RESEARCH ARTICLE

Development of In-House Multiplex Real Time PCR for Human Papillomavirus Genotyping in Iranian Women with Cervical Cancer and Cervical Intraepithelial Neoplasia

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Abstract

Background: HPV related cervical cancer as one of the most common women cancers in developing countries. Regarding accessibility of commercial vaccines, any long or short term modality for integrating preventive immunization against HPV in a national program needs comprehensive information about HPV prevalence and its genotypes. The important role of selecting most accurate diagnostic technologies for obtaining relevant data is underlined by different assays proposed in the literature. The main objective of the present study was to introduce an in-house HPV typing assay using multiplex real time PCR with reliable results and affordable cost for molecular epidemiology surveys and diagnosis. Materials and Methods: 112 samples of formalin fixed paraffin embedded tissues and liquid based cytology specimens from patients with known different grades of cervical dysplasia and invasive cancer, were examined by this method and the result were verified by WHO HPV LabNet proficiency program in 2013. Results: HPV was detected in 105 (93.7%) out of 112 samples. The dominant types were HPV 18 (61.6%) and HPV 16 (42.9%). Among the mixed genotypes, HPV 16 and 18 in combination were seen in 12.4% of specimens. Conclusions: According to acceptable performance, easy access to primers, probes and other consumables, affordable cost per test, this method can be used as a diagnostic assay in molecular laboratories and for further planning of cervical carcinoma prevention programs.

Keywords: HPV - cervical cancer - real time PCR - Iran

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Introduction

Human Papillomaviruses (HPVs) that comprise a large genus belonging to the Papillomaviridae, is a non enveloped virus with a circular double stranded DNA (ds DNA) genome approximately 7.9 kb in size. The genome encodes 6 early genes (E1, E2, E4, E5, E6, and E7) and 2 late genes (L1 and L2). L1 is the major of viral capsid that targeted at a conserved region is used for phylogenetic classification and molecular diagnosis. It has more than 100 subtypes that are capable of infecting the epithelial cells. Depending on the oncogenic potential, its genotypes are categorized into High Risk (HR) and Low Risk (LR) groups. HPVs can cause a variety of clinical manifestations such as warts, anogenital malignancies, cervical cancer and etc. Cervical cancer is the third most common cancer in women worldwide (Laudadido, 2013; Shayanfar et al., 2013). Globally, cervical cancer accounts for more than 529,000 new cancer cases and more than 275,000 cancer deaths in women annually (Jemal et al., 2011), with more than 80% of these cases occurring in developing countries (Li et al., 2013). Different methods are available for the HPVs screening, detection and genotyping such as cytologic evaluations (e.g. Pap Tests), PCR Hybridizations, PCR and Real Time PCR. HPV vaccination with cervical cancer screening (via both cytology and HPV molecular assays) can be effective in preventing cervical cancer and decrease its mortality (Jemal et al., 2011; de Freitas et al., 2012; Adams and Carnright, 2013). Based on the data reported by WHO, the crude incidence and mortality rates of HPV related cervical cancer until 2010 in Iran are about 1.8 and 0.8 per 100,000 per year, respectively. HPVs 16(HR), 18(HR), 31(HR), 6(LR), 11(LR), 33(HR) are the most common HPV genotypes in Iranian women with and without cervical dysplasia. Comparing the alternate national cancer registry reports in Iran shows a slow increase in rate of cervical cancer in country and health authorities have a great concern to conduct country wide programs for estimating the latest prevalence of HPV infection in

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general population (WHO, 2010). In parallel, there is an increasing request for HPV detection and genotyping by clinicians that has led to considerable rise in new introduced commercial and in-house molecular assays for HPV diagnosis. Regarding the above mentioned explanation access to accurate diagnostic methods with a reasonable cost is a necessity in Iran as developing country. This survey was conducted to design and develop a HPV genotyping assay by In-House Multiplex Real Time PCR in Iran. The quality and performance of developed assay is assessed based on an evaluation protocol provided in national reference laboratory.

