Human Papillomavirus and Cervical Cancer: Use of Molecular Diagnostic Techniques

Hammoudah S.A.F.[1,2,3], Hannan M.A.[1,3], Al Harbi A.E. [1,3] and Al Harbi K.M. [1,3]

[1]Centre of Genetics and Inherited Diseases, Taibah University, Saudi Arabia
[2]Faculty of Medicine, Tanta University, Egypt
[3]Faculty of Medicine, Taibah University, Saudi Arabia
saharfathi2011@yahoo.com

Abstract :Human papillomavirus (HPV) infection is associated with a number of diseases that vary from self-limited warts to life threatening types of cancers(head and neck, ano-genital, oropharengeal and cervical cancers). Cervical cancer is considered the third common cancer among women and the fourth cause of increased mortality. The etiology of cervical cancer has been attributed to Human Papilloma Virus (HPV) in >99% of cases. While the genotypes of HPV linked to cervical cancer may vary in different parts of the world, almost 70% of cases around the world have been found to be due to two types of HPV namely HPV 16 and HPV 18. Molecular techniques using target specific HPV DNA amplification by PCR is an essential step for genotyping HPV as HPV DNA quantity in most samples is very low. There are three main techniques used for HPV detection and genotyping: Target amplification [amplify a specific DNA sequence from a targeted gene e.g. polymerase chain reaction (PCR)], signal amplification (increase the DNA-proportional signal to detectable levels using branched DNA or hybrid capture technology) and probe amplification (amplify the probe e.g. ligase chain reaction). Other traditional, non-amplified based molecular techniques which include Southern blot hybridization, in situ hybridization (ISH), and dot blot are hardly used nowadays. The accurate detection of HPV genotypes has played a pivotal role in both molecular and epidemiological studies. Determining the prevalence and genotypes of HPV infection in women is mandatory for the development of vaccines targeting the oncogenic types of HPV. To achieve these goals, the HPV DNA test needed to be designed with the highest analytical sensitivity and specificity.

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1- Introduction

In developing countries, the leading cause of cancer mortality among women is believed to be cervical cancer. [(1)] Worldwide, cervical cancer is considered the third common cancer among women and the fourth cause of increased mortality. [(2)] Studies on the etiology of cervical cancer showed Human Papilloma Virus (HPV) to be associated with >99% of this cancer. [(3)] While the types of HPV linked to this disease may vary in different parts of the world, almost 70% of the tumor samples around the world have been shown to contain two types of HPV namely HPV 16 and HPV 18. [(4)]

The establishment of a causal relationship of HPV with cervical carcinoma led to the development of anti-HPV vaccines as a method of controlling HPV infection and hence, reducing the incidence of cervical cancer. In such a preventive strategy, the current world-wide vaccine formulation has targeted two most prevalent types of HPV (i.e HPV16 and HPV18) . [(5)] It is believed that by protecting women against infection with these two types of HPV, most cervical cancers would be prevented. Type specific anti-HPV vaccines is now a major strategy for preventing cervical cancers in women across the world. [(2,5)]

The accurate detection of HPV genotypes has played a pivotal role in both molecular and epidemiological studies. Determining the prevalence and genotypes of HPV infection in women is mandatory for the development of vaccines targeting the specific oncogenic types of HPV. To achieve these goals, the HPV DNA test needed to be designed with the highest analytical sensitivity and specificity. [(6,7)]

Molecular techniques using target specific HPV DNA amplification by PCR is an essential step for genotyping HPV as HPV DNA quantity in most samples is very low. [(5)] For genotyping HPV, while different methods are available, the INNO-LiPA assay is very useful in identifying oncogenic HPV-positive women. This assay is reliable, relatively cost-effective and easy to perform. [(7)]This review discusses the usefulness of different molecular techniques for detecting HPV genotypes.

History of HPV:

An outstanding work done by Jablonska and Gerard Orth at the Pasteur Institute in 1978 had led to the discovery of HPV-5 in skin cancer. [(8)] A documented hypothesis published in 1976 by Harald zur Hausen stated that the human papilloma virus plays an important role in the cause of cervical cancer. [(9)]

Human papillomavirus (HPV):

HPV is a double stranded DNA virus (dsDNA)-which infects human by infecting keratinocytes in

the mucus membrane or in the skin. [10] In persistent HPV infection, HPV viral sequences are always integrated into the cellular DNA of most HPV-induced cancers. [11]

HPV is replicated only in the basal cells of stratified epithelium, so HPV infection is restricted to such cells. The slow infectious process allows for the development of antibodies which play the most important neutralizing role while the virions still inhabit the basement membrane and cell surfaces. [12]

The genome of HPV, which is ds-DNA, contain Open Reading Frame (ORF) genes coding for eight types of proteins responsible for viral replication. These ORF gene products have been classified into 6 early stage (E1, E2, E3, E4, E5, E6) and 2 late stage (L1, L2) proteins . Once the HPV infects the host cells, E1 and E2 HPV proteins are expressed. When HPV dsDNA incorporats itself into the host DNA, the E2 HPV protein function is interrupted. Normally E2 suppresses the function of E6 and E7. The disruption of E2 function consequently leads to the activation of E6/E7. Persistent expression of oncoproteins E6 and E7 result in malignant transformation of infected cells. [2,13] E6 inactivates p53 while E7 inactivates pRb. The inactivation of these two very important tumor suppressor genes (p53 and pRb) might be the main etiological cause for cancer development. [14]

HPV genotypes and diseases:

It has been reported that, there are more than 200 genotypes of HPV. Fortunately most of these genotypes do not cause symptoms in people. Some types can cause warts and very few genotypes were related to cancer mainly cervical cancer or anogenital cancer. High-risk HPV genotypes include type 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, and 59 which might lead to the development of various types of cervical, vulvar, and/or anal neoplasia. [15]

WHO/ICO Information Centre on HPV and Cervical Cancer (HPV Information Centre) had reported that, more than 99% of cervical cancers are associated with HPV infection. [16] There is a wide variation in cancer incidence, type and mortality among different ethnic groups, which might be due to a difference in socio-economic status, availability of health care, pathogen exposure and carcinogen exposure. Worldwide differences in cancer epidemiology might be also due to an interaction of genetic factors, environmental changes and various tumor biology. [17,18]

Although HPV genotypes directly linked to the pathogenesis of cervical cancer are somewhat variable worldwide, there are other diseases than cervical cancer that have been attributed to HPV. [13,14] For example, high risk HPV is also linked to the pathogenesis of anogenital and oropharengial cancers which might affect both males and females. International Agency for Research on Cancer (IARC) reported that although cervical cancer almost exclusively caused by HPV but other sexually transmitted (STD) diseases might be caused by HPV as well. [19]Non-cervical HPV-related cancers is reported to be linked to the risk HPV genotypes (HPV16 and HPV18) which is detected in 5.2% and 3.7% of the world population respectively. [20]

Infections by low risk HPV genotypes were found to be associated with benign genital warts (condyloma acuminata) in women and the penis, scrotum or anus in men. These low risk genotypes were also associated with juvenile respiratory papillomatosis or recurrent respiratory papillomatosis (JRP-RRP). [21]

HPV type 16, among other types of HPV has been reported to be associated with HPV-positive oropharyngeal cancer (OSCC). [11]

Due to large number of new cervical cancer cases caused by HPV globally every year, HPV is considered as an important transmittable cause of cancer. [22]

It is believed that, the early detection of high risk HPV genotypes and the use of vaccines against them will reduce both cervical cancer and other HPV triggered diseases.

2-Transmission

Genital infections

Sexual activity is the main cause for HPV transmission. Approximately 40 identified HPV genotypes infect the genital tract. The risk of infection is reported to be markedly increased in women having more than one partner. Condoms could not be considered as a protection from HPV infection as the areas around the genitals are not covered. [23,24] Male circumcision is reported to have protective effect against HPV infection and cervical cancer incidence in female partner. [25]

Prenatal transmission:

Juvenile-onset of recurrent respiratory papillomatosis (JORRP) can be caused by prenatal transmission of HPV types 6 and 1. JORRP represent a relatively rare disease in the United States. [26]

3-Epidemiology and prevalence of HPV globally:

Most cases of genital HPV infections do not cause any obvious symptoms and the immune system is able to get rid of the infection in a few months. Immunity is believed to be type specific especially for cutaneous HPVs. This immunological control and clearance may fail in some infected individuals. Persistent infection with high-risk HPV types might be responsible for the pathogenesis of cervical cancer or other types of cancer. [27]

High-risk HPV types 16 and 18 were reported to be the main cause of about two-thirds of cervical cancer cases. [28] Type 16 accounts for the vast majority of HPV-induced vaginal/vulvar cancers, anal cancers, penile cancers, and head and neck cancers. [29]

Lee et al., [30] investigated the prevalence and the genotypes of HPV among Korean women in a total sample of 60.775 (aged 18-79 yr, median 44). They reported that HPV positive rate was 34.2% of which 87.7% were found to be due to single type infections, while multiple HPV types were reported in 12.3%. As regard the genotype, HPV-16 was found to be the most prevalent, followed by HPV-52, HPV-58 and HPV-18 respectively.

In a meta-analysis review published by Shi et al.[31] collecting evidence for the load of cervical cancer in China, it was reported that HPV infection incidence in China was less than 5/100.000 which is considered to be low compared to other countries. These findings are intriguing and need to be interpreted cautiously as the registries examined were only from high socioeconomic status regions without a consideration of the low-socioeconomic rural areas which might have higher incidence of HPV.

Rivera et al.,[32] investigated the prevalence and genotype distribution of HPV in 929 apparently healthy women from Mexico City using Pap smear cytological examination and multiplex PCR for HPV DNA detection. HPV infection was detected in 9.1%. High risk genotypes (16-18) were detected in 99% of those cases. Only 15% of the HPV positive cases showed abnormal cervical cytology. Their study highlights the importance of HPV screening and genotyping in healthy women. Also, it showed increased frequency of high risk HPV genotypes among Mexican women. These results warrant further investigations as the high frequency of high risk HPV genotypes (16-18) among healthy women is unexpectedly high (99%).

In USA more than 24 million persons are infected with HPV, and annually reported new cases is about 4 millions. [33]

Raza et al., [34] reported that in Karachi, Pakistan, the prevalence of HPV among general population was 2.8%, and the most common genotypes were HPV16 and 18. Young women showed no evidence of higher prevalence.

In Iran, the prevalence of HPV has been reported to be 5% and, seven different HPV genotypes have been detected, six of them being high risk genotypes (16, 18, 31, 33, 51 and 56) beside HPV-66 which was "probably carcinogenic." [35] In Algeria, the prevalence and genotypes of HPV in cervical cancer cases and control women were similar to that reported in Europe unlike the one in sub-Saharan Africa, where HPV 16 is less prevalent. [36]

A population-based cohort study of HPV in Chilean women to evaluate the prevalence of HPV for five years had shown that, high risk-HPV prevalence increased by 43%. Incidence was the highest in women < 20 years of age. [37]

In Denmark, a large population-based study was done to determine the overall and age-specific HPV prevalence, and HPV type distribution in women. The HPV prevalence was found to be 26.4% with a peak in women with an age range of 20-24 years. HPV 16 was the most prevalent type followed by HPV 18. [38]

Cervical HPV DNA prevalence among general women population in Guatemala was reported to be 38.1% while 67.3% in sex worker women. The most prevalent genotypes were HPVs 51 66 and 16. [39]

The published data regarding the prevalence of HPV genotypes in invasive cervical cancers in Italy from 1996 to 2008 was reviewed by Rossi *et al.,* [40] They performed pooled and multivariate analysis and showed that, the proportion of HPV 16 and/or 18 decreased with age, although it increased in cancers diagnosed in more recent years. Their results support the theory that, HPV 16/18 vaccination has beneficial effect on early onset cervical cancers.

A systematic review was conducted for the U.S. Preventive Services Task Force to compare liquid based cytology versus HPV detection and genotyping for primary screening of cervical cancer in 141 566 participants. [41] It concluded that, primary HPV screening detected more cases of CIN3 or cancer in women older than 30 years. The limitation of this study was that, short –term trial data that have been included might cause ascertainment unfairness.

Tricco et al., [42] systematically reviewed the prevalence of oncogenic cervical HPV infection among Canadian females in order to establish most advantageous vaccination strategies. The data collected from all investigated studies support the previous published data regarding those high risk genotypes HPV16 and 18 as the most prevalent genotypes. Also they reported that, vaccination might enhance cancer protection for female under age of 20 years.

Martín et al., [43] investigated the prevalence and genotype of HPV infection among 2.461women aged15-60 years living in Madrid (Spain) with normal and abnormal cytology. They reported HPV-16 as the most frequent type, followed by HPV 53 and HPV 31, but HPV 18 showed low prevalence. The commercially available vaccination targeted HPV16 and 18. These vaccines could decrease cervical cancer by 50%. HPV genotypes that are not covered by the available vaccines were reported to be frequent in women from Spain. So, before introducing HPV vaccination to any population detailed studies on the most prevalent genotype distribution should be considered to target the relevant HPV genotype.

The possible etiological role of persistent infection with HPV in high-grade cervical neoplasia was systemically reviewed by Koshiol et al., [44] in more than 22.500 women. They concluded that, persistence infection with HPV was constantly and highly associated with high grade cervical

neoplasia. The duration of persistency, HPV test interval and sampling procedure should be standardized before validation of the concept that HPV persistency is associated with high grade cervical neoplasia.

To evaluate the prevalence and genotypes of HPV DNA in women with normal cytology de Sanjosé et al., [45] systematically reviewed all published literature. They reported that, in all studied reviews globally 291 million women were found to be carriers of HPV DNA , of them 32% were carriers of HPV16 or HPV18, or both. These detected HPV types were similar to those described in pre-neoplastic and neoplastic cervical lesions. It should be mentioned that, the role of HPV16 and HPV18 is significantly lower in women with normal cervical cytology.

HPV prevalence and genotyping in Saudi Arabia:

Al-Muammar et al., [46] studied the prevalence of HPV16/18 in 120 Saudi and non-Saudi women living in Riyadh, during routine gynecological examination. They reported a high prevalence of HPV-16/18 among examined women. The progression rate of cervical intraepithelial neoplasia in those cases was reported to be low in a follow-up period of 4 years.

Sait and Gazzaz in 2011 conducted a study to detect HPV in cervical biopsy samples from 45 women Saudi women diagnosed with cervical cancer or cervical dysplasia, attending department of Obstetrics and Gynecology, Faculty of Medicine, King Abdulaziz University. They stated that, the most common genotype detected was HPV 16 .However, when they included cases with mixed infection, HPV 18 was found to be the second most common. [47]

Sait et al., [48] published a review using the Pub Med database between January 2000 till June 2011, including all the publications concerned with cervical cancer and cervical dysplasia in Saudi Arabia. They concluded that the distribution of HPV subtype in cervical cancer in Saudi Arabia still has not been extensively studied. Their data showed that, the absence of proper screening programs deprive women for their right of early detection of cytological changes. They stated that, cervical cancer could be prevented by proper HPV screening and genotyping. [48]

A WHO report on Human Papillomavirus and related cancer in Saudi Arabia states that: [15] **1-** No data are available from cancer registry about the incidence of cervical cancer, or age standardized incidence rate by histological types among Saudi women.

2- There is no data available on the genotype-specific HPV prevalence among Saudi women with and without cervical lesions.

3- There is no data available regarding the predictable reports of cervical cancer screening in Saudi Arabia, by age and study.

Screening and genotyping of HPV in a large number of native Saudi women with or without cervical cancer is now considered a high priority. This will provide data regarding the incidence and genotype of HPV among Saudi women, which is not available till now. A service platform based on these results is required to provide the clinical and genetic data for population screening service which is urgently needed in Saudi Arabia. Data on the HPV prevalence and genotypes will be helpful for the Ministry Of Health (MOH) before vaccination against HPV is introduced at the population level.

4-HPV infection, cervical cancer and vaccine development:

HPV infections in young females are almost temporary, with little or no long-term significance. Roughly after one year of infection, about two thirds of such infections will disappear and about 90% in two years. [18] Only 50–60% of women infected with HPV express HPV serum antibodies after natural infection. [49]

The approximate annual record of worldwide new cases of cervical cancer is about 500, 000 of which almost 80% have been diagnosed in developing countries. HPV is reported to be the main cause of cervical cancer. Standard gynecological screening combined with HPV DNA testing is believed to be of great value in preventing or reducing neoplastic transformation by discovery and treatment of precancerous lesions. [21]

The accurate detection of HPV genotypes has played a pivotal role in both molecular and epidemiological studies. Defining the prevalence and genotypes of HPV infection in women is mandatory for the development of vaccines targeting the oncogenic types of HPV. To achieve these goals, the HPV DNA test needed to be designed with the highest analytical sensitivity and specificity. [6] A relatively inexpensive but a reliable method of genotyping like INNO-LiPA can be routinely used for molecular epidemiological studies aimed at classifying the HPV positive women as well as for specific vaccine development. [7]

5- The current consideration for vaccines:

The current world-wide vaccine formulation has targeted two most prevalent types (i.e HPV16 and HPV18) associated with cervical cancer. It is believed that by protecting women against infection with these two types of HPV, most cervical cancers would be prevented. [5]

In April 2009, WHO published a paper on HPV vaccines. [50] The following quotation summarizes its recommendations: **"WHO recognizes the importance of cervical cancer and other HPV-related diseases as worldwide public-health trouble and recommends that regular HPV**

vaccination must be included in nationalized immunization programs. "

Types and use of HPV vaccines :

HPV vaccines besides being cost effective, is expected to decrease the incidence of cervical cancer, particularly in low-resource settings. In fact, model studies suggest that combining HPV vaccination and organized screening programs may have the highest impact on the disease control worldwide. (51)

HPV vaccines might be categorized into two groups, prophylactic vaccines which has been developed since 2006 and therapeutic which need to be developed in the near future. The prophylactic HPV vaccines include two types which were commercially available to avoid infection by some HPV types: quadrivalent vaccine (Gardasil, marketed by Merck against HPV 16, 18, 6, 11) and bivalent (Cervarix, marketed by GlaxoSmithKline against HPV 16 and 18). These two types of vaccines protect against preliminary infection with high risk genotypes 16 and 18. Gardasil is reported to be effective also towards low risk genotypes 6 and 11, which is the main causative agent for genital warts. (52)

These commercially available HPV vaccines were designed to be prophylactic (i.e. to prevent infection and consequent disease). Some reports stated that, sexually active women who have been infected with high risk HPV genotypes will get no or little benefit from these vaccines, but those who still are not sexually active will be protected by such vaccinations. (53)

In many countries, the vaccines are recommended to be given to females only. Only USA and UK have supported male HPV vaccination. No therapeutic effects of the vaccines on already HPV infected people have been reported. In 2010 >60% of teens in the US had gotten vaccination against meningitis and DPT and only about 49% have receive HPV vaccinations. (54)

After receiving the vaccine women should continue to undergo cervical screening, such as Pap smear testing. Recommendations for cervical cancer screening have not changed for females receiving HPV vaccine. Combination of both vaccination and continued Pap smear scanning might decrease the risk of cervical cancer development. