPV type with concordant results between NGS and one of the other methodologies. Samples were positive for a given HPV type when the number of reads mapped to the given HPV type reference was greater than both established cutoffs.

2.6. Sequencing methodology selection

The methodology used in each sample was selected based on the RT-PCR (Cobas®) results. Samples identified by RT-PCR as co-infected with HPV16 and/or HPV18 and at least one of the other 12 hr-HPV types (HPV16/18 + HR) were also tested by Sanger sequencing and NGS. Samples positive for the 12 hr-HPV types pool and negative for HPV16/18 (HR-HPV) were submitted to Sanger sequencing, reverse hybridization and NGS. Seventy-seven samples classified as negative for both HPV16/18 and hr-HPV types pool by Cobas® (HPV-NEG) were analyzed by reverse hybridization, and those that tested positive were additionally analyzed by NGS. The remaining four samples negative for HPV by Cobas® (HPV-NEG) were not analyzed due to unsatisfactory DNA quality determined by the lack of *TP53* PCR amplification.

2.7. Statistical analysis

The frequency of HPV, hr-HPV and multiple HPV type infections were calculated for each test and group of samples. Chi-square or Fisher's exact test was used to compare these detection rates between the different tests employed. The concordance between HPV tests was calculated considering all the samples analyzed by both tests independent of the sample group. Cohen's kappa coefficient was calculated using SPSS (IBM Corp., Chicago, IL) to determine the agreement between each two different HPV detection methods. HPV types classified as carcinogenic or probably/possibly carcinogenic by the International Agency for Research on Cancer [36] were assumed as high-risk HPV, while the other HPV types were classified as undetermined or low-risk HPV.

3. Results

Samples of four women were excluded from the study due to non-amplification of the *TP53* gene fragment. The median age of the

110 remaining women was 38 years (interquartile range (IQR): 29–48 years) and their samples were tested by at least one methodology in addition to the gold standard real-time PCR HPV test (Cobas® 4800 HPV System), as described in the Methods section.

All seven samples in the group of HPV co-infection with HPV-16 and/or HPV-18 and at least one of the other 12 hr-HPV types (HPV16/18 + HR) were also positive for hr-HPV by Sanger and NGS, but the presence of HPV16 or 18 were not identified in one and three of the samples, respectively (Fig. 1A and B; Table 1). High-risk HPV types were found only in part of the samples classified by Cobas® as positive for the 12 hr-HPV types pool and negative for HPV16/18 (HR-HPV), regardless of the methodology used (Fig. 1B). In this group of samples, NGS showed a lower rate of hr-HPV type identification (62 %) compared to Sanger sequencing (96 %; $p = 0.002$) and to reverse hybridization (88 %; $p = 0.024$; Table 1). This result was similar for HPV detection, with a difference of just one sample between them (Fig. 1C). Reverse hybridization found HPV in eight samples (10 %) negative for both HPV16/18 and hr-HPV types pool by Cobas® (HPV-NEG), but only one sample (1 %) showed hr-HPV (Fig. 1D). Six of those eight HPV-positive samples by reverse hybridization were also submitted to next-generation sequencing, which found only lr-HPV present in three samples (Table 1; Fig. 1D). The two remaining samples were not analyzed by NGS because the first attempt to prepare the libraries was not successful and there was no DNA available to repeat the experiment.

Despite the different results obtained by the four genotyping methods, all but one sample presented at least one other methodology result that corroborated the RT-PCR (Fig. 1).

Among the HPV16/18 + HR samples, HPV16 was the most frequent HPV type identified both by Sanger and NGS (Table 2). Another eight hr-HPV types were identified by NGS, while Sanger sequencing only identified HPV18 and HPV58 in addition to HPV16. The distribution of HPV types found in the HR-HPV group of samples was clearly concentrated in hr-HPV types for all methods, but the

