Original Research Article

A study on Rapid real time PCR and Cytological examination to detect the high risk human papillomavirus types 16 and 18

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Abstract

Background: Persistent infection with HPV is the necessary cause for development of cervical cancer. HPV 16 and 18 are the most prevalent genotypes worldwide. Based on WHO factsheets, India is noted to have high burden of cervical cancer. Standardized cervical cancer screening guidelines and cervical screening programmes worldwide follow PAP smear cytology as accepted screening tool. But cervical cytology lacks in sensitivity compared to HPV DNA testing which is gaining importance as a primary screening tool. However, performing PCR on a large scale is expensive. **Aim & Objective:** The study will be performed by cytology and polymerase chain reaction (PCR) to detect the HPV genotypes 16 and 18 in our region. **Results:** Of the 150 cases, cytology was positive in 2 cases and PCR was negative in all cases. The pap test positive cases represented CIN 1 / LSIL (low grade squamous intraepithelial lesion). These 2 cytology positive samples probably represent false-positive results or may represent HPV infection with genotypes other than 16 and 18. Low sensitivity of PCR in our study could be due to low prevalence in our region, small study population and short study period. **Conclusion:** Real time PCR is a rapid, efficient method for the detection of HPV with the separation of HPV-16 and HPV-18 on the basis of differential T_m. Preliminary results suggest it could prove useful if HPV testing is added to cervical screening programmes.

Keywords: Real time polymerase chain reaction, cervical screening, human papillomavirus types 16 and 18, cytology

Introduction

More than 40 types of human papillomavirus (HPV) infect the genital epithelium and several high risk types including HPV types 16, 18, 31, 33, and 45 are found in almost all cases of high grade cervical intraepithelial neoplasia and cervical cancer[1,2]. In Europe, the most prevalent type is HPV-16,[1] but there are several reports that HPV-18 infection can lead to the development of more clinically aggressive disease[3-5].

Laboratory diagnosis of HPV infection is dependent upon molecular techniques such as DNA hybridisation or nucleic acid amplification.

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Several polymerase chain reaction (PCR) methods have been developed to detect a broad spectrum of mucosotropic HPV types using either degenerate or consensus primers[6-9]. A second generation commercial hybridisation assay, Hybrid Capture™ (HCA II), is also available for the detection of HPV DNA in cervical swab samples,[10] and has been used widely in epidemiological studies[11-13]. However, both consensus PCR and HCA II have important limitations. They are costly and labour intensive and, without additional procedures, neither technique can differentiate between individual types or detect infection with more than one type. Furthermore, HPV infections are often transient, frequently cleared by immunocompetent people, and require interaction with cofactors for the progression of disease. Thus, the development of highly sensitive detection tests for high risk HPV raises problems of clinical interpretation.

The potential use of HPV testing in cervical screening programmes is dependent on a rapid sensitive test that can distinguish high risk HPV types present in clinical samples. In most conventional PCR assays, amplification is performed by automated temperature cycling, but product analysis requires a subsequent manual operation.

Materials and methods

Study design

A prospective cross-sectional study

Study group
Women of the reproductive age group aged between 18 to 40 yrs attending the obstetrics and gynaecological out patient department at Sri Venkateswaraa Medical College Hospital and Research Centre, Ariyur, Puducherry were included in the study.

Study group volume/population
This study involved 150 outpatients
Study duration
The study was performed over a period of 1 year, between November 2013 to November 2014.

Patient Selection
Inclusion criteria
1) Women aged between 18 to 40 years attending the Obstetrics and gynaecological out patient department
2) Women who provided informed consent

Exclusion criteria
1) Women younger than 18 years and older than 40 years of age
2) Women who presented during their menstrual period
3) Women with history of cervical carcinoma
4) Women on treatment or during follow up period for cervical carcinoma

Institutional Ethical Committee and scientific research committee clearance was obtained before the commencement of the study. An informed consent was obtained and detailed questionnaire were given to the subjects included in the study.