Materials and Methods

Patients' Specimens

From March 2012 to April 2013, a total of 112 samples (48 ThinPrep and 64 Archival Formalin-Fixed Paraffin-Embedded Tissue (FFPE) specimens) from women with known cervical precancerous and cancer referred to Tehran Women Central hospital, Imam Khomeini Hospital and Imam Hossein Hospital in Tehran, were collected. All the diagnoses were confirmed in histopathology examinations. A questionnaire was designed and filled out for each patient and necessary clinical data was recorded. In order to meet ethical considerations, each patient was informed about the objectives of study and a signed a consent form before entering the study. After cytologic examination of specimens and confirming the histopathologic diagnoses, collected Liquid Based Cytology (LBC) and FFPE blocks were transported to Molecular Biology department in Health Reference Laboratory of Ministry of Health and Medical Education, Iran. The LBCs were stored at -20˚ C and FFPE tissue blocks in a proper storage area until experimental phase.

HPVs standard plasmids

HPV DNA Plasmids for fifteen genotypes: 6, 11, 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68 containing partial length L1 sequence (20 $e10^4$ copy/ μl) were provided and synthesized separately by TIB MOLBIOL® ,Germany, to be used for preparing standard samples necessary for evaluation studies.

Extraction of HPV DNA

Viral DNA from Thin Prep preservatives and 20-30 mg FFPE specimens was extracted using the QIAamp DNA Mini kit and QIAamp DNA FFPE Tissue kit (Qiagen, Germany), according to the manufacturer's instructions.

Design of primers and probes

We developed and designed 15 set of specific primers and TaqMan probes on the partial region of L1 HPV genotypes sequences by AlleleID 7.5 software. All of sequences comprehensive aligned by Mega5 software and blasted in NCBI database (www.ncbi.nlm.nih.gov/nucleotide). Analysis of extensive bioinformatics was done on primers and probes designed by Oligo7, Primer Express, Generunner, CLC Main Workbench 5 software's and internet online sites. So, β globin endogenous gene as internal control primers and probe were considered in Multiplex Real Time PCR for ruling out possible PCR inhibition and quality of extracted DNA.

In-house multiplex real time PCR for HPV genotyping

We used 6-FAM, Yakima Yellow, Cy5, Texas Red as fluorescent reporter dye at the 5'end and BHQ-1, BHQ-2 as quencher dye at the 3' end of probes sequences. For the multiplexing, four reaction mixtures were designed: Mix1 (HPV16, 18, 31, 45), Mix2 (HPV 6, 11, 52, 68), Mix3 (β globin, HPV 56,58,59) and Mix4 (HPV 33, 35, 39, 51). The Multiplex Real Time PCR amplifications were carried out in a 25μl reaction volume containing 12.5μl 2X Premix ExTaq (Probe qPCR)(TaKaRa, Japan), 10pmol/ μl each primers and probes (TIB MOLBIOL, Germany), and 5 μl DNA extracted. These reaction mixtures were amplified in a Rotor Gene 6000 (Corbett Life Science), by using the following conditions: 95ºC; 90s for initial enzyme activation, for 50 cycles 95ºC; 5s, and 60ºC; 60s (with fluorescence detection in Green, Yellow, Red, Orange Channels). Furthermore, all of the four mixtures were tested against known positive specimens of HBV, HCV, HSV1 and 2, VZV, BKV, CMV, Streptococcus pneumonia, Klebsiella pneumonia and Ecoli to evaluate the specificity of primers and probes. Four-fold serial dilutions of HPV plasmids were prepared from 1 to 1000 copy/ μl as separated and mixed samples.

Results

Analytical sensitivity, specificity and accuracy

The quality and performance of developed assay was evaluated focusing on analytical sensitivity (detection limit), specificity and accuracy.

Analytical specificity: the extracted genome of HBV, HCV, HSV1 and 2, VZV, BKV, CMV, Streptococcus pneumonia, Klebsiella pneumonia and Ecoli, 10 HPV negative and 20 HPV positive samples (both as single specimens and mixed cocktails with different copy numbers of each genotype per micro liter from 1 to 1000) were selected. There was no non specific amplification signal in all samples.

Analytical sensitivity: 20 HPV positive samples (15 high risks and low risks HPV genotypes) were tested and only one sample (genotype 52) was falsely negative.

Detection limit: serial dilutions of HPV plasmids were prepared (from 1 to 1000 copy /μl) and the assay could detect 5 copy/μl of each HPV genotypes in single and mixed cocktails. Data for these evaluations are not shown.