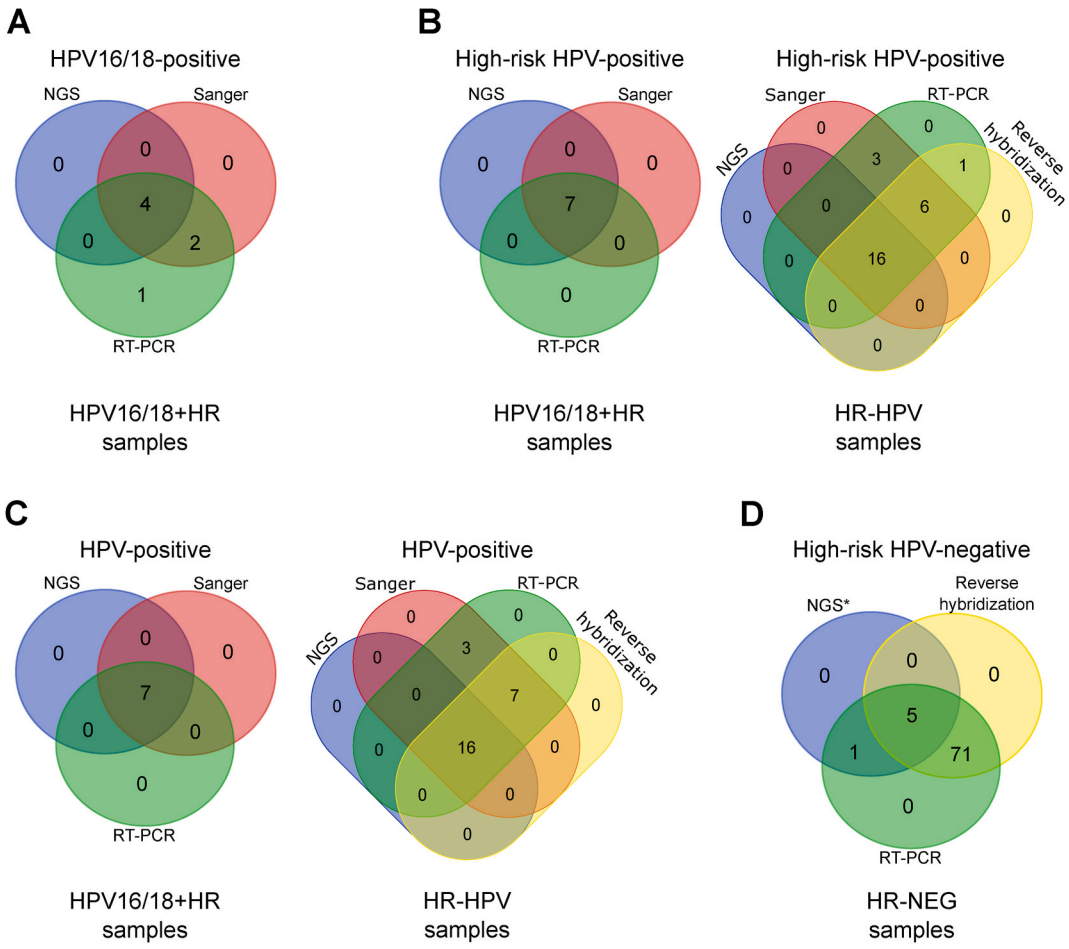

Fig. 1. Venn diagram illustrating the HPV identification overlaps among the methodologies for each group of samples. A) HPV16/18 detection in the HPV16/18 + HR group. B) High-risk HPV detection in the HPV16/18 + HR and HR-HPV groups. C) Overall HPV detection in the HPV16/18 + HR and HR-HPV groups. D) High-risk HPV absence in the HPV-NEG group. HPV16/18 + HR: samples identified by RT-PCR as co-infected with HPV16 and/or HPV18 and at least one of the other 12 established high-risk HPV types (HPV31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66 and 68); HR-HPV: samples positive for the 12 high-risk HPV types pool and negative for HPV16/18; HPV-NEG: samples classified as negative for HPV16/18 and high-risk HPV types pool. NGS: next-generation sequencing; Sanger: Sanger sequencing; RT-PCR: HPV-HR + GT 16/18 test on a Cobas® 4800 HPV system (Roche); reverse hybridization: High + Low-Risk PapillomaStrip kit (OPERON S.A. Immunodiagnostics). * For this group, NGS was only performed in six samples.

Table 1
HPV detection rates by different genotyping methods.