Sample collection, storage & processing
HPV sample collection kit for HPV DNA PCR containing spatula, cyto-brush & tube containing 100% ethanol were used.
1) HPV DNA extraction kit

Sample collection - Overview
The sample was collected in Obstetrics and Gynaecology outpatient department in Sri Venkateswaraa Medical College Hospital and Research Centre. Sample collection was done on the basis of inclusion and exclusion criteria. The importance and purpose of this study was explained to the patient and informed consent was obtained from the patient before the procedure. The clinical details regarding this topic were collected. PAP smears was done free of cost for the patient and processed in pathology department.
HPV DNA extraction kits for types 16 & 18 was purchased and used as per the manufacturer's guidelines. Polymerase chain reaction was done as per standard protocols and the serotypes were identified. Required statistical analysis of the observations was done by SPSS package (Statistical Package for Social Sciences version-17). DNA extraction was done for all 150 samples irrespective of the PAP smear results. Extracted DNA was amplified using E6 and E7 region primers.

DNA Extraction
Principle: Cells are lysed during a short incubation with chaotropic salt, which inactivates all nucleases. Cellular nucleic acids bind immediately to special glass fibres pre-packed in the purification filter tube. Bound nucleic acids are purified in a series of rapid wash and spin steps to remove containing cellular components. A special inhibitor removal buffer has been included which removes inhibitors from the preparation. Finally low salt elution releases the nucleic acids from the glass fibres. This simple method eliminates the need for organic solvent extractions and nucleic acid precipitation, allowing for rapid purification of many samples simultaneously.

Kit components: [stored at -20°C]
1) Carrier RNA
2) Proteinase K
3) Lysis buffer
4) Elution buffer
5) Spin columns with collection tube

Procedure
1) DNA extraction was performed under aseptic conditions. Micro centrifuge tubes were labelled for each sample.
2) Samples were thawed and vortexed thoroughly.
3) Pellets were obtained after centrifuging at 8000 rpm for 5 minutes. Supernatant was discarded.
If the sample volume is less than 200µl, the sample volume was adjusted with PBS and vortexed to mix.
5) 200µl of Lysis buffer was added to a nuclease free 1.5ml centrifuge tube.
6) 5µl of carrier RNA was then added.
7) Then 200µl of the sample was added and mixed by inverting several times.
8) Following that 20µl of proteinase K and 5µl of internal control template were added and mixed immediately by brief vortex.
9) Then it was incubated at 56°C for 15 minutes.
10) Then 300µl of 100% ethanol was added and mixed well by vortex for 10 seconds. Spinning down for few seconds to bring down drops to bottom of the tube was performed.
11) Entire sample was pipette into the pure fast spin column and it was centrifuged at 12000rpm for 1 minute. Flow-through was discarded and the column was placed back into the same collection tube.
12) Then 500µl of 70% ethanol was added to the pure fast spin column which was centrifuged at 12000rpm for 1 minute and the flow-through was discarded.
13) 70% ethanol wash was repeated once.
14) Centrifugation of the empty spin column attached with collection tube at 12000rpm for an additional 2 minutes was done to avoid residual ethanol. Collection tube was discarded.
15) The pure fast spin column was transferred into a fresh 1.5ml micro-centrifuge tube.
16) 50µl of elution buffer was added to the centre of pure fast spin column membrane and incubated for 2 minutes at room temperature.
Then it was centrifuged at 12000rpm for 1 minute and the pure fast spin column was discarded.
18) Micro centrifuge tube now contains the eluted nucleic acid which can be directly used for PCR or stored at -80°C for later analysis.

Polymerase Chain Reaction (PCR)
Principle: Polymerase chain reaction amplifies a target region specific for each gene in a DNA strand. E6 and E7 region primers were used to identify HPV DNA 16 and 18 types.
Components of Detection mix
1) RedDye PCR Master Mix
2) HPV 16/18/IC Primer mix
All reagents are thawed completely and centrifuged briefly before each use.

