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HUMAN PAPILLOMA VIRUS GENOTYPING IN A GROUP OF IRAQI WOMEN BY USING REPRODUCIBLE DNA EXTRACTION FROM FORMALIN FIXED PARAFFIN EMBEDDED SECTIONS

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ABSTRACT

HPV detection and genotyping from formalin-fixed, paraffin-embedded (FFPE) tissues by using molecular analysis has become of great interest in recent years. The subjects included in this part of study were represented as formalin - fixed paraffin - embedded (FFPE) tissue blocks. The collection of tissue blocks of this study was carried out during the period from September 2013 to March 2014. A total of (100) samples were collected from the archives of histopathology department / Teaching Laboratories / Baghdad Medical City with different age ranged from (<31 years to >60 years old). Using a microtome serial tissue sections were cut into (3-10 µm) thickness, from each tissue block. Total DNA was extracted from the replicate tubes by two different methods, based on the DNeasy blood and tissue kit (Qiagen, Valencia, CA). The extraction done with two methods the first by paraffin removal by xylene and the other was processed by using a high-heat treatment. The prevalence of infection was 70% in FFPE samples, most of them are mixed infections (84.3%) and (15.7%) were single infections by types 16 (97.1%) and 18(72.9%) respectively. Of the total number 70/100 (70%) samples were positive in genotyping test. Recent finding showed that HPV 16 are responsible for (97.1) % of cases followed by HPV18 with (72.9%), HPV 31(45.7%), HPV 45 (11.4%), HPV 59 (8.6%), HPV 51 (4.3%), HPV 35 (2.9%), HPV 36 (1.6%) respectively. The result of HPV typing showed that most of them were mixed infections by more than one type with majority to type 16 as it

founded in 68 (97%) of samples . Of 70 samples that were PCR positive followed by type 18 (51) (72.9%).

KEYWORDS: Human Papilloma Virus, Real Time PCR typing, DNA extraction.

INTRODUCTION

HPV detection and genotyping from formalin-fixed, paraffin-embedded (FFPE) tissues by using molecular analysis has become of great interest in recent years. Although the DNA in archived tissues is generally preserved over long periods of time, fragmentation and DNA-protein cross-linking by formaldehyde exposure, as well as the presence of paraffin, can have significant negative effects on DNA yield and amplification efficiency in subsequent PCR applications.[1] Genital HPV types are classified by risk level. The majority of those infections have no symptoms and clears away without clinical treatment. Low-risk types of infections (e.g., types 6 and 11) can cause nonmalignant or low-grade cervical cell changes and genital warts. Most commonly, HPV 16 and HPV 18 types are isolated from cervical cancer worldwide, but there are several oncogenic types of considerable importance, i.e. types 33, 45, 31, 58, 52, 35, 59 and 51.[2] As new or refined and more sensitive analytic methods become available, FFPE tissue collections provide vast sample repositories for research projects and retrospective epidemiology studies. Although the DNA in archived tissues is generally preserved over long periods of time, fragmentation and DNA-protein cross-linking by formaldehyde exposure, as well as the presence of paraffin, can have significant negative effects on DNA yield and amplification efficiency in subsequent PCR applications.[1] In Iraq there is an evidence of increasing risk of infection with Human Papilloma Virus and therefore the aim of this study came to to apply the most effective way of typing the high risk types in order to determine the most appropriate types of vaccine that may be needed.

MATERIAL AND METHODS

The subjects included in this part of study were represented as formalin - fixed paraffin - embedded (FFPE) tissue blocks. The collection of tissue blocks of this study was carried out during the period from September 2013 to March 2014. A total of (100) samples were collected from the archives of histopathology department / Teaching Laboratories / Baghdad Medical City with different age ranged from (<31 years to >60 years old). Using a microtome serial tissue sections were cut into (3-10 µm) thickness, from each tissue block.

The eppendorf tubes sections used for DNA extraction for molecular analysis, each tube had (3-5) sections with the thickness of 5µm according to the kit of Qiagen. The disposable knife was changed for each sample to prevent carry-over DNA contaminations from one tissue sample to another.