	HPV 16/18 + HR (n = 7)			HR-HPV (n = 26)				HPV-NEG (n = 77)		
	NGS	Sanger	RT-PCR	NGS	Sanger	Reverse hybridization	RT-PCR	NGS*	Reverse hybridization	RT-PCR
HPV-positive (%)	100	100	100	61.5	100	88.5	100	50	10.4	0
High-risk HPV-positive (%)	100	100	100	61.5	96.2	88.5	100	0	1.3	0
HPV16/18-positive (%)	57.1	85.7	100	0	0	0	0	0	0	0
Multiple infection	6	–	7	5	–	10	–	1	3	–
No. HPV types detected	11	3	–	15	12	18	–	4	7	–

HPV16/18 + HR: samples identified by RT-PCR as co-infected with HPV16 and/or HPV18 and at least one of the other 12 high-risk HPV types (HPV31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66 and 68); HR-HPV: samples positive for the 12 high-risk HPV types pool and negative for HPV16/18; HPV-NEG: samples classified as negative for HPV16/18 and high-risk HPV types pool. NGS: next-generation sequencing; Sanger: Sanger sequencing; RT-PCR: HPV-HR + GT 16/18 test on a Cobas® 4800 HPV system (Roche); reverse hybridization: using the High + Low-Risk PapillomaStrip kit (OPERON S.A. Immunodiagnostics). * For this group, NGS was only performed in six samples that tested positive by reverse hybridization.

Table 2
HPV type distribution by different methods.

		HPV 16/18 + HR (n = 7)		HR-HPV (n = 26)			HPV-NEG (n = 77)	
		NGS (%)	Sanger (%)	NGS (%)	Sanger (%)	Reverse hybridization (%)	NGS* (%)	Reverse hybridization (%)
High-risk HPV type	HPV16	42.9	71.4	0	0	0	0	0
	HPV18	14.3	14.3	0	0	0	0	0
	HPV26	14.3	0	3.8	0	3.8	0	0
	HPV31	28.6	0	15.4	7.7	11.5	0	0
	HPV33	0	0	0	11.5	15.4	0	0
	HPV35	14.3	0	3.8	7.7	7.7	0	0
	HPV45	0	0	0	3.8	7.7	0	0
	HPV51	14.3	0	3.8	0	3.8	0	0
	HPV52	42.9	0	3.8	3.8	7.7	0	0
	HPV53	0	0	0	0	3.8	0	0
	HPV56	0	0	15.4	11.5	11.5	0	0
	HPV58	14.3	14.3	7.7	19.2	11.5	0	0
	HPV59	14.3	0	3.8	7.7	7.7	0	0
	HPV66	0	0	7.7	15.4	15.4	0	0
	HPV67	14.3	0	0	0	0	0	0
	HPV68	0	0	15.4	3.8	11.5	0	1.3
	HPV70	0	0	3.8	3.8	0	0	0
	HPV73	0	0	3.8	0	3.8	0	0
Undetermined or low-risk HPV type	HPV6	0	0	0	0	0	16.7	0
	HPV32	0	0	7.7	0	–	0	–
	HPV42	0	0	0	0	0	16.7	3.9
	HPV44	0	0	0	3.8	0	0	0
	HPV54	0	0	3.8	0	11.5	0	0
	HPV61	0	0	0	0	0	16.7	2.6
	HPV62	0	0	0	0	0	16.7	1.3
	HPV71	0	0	0	0	3.8	0	0
	HPV74	0	0	0	0	0	0	1.3
	HPV81	0	0	0	0	0	0	2.6
	HPV83	0	0	0	0	0	0	5.2
	HPV84	0	0	0	0	3.8	0	0
	HPV90	14.3	0	0	0	–	0	–
	HPV91	0	0	3.8	0	3.8	0	0

HPV16/18 + HR: samples identified by RT-PCR as co-infected with HPV16 and/or HPV18 and at least one of the other 12 high-risk HPV types (HPV31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66 and 68); HR-HPV: samples positive for the 12 high-risk HPV types pool and negative for HPV16/18; HPV-NEG: samples classified as negative for HPV16/18 and high-risk HPV types pool. NGS: next-generation sequencing; Sanger: Sanger sequencing; RT-PCR: HPV-HR + GT 16/18 test on a Cobas® 4800 HPV system (Roche); reverse hybridization: using the High + Low-Risk PapillomaStrip kit (OPERON S.A. Immunodiagnostics). * For this group, NGS was only performed in six samples that tested positive by reverse hybridization.

most prevalent types were different for each method applied. The most prevalent HPV types were HPV31, 56 and 68 by NGS; HPV58 followed by HPV66 by Sanger sequencing and HPV33 and HPV66 were the most prevalent by reverse hybridization (Table 2). In the HPV-NEG group of samples, reverse hybridization evidenced hr-HPV type in only one sample (HPV68) and, similarly to NGS, identified several lr-HPV types distributed in these samples.