Standard curves obtained from HPV plasmids serial dilutions in HPV Mix 1 to HPV Mix 4 showing dynamic ranges from 10 Copies/μl to 1000 Copies/μl are indicated in Figure 1.

Human papillomaviruses genotyping

HPV genotyping was assessed by testing 112 samples (48 Liquid Based Cytology and 64 Archival FFPE) collected from patients with confirmed diagnoses of cervical dysplasia and cancer. 105 out of 112 specimens (93.7%) were positive for different Human Papillomavirus genotypes and 7 samples (6.3%) were negative. Detected HPV genotypes are shown in Figure 2. Among detected

genotypes and 7 samples (6.3 %) were negative. Detected HPV genotypes are shown in Figure 2. Among detected HPV genotypes, HPV 18 (69/61.6%) and HPV 16 (48/42.9%) were dominant ones. Demographic data of selected patients consist of Age, Marital status, Educational level and history of abortion in previous pregnancies are shown in Table 1 and Figure 3. Furthermore, distribution of HPV genotypes in comparison to histopathology diagnoses are mentioned in Table 2. From the 105 HPV positive specimens, 21 cases (20%) have single HPV infection (HPV 18; 12/105(11.4%), HPV 16; 3/105 (2.9%), HPV 31; 2/105 (1.9%) and each of the HPV6, 58, 59, 68; 1/105 (0.96%)), respectively. Among samples with the HPV mixed genotypes, HPV 16, 18 (13/105 (12.4%) were the prevalent combination genotypes.

We also participated in 2013 WHO HPV LabNet

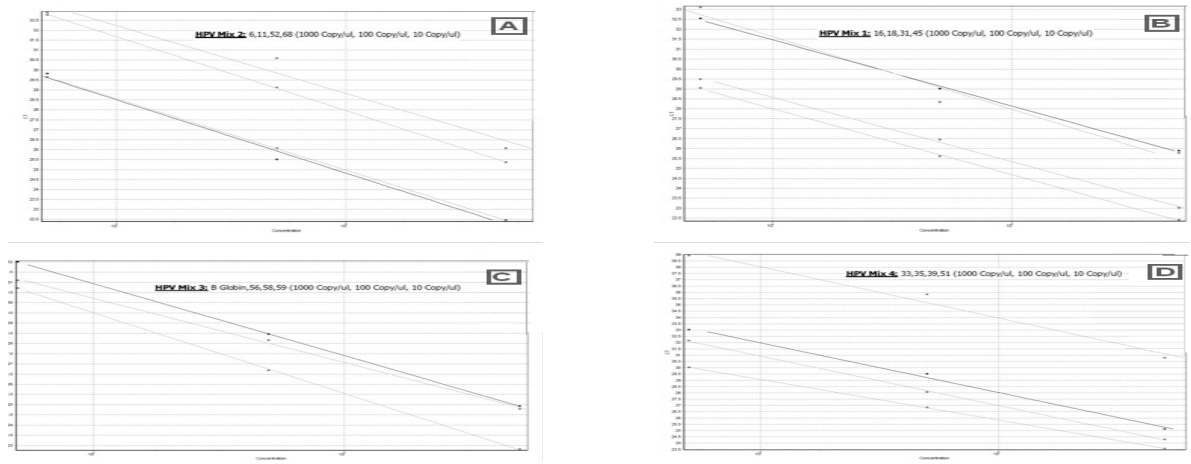

Figure 1. A) Standard Curves of HPV Plasmids Mix 1 (HPV 16, 18, 31, 45) as Positive Controls by Serial Dilutions from 10 copies/μl to 1000 copies/μl in Order to Analysis of the Assay LOD and Analysis of Linearity Range. B) Standard Curves of HPV plasmids Mix 2 (HPV 6, 11, 52, 68) as positive controls by serial dilutions from 10 copies/μl to 1000 copies/μl in order to analysis of the assay LOD and analysis of linearity range. **C)** Standard Curves of HPV plasmids Mix 3 (β Globin, HPV 56, 58, 59) as positive controls by serial dilutions from 10 copies/μl to 1000 copies/μl in order to analysis of the assay LOD and analysis of linearity range. **D)** Standard Curves of HPV plasmids Mix 4 (HPV 33, 35, 39, 51) as positive controls by serial dilutions from 10 copies/μl to 1000 copies/μl in order to analysis of the assay LOD and analysis of linearity range