DNA Extraction

DNA extraction from paraffin embedded blocks (Moodified Method)

The DNA extraction from FFPE samples was done with QIAamp DNA FFPE Tissue Kit (Qiagen/Germany) which used for DNA extraction.

Modification had been done according to[1] and as following:

Total DNA was extracted from the replicate tubes by two different methods, based on the DNeasy blood and tissue kit (Qiagen, Valencia, CA). One tube was processed according to the manufacturer's protocol, using xylene treatment to remove paraffin as outlined in the DNeasy user manual ("Purification of Total DNA from Animal Tissues" as found in DNeasy Blood & Tissue Handbook, Qiagen, Valencia, CA; July 2006). Briefly, the paraffin was removed by vortexing and 10 minutes incubation with 1.2 mL xylene, followed by two washes with pure (200 proof) ethanol. The air-dried pellet was then incubated with 20 µL proteinase K and 180 µL ATL lysis buffer (from the DNeasy kit) for 16 hours in a heat block at 56°C. The lysed emulsion was further purified with the DNeasy spin-column kit. DNA was finally recovered in a single elution step with 100 µL AE solution from the kit.

The other tube was processed using a high-heat treatment. Instead of xylene extraction, 180 µL ATL lysis buffer from the DNeasy kit was added directly to the paraffin sections, followed by incubation of the tightly closed tube at 120°C for 20 minutes. Within 5 minutes after the paraffin had melted, the emulsion was mixed by finger-flicking, to ensure that all tissue fractions were submerged in the buffer. After this initial incubation, tubes were briefly centrifuged to remove condensate. Proteinase K (20 µL) was added to the liquid, and the closed tubes were incubated at 65°C for 16 hours in another heat block. Subsequent steps with DNeasy and DNA elution were identical to the first protocol described above.

Quantitation of Isolated Genomic DNA

The DNA concentration was measured by using Nano Drop system to determine DNA quantitative and purity in which 1µl of the patient Genomic DNA is loaded in order to

measur the optical density (OD) at wave length 260nm and 280nm. The final concentration of DNA could calculate according the formula:

DNA concentration µg/ml = O.D 260nm × 50µg/ml × Dilution factor

Also the purity calculated according to the formula:

DNA purity = O.D. 260 nm/O.D.280 nm (range = 1.8-2)

All these equation calculated digitally by Nano drop setup.

HPV High Risk Typing

3.2.6.1. PRINCIPLE OF ASSAY

Kit HPV High Risk Typing Real-TM is based on two major processes: isolation of DNA from specimens and multiplex Real Time amplification of 4 tubes for each sample. Each tube contains primers directed against regions of three *HPV* types and b-globine gene used as Internal Control to control the presence of cellular material in the sample and avoiding false-negative results.

The steps of the RT PCR typing had been done according to the manufacture.

Data Analysis

1-The experiment may be considered valid if:

- the Negative Amplification Controls don't have any positive fluorescence signal;
- in each of the Positive Controls are determined 3 types of HPV.

2- The result of the sample is considered:

- *Invalid* in case of absence of any fluorescence signal (positive or internal)
- *Negative* if all 4 tubes contain only the positive signal in the Cy5 (Red) channel;
- *Positive* if it contains the positive signal in the Fam (Green) e/or Joe (Yellow) e/or Rox (Orange) channel regardless of the Cy5 (Red) channel result.

RESULTS

A direct comparison of the overall HPV results obtained from xylene-treated versus heat-treated extracts is noticed, The xylene method was more susceptible to inefficient results than the heat-treated method: (6%) vs. (70%) HPV was detected in more of the heat-extracted DNA samples.

Since the human Papilloma virus is an intracellular agent, there is need to monitor the presence of cellular material in the sample, in order to avoid false-negative results. HPV High Risk Screen Quant kit contains the internal control (human beta-globine gene), which allows to control the presence of cellular material in the sample.