Only three samples of this study were associated with abnormal cytology. ASCUS (atypical squamous cells of undetermined significance) was present in one sample positive for HPV18 by all three methodologies analyzed (Sanger sequencing, NGS and RT-PCR), and also positive for HPV59 by NGS and for the hr-HPV pool by RT-PCR. Two LSIL (low-grade squamous intraepithelial lesion) cases were present. One was positive for HPV16 and for the hr-HPV pool by RT-PCR, but Sanger sequencing only identified HPV58, and NGS identified HPV58 and HPV26. The other LSIL sample was negative for HPV16/18 and positive for hr-HPV pool by RT-PCR. The three other methodologies applied (NGS, Sanger sequencing and reverse hybridization) identified HPV58, and reverse hybridization additionally identified HPV84.

The concordance between the different methodologies was calculated (Table 3). NGS showed the lowest concordance rates of HPV and hr-HPV infection compared to the remaining methods (*vs* RT-PCR: HPV – 67 %, hr-HPV – 74 %; *vs* Sanger sequencing: HPV – 70 %, hr-HPV – 73 %; *vs* reverse hybridization: HPV – 69 %, hr-HPV – 75 %; Table 3). RT-PCR and Sanger sequencing showed the highest rates of concordance: 100 % for HPV and 97 % for hr-HPV infection. We could not calculate the Cohen's kappa coefficient between these methods because all samples analyzed by Sanger sequencing were positive for hr-HPV by RT-PCR and this variable became a constant. RT-PCR and reverse hybridization showed a concordance rate of 89 % for HPV and 96 % for hr-HPV, with a high agreement (kappa: HPV = 0.734; hr-HPV = 0.894).

Sensitivity and specificity were determined for HPV and hr-HPV detection separately. Sanger was the most sensitive method (HPV – 100 % and hr-HPV – 96.97 %) compared to reverse hybridization (HPV and hr-HPV – 88.46 %) and NGS (HPV and hr-HPV – 69.7 %). Reverse hybridization specificity was 89.61 % for HPV and 98.7 % for hr-HPV detection. Only six samples classified as HPV-negative by RT-PCR were analyzed by NGS and this method showed a 50 % specificity for hr-HPV and a 100 % for HPV identification. Specificity was not calculated for Sanger sequencing because we did not test samples identified as HPV-negative by RT-PCR using this methodology.

4. Discussion

Since HPV infection is associated with cervical cancer development, HPV detection and genotyping are effective tools for screening, complementing the diagnosis and preventing cervical cancer [27]. Despite high coverage of cervical cytology screening, this technique has a low sensitivity and sometimes provides confusing interpretation with unclear meaning [37–39]. Sample high quality requirement and subjectiveness in reading results are well-described limitations of cytology, which can lead to errors in reports [38]. In this sense, HPV molecular testing has greater advantages, like its high reproducibility and the potential to be automated. The main advantage associated with the use of HPV genotyping techniques is the accuracy to determine the HPV types, leading to investigation of novel types and multiple infections [40–42].

HPV type-specific detection can provide information to epidemiological studies and vaccine development, besides contributing to allow individual risk stratification and subsidize therapeutic decisions [41,43]. The variable HPV type distribution worldwide can highlight differences that should be taken into consideration in vaccine design, with certain types being most commonly associated with cervical cancer in specific regions [44]. In the Brazilian scenario, the quadrivalent vaccine against HPV (including HPV16, 18, 6 and 11) was approved in 2006, followed by the bivalent vaccine (HPV16 and 18) in 2008 [45]. The former is freely available since 2014 to females aged 9–14 years and, since 2017, also became available to males and to immune-compromised adults.

The identification of HPV types infecting Brazilian women will increase the knowledge on viral epidemiology, mainly at the regional level, allowing the evaluation of the prevalence of HPV types covered by the vaccine and the development of novel strategies for vaccination and patient management. This study aimed to evaluate different HPV genotyping methods, allowing us to compare and highlight pros and cons of each method (Table 4).

Our data show heterogeneity among the four methods used for HPV identification, with concordance rates ranging from 67 to 100 %. All methods differ in their sensitivity and specificity. Techniques that use probes such as *in situ* hybridization and Southern blotting

Table 3
Concordance rates between HPV detection methods.