Preparation of Detection Mix
Before starting the procedure, a workbook was maintained which indicates the sample positioning in gel loading tray. PCR tubes were marked accordingly.
1) Red Dye PCR Master Mix - 10µl
2) HPV 16/18/IC Primer Mix - 15µl
3) Purified DNA sample - 5µl
Total reaction volume - 30µl
Negative control - Nuclease free water
Positive control - 5µl of Positive control provided in the kit

Steps to do Polymerase Chain Reaction
1) All samples were taken out from deep freezer, brought to the room temperature and centrifuged briefly.
2) PCR tubes were numbered accordingly.
3) Master mix was distributed in all the PCR tubes which contains 25µl each.

4) 5µl of sample DNA was added in each tube and so the total reaction volume is 30µl.
5) Briefly centrifugation of the tubes was done before placing in thermocycler.
6) PCR tubes were placed in the thermo cycler.
7) Amplification Protocol:

- Initial Denaturation - 95°c for 9 minutes
- Denaturation - 95°C for 30 seconds
- Annealing - 58°C for 30 seconds
- Extension - 72°C for 30 seconds
- Final extension - 72°C for 5 minutes

8) Totally 40 cycles was run.
9) 2X Tris buffer was prepared for 300ml, out of which 294ml was distilled water and 6ml was TE buffer. This solution was poured in the gel electrophoresis apparatus.
10) 2.5% agarose gel was prepared for 100ml. This was prepared by adding 2 grams of
agarose powder in 100ml of 2X Tris buffer. It was then boiled until it was completely dissolved which was mixed well while heating.
11) After it cooled, Ethidium bromide 10µl was added and poured in the comb tray.
12) When it is set, tray was placed in the gel electrophoresis apparatus. Then the comb was removed.
The final PCR product was loaded into gel. Positive 15µl and Negative control 15µl was also loaded in gel.
14) 10µl of DNA ladder which was also loaded.
15) Electrophoresis was carried out at 50 watts for one hour and 30 minutes.
16) Gel documentation was done by gel document software under UV light.

Gel Electrophoresis Interpretation:

Expected PCR product size:
Internal control: 400bp
HPV 16 : 190bp
HPV 18 : 290bp
The sample was considered as positive if a band was seen at the level of positive control band at 190bp for HPV16 and 290bp for HPV18. Other samples were considered as negative.

Results

Cytology and PCR DNA analysis

Of the 150 women who underwent PAP smear, the cytology was positive in 2 women (1.33%) and it was negative in the remaining subjects (98.66%). Genotyping for HPV 16 & 18 was performed in all the 150 cervical samples inclusive of the two samples in which the cytology was positive. But HPV DNA test was negative for all the 150 cervical samples.
The histology of both the patients showed hypertrophied cervix comprised of squamous epithelium showing parakeratosis and koilocytic changes and the deeper tissue showed fibrocollagenous tissue and stromal cells. The features were associated with cervical hyperplasia and koilocytic (WHO description) changes which is consistent with CIN I and LSIL (low grade squamous intraepithelial lesion) of Bethesda system.
The Chi-square statistic test to see the significance of row/column association between positivity in PAP smear and PCR, showed a one-sided p-value of 0.239 and the association was inferred to be not statistically significant.