Table 1. Information of Patients Demographic Variables in Present Survey

Age (n=112)			Marital Status (n=46)			Education Condition (n=45)				Abortion history (n=46)		
Min	Max	Average	Married	Widow	Single	Illiterate	Under Diploma	Diploma	Graduated	No Abortion	Once Abortion	Twice Abortion
23	90	42.73±11.36	38 (82.6%)	8 (17.4%)	0	12 (26.7 %)	18 (40%)	8 (17.8 %)	7 (15.6 %)	31 (67.4 %)	13 (28.3 %)	2 (4.4 %)

Table 2. Analysis of Relationship between the HPV Genotypes and Cytopathology Reports

Histopathology Report	No. of Sample (%)	No. of HPV Positive (%)	No. of HPV Negative (%)	No. of Most HPV Genotypes (%)	Other HPV Genotypes
Cytology Normal	7 (6.2%)	6 (85.8%)	1 (14.3%)	HPV 16, 18; 5 (71.5%), HPV 6; 1 (14.3%)	HPV 6, 59, 31
Genital Warts & Condyloma Acuminata	2 (1.8%)	2 (100%)	0	HPV 18; 2 (100%)	HPV 6, 56, 59
CIN I/ ASUS/LSIL	33 (29.5%)	29 (87.9%)	4 (12.1%)	HPV 16, 18; 23 (69.7%)	HPV 31, 11, 35, 58, 59, 56, 52, 68, 33, 45, 51
CIN II/ LSIL	24 (21.5%)	22 (92%)	2 (8.3%)	HPV 16,18; 16 (66.7%)	HPV 35, 68, 31, 39, 6, 52, 11, 33, 58
CIN III,IV/SCC/HSIL	46 (41 .1%)	46 (100%)	0	HPV 16, 18; 41 (89.2%)	HPV 31, 56, 58, 45, 52, 68, 35, 6, 39, 11, 59, 51
Total	112	105 (94%)	7 (6.3%)	87 (77.7%)	

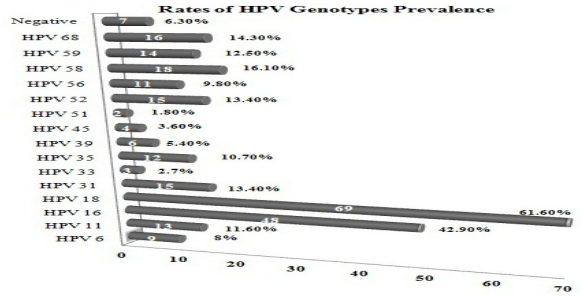

Figure 2. Rates of HPV Genotypes in Women with Cervical Cancer and Cervical Intraepithelial Neoplasia

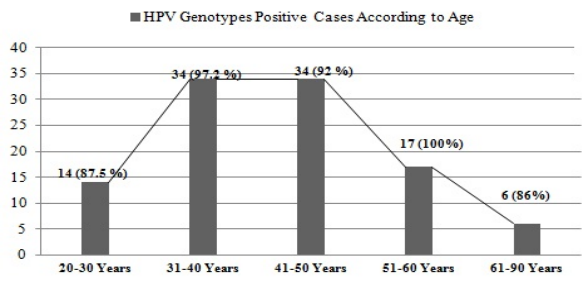

Figure 3. Frequency Prevalence of HPV Genotypes in Different Age Groups

proficiency study with our developed assay. The panel of specimens in this program consists of different single and mixed cocktails of HPV plasmids containing HPV genotypes 6, 11, 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66 and 68b. To be considered as proficient in this program, the assay shall detect 50 International Units (IU)/5 μl of HPV 16 and HPV 18 DNA, and 500 genome equivalents (GE)/5 μl of the other HPV types included in the panel both in samples contains single and multiple plasmids and no more than one false positive result is accepted. Accordingly, our data set using the In-House Multiplex Real Time PCR genotyping assay is recognized as proficient for detection of HPV 6, 11, 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66 and 68b with no false positive result.

Discussion

To identify HPV genotypes in positive samples detected in cervical screening (i.e., Pap tests), molecular based methods are the most common tests and they can differentiate various high and low risk genotypes. Real Time PCR is one of the revolutionized molecular diagnostic methods that is used as a routine diagnostic assay for rapid detection and genotyping of clinical samples.

In-House Multiplex Real Time PCR designed in present study which is composed of 15 pairs specific primers and 15 TaqMan probes and 1 pairs primers and 1 probe for β globin as internal control can detect 5 copy/5 μl of HPV 6,11(Low Risks), 16, 18, 31, 45, 51, 52, 56, 58, 59, 68 (High Risks) in quadruplex reactions. On the other hand, the validation of method was also confirmed according to results of 2013 WHO proficiency testing program. Our developed assay has high sensitivity (95%), specificity (100%), a wide dynamic linear range (detection limit is 5 Copy/μl) and a high degree of repeatability (at least three times). No evidence of cross-reactivity with other various HPV genotypes and microorganisms was observed. According to estimated price of each test (~$20), it seems that the assay developed in our research is an affordable method in comparison to other commercial kits in Iran. Rapid, reliable results and the capability of simultaneous detection of 15 common HPV genotypes are other advantages of developed assay considering other available commercial technologies. Determination of the different human Papillomavirus genotypes prevalence in women with cervical precancerous and cancer can help to plan and implement national immunization program and also treatment protocols. In our study, HPV genotypes were detected in 105 from 112 samples (93.7 %) and HPV18 (69/61.6%), HPV16 (48/42.9%), HPV58 (18/16.1%), HPV68 (16/14.3%), HPV31 (15/13.4%), HPV 52 (15/13.4%), HPV 59 (14/12.5%), HPV11 (13/11.6%), HPV35 (12/10.7%), HPV56 (11/9.8%), HPV6(9/8%), HPV39 (6/5.4%), HPV45 (4/3.6%), HPV 33 (3/2.7%) and HPV 51 (2/1.8%) were the most common genotypes, respectively. HPV 16, 18 (12.4%) were the most common genotypes in mixed infection. This finding is almost similar to studies that have been performed during the last decade in different regions of Iran and other countries such as Brazil, Turkey, Canada, Kuwait, Germany, New Zealand, India, Saudi Arabia, Malaysia, China and Thailand (Hamkar et al., 2002; Zandi et al., 2010; Allameh et al., 2011; Shahsiah et al., 2011; Eghbali et al., 2012; Kim et al., 2012; Yuce et al., 2012; Afshar et al., 2013; Akcali et al., 2013; Al-Awadhi et al., 2013; Das et al., 2013; Hamzi Abdul Raub et al., 2013; Natphopsuk et al., 2013; Petry et al., 2013; Rocha et al., 2013; Severini et al., 2013; Simonella et al., 2013; Turki et al., 2013; Wang et al., 2013). Considerable prevalence of HPV genotypes 58, 68, 59, 31 and 11 (more than 10%), suggest that epidemiological evolution of other HPV genotypes in cervical cancer and other cancers associated with papillomaviruses should be considered. As mentioned above comparing the results of these studies represent that HPV genotypes especially HPV 16 ,18 are the most common genotypes in full spectrum of cervical dysplasia's from Cervical Intraepithelial Neoplasia I to invasive squamous cell carcinoma in different age groups especially women with 30-50 years (Clifford et al., 2005; Lee et al., 2011; Kasamatsu et al., 2012; Manjari and Sweta, 2012; Panjgoro et al., 2013; Othman and Othman, 2014). It is proposed that according to numerous studies in worldwide, screening assays such as pap tests as a single test in screening strategies can't be helpful (Jones et al., 2011; Gichki et al., 2012; Saxena et al., 2012; Heidegger et al., 2013; Junyangdikul et al., 2013).

In Conclusions, It seems that molecular methods e.g. PCR and Real Time PCR as high throughput, reliable methods with capability of fast detection and genotyping must be developed and used in combination of pap smear in order to proper screening and diagnosis in women with cervical epithelial lesions. Certainly, reliable results obtained from valid diagnostic methods have an important role in selecting best national policies particularly for immunization and prophylactic programs.

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