	HPV		High-risk HPV	
	%	Kappa	%	Kappa
RT-PCR *vs.*				
NGS	66.7 % (26/39)	0.214	74.4 % (29/39)	0.414
Reverse hybridization	89.3 % (92/103)	0.734	96.1 % (99/103)	0.894
Sanger	100 % (33/33)	NC*	97.0 % (32/33)	NC*
NGS *vs.*				
Reverse hybridization	68.8 % (22/32)	0.263	75.0 % (24/32)	0.500
Sanger	69.7 % (23/33)	NC*	72.7 % (24/33)	0.134
Reverse hybridization *vs.*				
Sanger	88.5 % (23/26)	NC*	84.6 % (22/26)	−0.061

HPV16/18 + HR: samples identified by RT-PCR as co-infected with HPV16 and/or HPV18 and at least one of the other 12 high-risk HPV types (HPV31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66 and 68); HR-HPV: samples positive for the 12 high-risk HPV types pool and negative for HPV16/18; HPV-NEG: samples classified as negative for HPV16/18 and high-risk HPV types pool. NGS: next-generation sequencing; Sanger: Sanger sequencing; RT-PCR: HPV-HR + GT 16/18 test on a Cobas® 4800 HPV system (Roche); reverse hybridization: using the High + Low-Risk PapillomaStrip kit (OPERON S.A. Immunodiagnostics). *NC: not calculated because all samples were HPV-positive for one of the methodologies.

Table 4
Pros and cons of each HPV detection methods evaluated in this study.

HPV detection method	Pros	Cons
RT-PCR	• Targets a highly conserved L1 region • Able to identify hr-HPV types with clinical relevance	• Only HPV16 and 18 identified individually • Limited to identifying multiple infections • Limited range of HPV types detected
Sanger sequencing	• Targets a highly conserved L1 region • Potential to detect all HPV types in a sample	• Detects preferentially the major viral population • Lowest variability of HPV types identified • Limitation to identify multiple HPV infections (except if previously submitted to cloning or multiplex PCR)
NGS	• Detects a greater variety of genotypes • Potential to detect all HPV types in a sample, including novel HPV types • Provides HPV complete genomes	• Lowest concordance rates with other techniques • Fails to detect integrated viruses (when using rolling circle amplification as a target enrichment method)
Reverse hybridization	• Targets the E6/E7 oncogenes • Identifies multiple HPV infections • Identifies and genotypes hr-HPV types with clinical relevance	• Time-consuming • Requires large amounts and integrity of the DNA • Limited range of HPV types detected

RT-PCR: HPV-HR + GT 16/18 test on a Cobas® 4800 HPV system (Roche); NGS: next-generation sequencing; reverse hybridization: High + Low-Risk PapillomaStrip kit (OPERON S.A. Immunodiagnostics).

are less sensitive for specifically detecting the viral DNA sequence, while the most sensitive techniques are those based on amplifying the target DNA, such as PCR and RT-PCR [46,47].

The Sanger sequencing method used herein was able to detect HPV in all samples through PCR amplification of the L1 fragment. For the samples classified as hr-HPV-positive by Cobas RT-PCR (HR-HPV), Sanger sequencing identified hr-HPV in 97 % of samples. However, among the samples identified with Cobas® as co-infected with HPV16/18 and at least one of the others 12 hr-HPV types (HPV16/18 + HR), HPV16 or 18 was not identified in only one of them by Sanger. In this specific sample the HPV16/18 were not identified using NGS, either (Fig. 1). We cannot determine if this result was a failure of the Sanger/NGS or RT-PCR. However, as we assumed RT-PCR as the gold standard, we considered it as a Sanger/NGS false-negative result. In addition, this could be associated with limitation of Sanger sequencing to detect only the major (most frequent) viral population, because this sample was also positive for high-risk HPV types and it identified HPV58. This limitation also includes the detection of low-frequency HPV types compared to the other methodologies [48]. Despite the large use of L1 sequencing for HPV genotyping, its limitation to identify multiple HPV infections by Sanger sequencing is well recognized [49]. HPV diversity can only be accurately assessed through cloning or multiplex PCR prior to sequencing, resulting in a clonal library composed of different HPV-L1 fragments [30,50,51]. These methods could circumvent the limitations observed in this study, allowing Sanger sequencing to properly identify all the HPV types present in a given sample. The correct identification of multiple infections can contribute to possible competitive or cooperative interactions that may exist among the co-infecting genotypes in a subject, an issue still under intense debate in the literature [52].

Next-generation sequencing had the lowest concordance rates of HPV and hr-HPV identification compared to the other methodologies. Interestingly, this method did not identify HPV16 or 18 in three samples classified as HPV16/18 + HR by RT-PCR, of which two were HPV16/18-positive by Sanger sequencing (Fig. 1). Moreover, NGS did not identify hr-HPV types previously identified by RT-PCR, Sanger sequencing and reverse hybridization. The lack of identification of certain HPV types has an important impact to cervical cancer screening and can result in an underestimated prevalence of those types based on that method. One possible explanation for the results observed may be associated to the rolling circle amplification (RCA) method used prior to NGS library preparations. RCA is based on the amplification of circular DNA, which could fail to detect viruses already integrated into the host genome. Another point that should be considered is the low viral load of some HPV types that could result in low read counts that are disregarded in further analyses. Next-generation sequencing protocols have an intrinsic limitation in that they lack internal controls as the other methods compared herein, which make interpretation of results more challenging.

The association between HPV genome integration and cytology status is still controversial. Some studies investigating HPV-16 integration status found a strong positive correlation with increasing disease severity [53–57]. However, no relationship between cervical disease grade and HPV16 integration status was identified by other studies [58–61]. Different associations were also found for HPV18, with some studies describing an association with disease and other not [54,57,62]. A work by Marongiu et al. found that the HPV integration status could distinguish between normal and abnormal cytology with a sensitivity of 72 % and a specificity of 50 % (OR 2.6, 95 % CI 1.0–6.8; $p = 0.054$) [63]. Thus, it is expected that some HPV genomes are already integrated in the host genome in our patients and will fail to be sequenced after RCA. To overcome this problem, other target enrichment methods can be used before the NGS, like PCR or hybridization capture, or even sequencing of the total DNA ("shotgun" sequencing). On the other hand, four HPV types were identified only by NGS (HPV6, 32, 67 and 90). Although they are HPV types of undetermined or low-risk for cancer, their identification is relevant for prevalence studies, especially for HPV6, which together with HPV11 is responsible for 90 % of the genital warts worldwide [64].

Differently from RCA, studies based on PCR amplification as input for NGS showed increased HPV detection, including hr-HPV, compared to multiplex-PCR or PCR followed by Sanger sequencing [21,23,48,65–67]. Da Fonseca et al. found an increase of up to 40 % for HPV detection (all types) when compared to other methods (multiplex-PCR and nested-PCR followed by Sanger sequencing)