The prevalence of infection was 70% in FFPE samples, most of them are mixed infections (84.3%) and (15.7%) were single infections by types 16 (97.1%) and 18(72.9%) respectively.

The PCR test was considered satisfactory when

(a) The Internal control gave positive result.

(b) The negative control probe gave negative result.

(c) Positive control gave positive result. The results of amplification plot are seen in figure (4-4).

Typing RT PCR of HPV in Cervical carcinoma samples

Samples that were initially RT PCR positive were retrospectively identified from the archives and 70 samples were targeted for genotyping as they were positive in the screen test of the total number 70/100 (70%) samples were positive in genotyping test. Recent finding showed that HPV 16 are responsible for (97.1) % of cases followed by HPV18 with (72.9%), HPV 31(45.7%), HPV 45 (11.4%), HPV 59 (8.6%), HPV 51 (4.3%), HPV 35 (2.9%), HPV 36 (1.6 %) respectively. Table 1. Figure 1.

Table 1: Compare the result of HPV genotyping.

Type	Positive		Negative		Total
type16	68	97.1%	2	2.9%	70
type18	51	72.9%	19	27.1%	70
type31	32	45.7%	38	54.3%	70
type35	2	2.9%	68	97.1%	70
type36	1	1.4%	69	98.6%	70
type45	8	11.4%	62	88.6%	70
type51	3	4.3%	67	95.7%	70
type59	6	8.6%	64	91.4%	70

The result of HPV typing showed that most of them were mixed infections by more than one type with majority to type 16 as it founded in 68 (97%) of samples. of 70 samples that were PCR positive followed by type 18 (51) (72.9%).

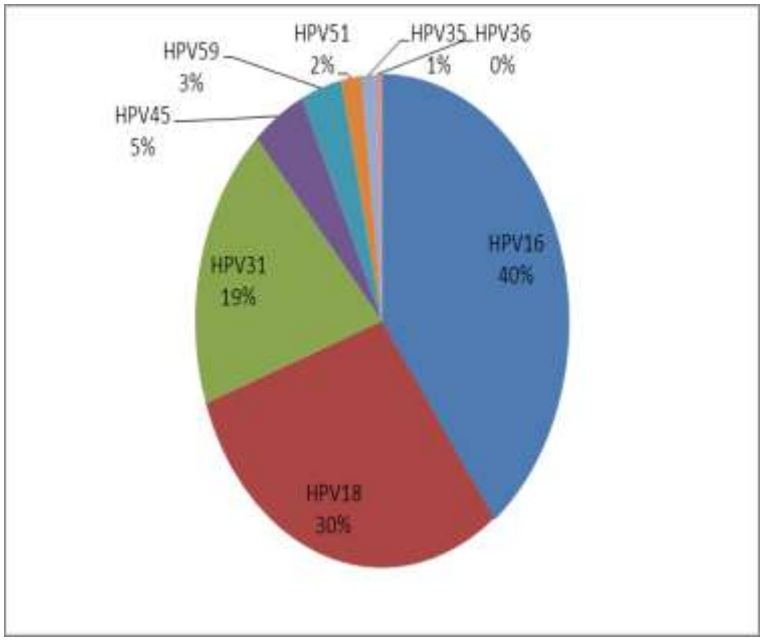

Figure 1: The different results of RT PCR genotyping.

To determine the frequency of individual HPV types among mixed infections, the type distribution among cervical specimens from the 70 Hpv positive samples had examined. HPV type distribution and the frequency of single and multiple HPV types for specimens also examined Using Multiplex Real Time PCR typing technology, results found that 84.2% of the HPV-positive patients were infected with multiple HPV.

From the total 70 HPV DNA positive samples there were 11(15.7%) with single infection, 24 (34.3%) with dual infection, 19 (27.1%) with triple infection, 15 (21.4%) with guad infection, and only one (1.4%) with penta infection. Table 2.

Table 2: Detection of mixed genotypes of HPV DNA using confirmatory RT PCR.

	Frequency	%
Single infection	11	15.7%
Dual infection	24	34.3%
Triple infection	19	27.1%
Quad infection	15	21.4%
Penta infection	1	1.4%
Total	70	100.0%

DISCUSSION

Cervical cancer consider both the fourth-most common cause of cancer and the fourth-most common cause of death from cancer in women worldwide. Nearly to 70% of cervical cancers found in developing countries.[3]

As stated by Iraqi cancer registration of period 1999-2004, cervical cancer incidence was 1.04%. And cervical carcinoma constitute 1.16% from the total malignant cases in 2005 and the Crude Incidence Rate per 100,000 population was 0.63%. While Iraq Cancer Board, Results of Iraqi Cancer Registry (1999-2004).[4] pointed that cervical cancer ranks the 8th among the most common female cancers in IRAQ accounting for 0.8% of total female malignancies.

HPV frequency in FFPE cervical cancer samples

Human Papillomaviruses (HPVs) are known to be causative agents of cervical cancer and have been found in 99.7% of women with high-grade cervical intraepithelial neoplasia (CIN) pre-cancer (such as CIN II/III and cancer).[5]

Tissues fixation also leads to the fragmentation of nucleic acids, such that (PCR) methods that amplify a smaller portion of the viral genome like RT PCR are most efficient.[6,7]

So, in the recent finding in order to generate DNA suitable for PCR a modification had been applied on the commercially available DNA isolation kit (DNeasy Blood & Tissue, Qiagen, Valencia, CA; July 2006) with a heat treatment instead of paraffin removal by xylene and tissue lysis at 56°C over night. And evaluating the resulting DNA with regards to HPV detection and typing methods.

HPV detection differed, with 6/100 (6%) positives in the xylene treated and 70/100 (70%) positive results in the heat-treated extracts. So, In the present findings demonstration of the advantage of incorporating high-heat pretreatment into the extraction protocol for FFPE tissue gave the result that tissues extracted. with heat method have yield with adequate results in the RT PCR assay, compared with the xylene method. As purity was (1-3) in xylene method and become (1.8-2) after applying heat method , this mean astep of purification had also been applied in addition to the stability of viral DNA.

Some advantages may be due to the increased heat at proteinase K treatment in the kit, which was previously found more effective for tissue lysis. The improvement might be also as acaues of reversal of formaldehyde-induced cross-linking of nucleic acids by the 120°C incubation.[6]

Close correlation between HPV infection and cervical cancer is well determined but there is a high difference between the prevalence of infection and the occurrence of real cancer[9] this

finding could be explained as that not every case infected with HPV can progress cancer it may depend on the type, also how do the viral load affect this subject.

HPV genotyping and type of mixed infection

Currently, HPV infections are increased significantly among Iraqi women without vaccineand many studies detected some HPV genotypes with different samples.[10]

Although correlation between HPV and cervical cancer had been well established but the severity of infection depend on the types included in the infection and as well-known that type 16 have the higher carcinogenicity followed by type 18 and this was very clear in this study as it represent (97.1%) 68/70 from the total types and most of them were mixed with other types mostly type 18, of them 10 single infections with type 16 and only one with type 18.

Of 24 cases dual infections,[20] cases were mixed with types 16 and 18and and represent the most frequent among the mixed in percent of 34.3%.

In contrast to Siddiqa *et al.*[11] that showed from a total of 94.81% of cervical lesions were positive for HPV. Single infections of HPV16 were detected in 24.68% of total samples and HPV18 was found in 25.97% samples 25.[11]

This finding match with study[12] which found that most common genotype was HPV-16, followed by HPV-18 and so these finding give a description about the importance of types 16 and 18 as important markers in causing cervical cancer.

In conclusion, HPV 16 and HPV 18 are responsible on most cervical cancer cases in Iraq. So the introduction of HPV vaccine is important to reduce the progression of cervical cancer and elimination of HPV infection among Iraqi women. In addition to that, the heat treated of FFPE samples are the best method for better yield of HPV DNA from FFPE samples.

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