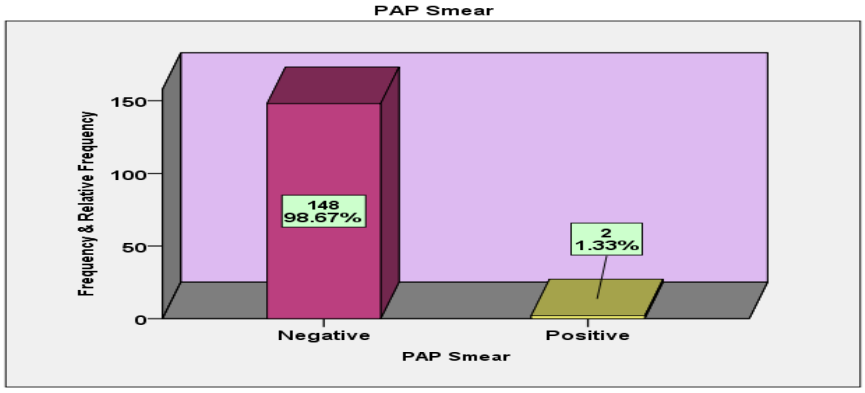

Fig 1: Results of Cytological examination

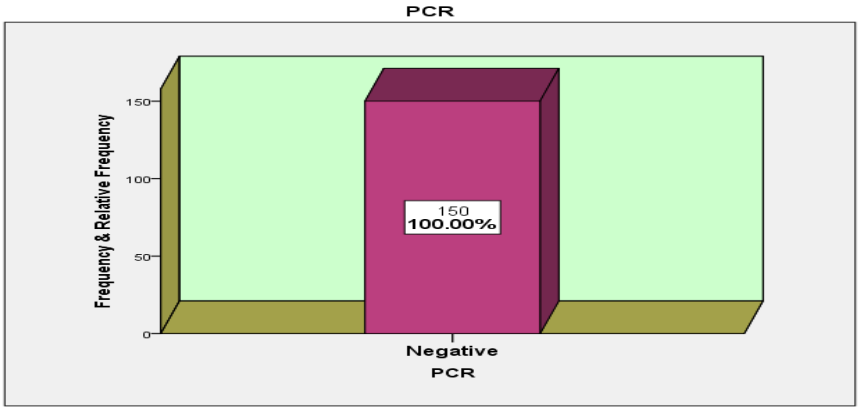

Fig 2:Results of Polymerase Chain Reaction for HPV 16 & 18

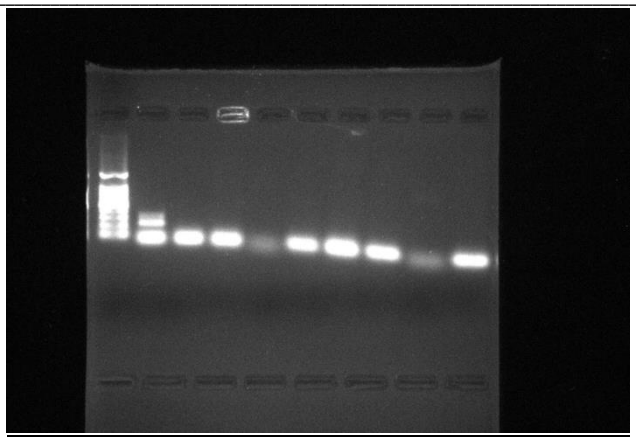

Fig 3: Documentation under UV light following amplification

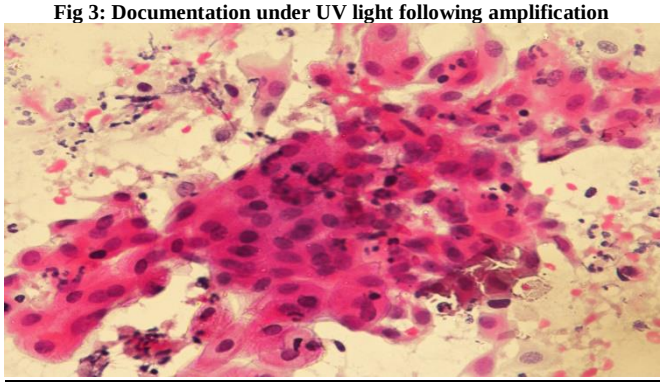

Fig 4: Microscopic image of the cervical intraepithelial neoplasia(CIN) 1 lesion

Discussion

Cytological evaluation of PAP smear was done for all cervical samples. Out of 150 cervical samples, two samples showed features of cervical hyperplasia and koilocytic changes. These changes are consistent with CIN 1 and LSIL (low grade squamous intraepithelial lesion) of Bethesda system categories. HPV DNA testing for HPV genotypes 16 and 18 was performed in the two cytology positive samples along with the other negative samples, in total 150 cervical samples. No HPV was identified in all the samples from the study group (n=150). The two samples which showed cytological abnormalities may represent false-positive results or might be HPV infection with genotypes other than 16 and 18.

Studies have shown that about 90% of women with CIN 1 changes show spontaneous resolution. It is known fact that most HPV infections produce only temporary changes in cervical cells and inordinate cervical screening could detect HPV infections or cervical cell changes that would never cause cancer[14, 15, 16]. HPV infections with normal cervical cytology and those with CIN 1 changes are considered the same for clinical purposes [17, 18].

There are not many studies in literature addressing the false-positive cytological results. The published false positivity rate ranged between 5.5% to 7% [19, 20, 21].Real time PCR technology has great potential for clinical and non-clinical development. Nevertheless, it is still a new technique and some technical problems have been reported, including the presence of primer dimer formation.

HPV DNA testing is recognized as an adjunct to cervical cytology for screening and diagnosis of cervical cancer. Although many PCR based methods for HPV detection and genotyping are available, its increased cost makes its routine use difficult. In India, a developing country with high prevalence of cervical cancer a cost effective test for HPV detection and genotyping is essential.

In developed countries, there is standard cervical cancer screening guidelines in practice. Under the National Health Service (NHS) cervical screening programme in United Kingdom women from ages 25 to 64 years are invited for cervical screening. Women aged between 25 to 49 years are screened every 3 years. After that, women are screened every 5 years until the age of 64[22].

Regular population based screening using Pap smear cytology is the internationally accepted screening method for cervical cancer.

In United States of America, according to the screening guidelines issued in 2012 by United States Preventive Services Task Force and jointly by the American Cancer Society, the American Society for Colposcopy and Cervical Pathology, and the American Society for Clinical Pathology the following recommendations were suggested [23]. Women aged between 21 to 29 years should be screened every 3 years with a Pap test, women aged between 30 to 65 years should be screened every 3 years with a Pap test alone or should be screened every 5 years with Pap and HPV co-testing.

In women less than 30 years of age, cervical cancer screening with HPV testing alone or in combination with cytology was not recommended.

It was also suggested that women with human immunodeficiency virus (HIV) infection, immunosuppression, exposure to diethylstilbestrol before birth and those treated for precancerous cervical lesion or cervical cancer might require more frequent screening and screening might be continued beyond 65 years of age.

At present there is no organized screening programme based on the Pap smear available in India. It's high time that a standardized national screening programme is implemented in India.

The limitations of our study include small study population which was primarily attributed to the short study duration. Restricting ourselves with screening of HPV genotypes 16 & 18 could have resulted in exclusion of other genotypes which might be prevalent in our region.

Conclusion

Based on WHO factsheets, India is noted to have high burden of cervical cancer. Standardized cervical cancer screening guidelines and cervical screening programmes worldwide follow pap smear cytology

as accepted screening tool. But cervical cytology lacks in sensitivity compared to HPV DNA testing which is gaining importance as a primary screening tool. However, performing PCR on a large scale is expensive. In India till date there is no standardized national level cervical cancer screening programme. In developing countries like India, taking into account the financial constrain it is impractical to use HPV DNA testing as a screening method. But there is need for a cost effective method for HPV testing and genotyping. A proposed approach could be to perform the cervical screening by pap smear test followed by genotyping as confirmatory tool. More importantly, health education to increase the awareness of the cervical cancer screening will act as a principle drive towards implementing the screening programme.

We screened all the study group population with cervical cytology and PCR methods for detecting the prevalence of HPV genotypes 16 and 18. The prevalence rate based on PCR was nil and no comment on the association of demographics with the HPV prevalence be made.

However further studies with large sample size is needed to determine the prevalence of high-risk HPV genotypes 16 and 18 in our region and to ascertain the sensitivity of HPV DNA testing as a primary screening tool in cervical cancer screening.

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