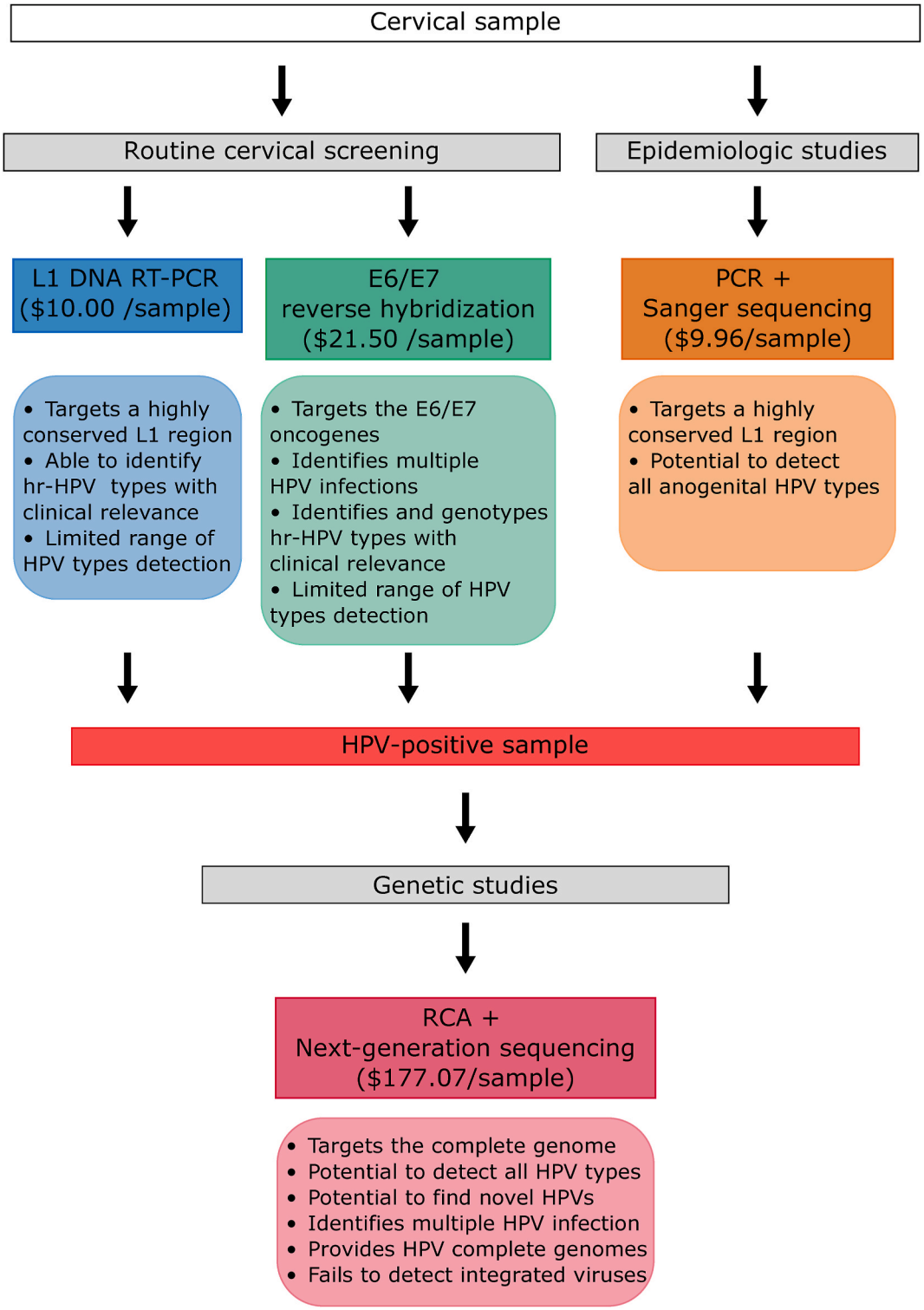

Fig. 2. Schematic flowchart suggesting the use of different HPV genotyping methods for clinical or epidemiological/genetic purposes. NGS: next-generation sequencing; Sanger: Sanger sequencing; RT-PCR: HPV-HR + GT 16/18 test on a Cobas® 4800 HPV system (Roche); reverse hybridization: High + Low-Risk PapillomaStrip kit (OPERON S.A. Immunodiagnostics).

[48]. NGS is also a useful method to obtain the complete sequence of previously unknown or only partially characterized HPV types [65,66,68,69]. Thus, NGS is an effective method for HPV detection and genotyping and a successful tool to determine the diversity of HPV genotypes. It can also reveal the dynamics of HPV infection, a characteristic that would otherwise remain unseen by traditional sequencing [23,67].

Reverse hybridization was used for HR-HPV and HPV-NEG groups and showed a strong agreement with the RT-PCR results. This technique also identified the widest range of HPV types and multiple infections. The hybridization method used in this study is based on amplification of E6/E7 regions. Methods targeting this region are more sensitive in detecting cancers [70] probably because E6/E7 are required during oncogenesis and are unlikely to be deleted in those cases. In this study, we found a strong agreement between the method that targeted E6/E7 (reverse hybridization) and the one that targeted L1 (RT-PCR), suggesting that the possibility of L1 deletion did not impact the HPV detection by RT-PCR in these samples, probably because only three samples studied had abnormal cytology. Despite not being a region conserved among the different HPV types, E6/E7 is conserved within each HPV type, what enables the design of E6/E7 reliable primers for each HPV type. The disadvantage of the hybridization process is time consumption and the relatively larger amounts of DNA required, relying on the integrity of the DNA sample [71]. Another important limitation of the technique concerns the use of probes, which limits the range of HPV types that can be identified. Our study identified two types (HPV32 and 90) that are not included in the reverse hybridization kit used herein.

Previous studies based on hybridization techniques reported that a wider range of genotypes can be detected when compared to PCR-based targeted amplified techniques [72]. However, our results based on RT-PCR, PCR followed by Sanger and reverse hybridization showed a great concordance between them, with similar detection of different HPV genotypes. The methodology used in the RT-PCR targets a highly conserved L1 region of the HPV genome and features automated sample preparation combined with real-time PCR technology to detect 14 HR-HPV through four different PCR reactions (HPV16, HPV18, 12 hr-HPV as a pool, and β-globin as a control for extraction and amplification viability) [73]. The ability of the RT-PCR Cobas® system to only genotype HPV16 and 18 individually, but not the other high-risk types present in the pool, limits its use in prevalence studies. The approach can only identify multiple HPV infections if a sample contains HPV16 and/or 18 and other hr-HPV; otherwise it is classified as positive for high-risk HPV type pool regardless of the number of distinct HPV types that are present. In addition, it does not identify other high (HPV73) or probable high-risk (HPV26, 53 and 67) HPV types with evidence of carcinogenicity [74,75] that were identified herein by others methods. White et al. [72] found that the sensitivity and specificity of the RT-PCR test for cervical intraepithelial neoplasia (CIN) grade 2-positive detection were 90 % (95 % CI 88.8–91.3) and 55.5 % (95 % CI 52.5–58.5), respectively. For CIN grade 3-positive samples, the sensitivity was even higher (100 %), but the specificity was of 44.5 % (95 % CI 42–47). The higher frequency of hr-HPV types included in the Cobas® system in CIN3+ samples explains such high sensitivity and highlights its potential in detection and prevention of cervical cancer.

There are several limitations to our study. Only one group of samples (HR-HPV) was subjected to all four HPV genotyping methods studied. This was due to unavailability of the sample and/or HPV genotyping test and may affect the results found herein. Furthermore, no positive or negative controls were included. The establishment of international standards to evaluate HPV typing techniques is yet a major challenge [76]. In this study, we used RT-PCR as the gold standard and to overcome the lack of controls we compared the results of the different methodologies and observed that in all but one sample the result found by RT-PCR was corroborated by at least one of the other techniques studied.

An unambiguous HPV genotyping pipeline requires the combination of typing methods, but the cost is a barrier for routine settings. Overall, we described pros and cons of four widely used HPV identification methods. Reverse hybridization and RT-PCR have limitations in genotyping, however they are able to identify the presence of hr-HPV types with clinical relevance and thus, to predict the development of pre-cancerous lesions. RT-PCR is the least expensive among them, but both may be used for cervical cancer screening (Fig. 2). On the other hand, Sanger sequencing and NGS, preceded by non-biased assays, would be indicated for HPV prevalence and genotyping studies (Fig. 2), mainly supported by their potential to identify most, if not all, HPV types present in a sample, notably in a multiple infection, and including novel HPV types. Through the improvement of HPV genotyping, clinical and epidemiological information can be obtained and will contribute to individual risk stratification, therapeutic decisions, epidemiological studies and vaccine development, supporting enhanced approaches in prevention, assistance and management of HPV infection.

Ethics statement

This study was approved by the Ethics Committee of the Instituto de Medicina Social of Universidade do Estado do Rio de Janeiro (UERJ) (reference protocol 0026.1.259.180-09).

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Data availability statement

The data that support the findings of this study are available from the corresponding author upon request.

CRediT authorship contribution statement

Juliana D. Siqueira: Writing – original draft, Validation, Methodology, Investigation, Formal analysis. **Brunna M. Alves:** Writing – original draft, Validation, Methodology, Investigation, Formal analysis. **Adriana B.C. Castelo Branco:** Validation, Methodology, Investigation, Formal analysis. **Kristiane C.D. Duque:** Methodology, Investigation, Data curation. **Maria Teresa Bustamante-Teixeira:** Methodology, Investigation, Data curation. **Esmeralda A. Soares:** Writing – review & editing, Conceptualization. **José Eduardo Levi:** Methodology, Investigation, Data curation. **Gulnar Azevedo e Silva:** Writing – review & editing, Funding acquisition, Conceptualization. **Marcelo A. Soares:** Writing – review & editing, Writing – original draft, Supervision, Resources, Project administration, Investigation, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:Marcelo A. Soares reports financial support was provided by Carlos Chagas Filho Foundation for Research Support of Rio de Janeiro State (FAPERJ). Marcelo A. Soares reports financial support was provided by National Council for Scientific and Technological Development (CNPq).

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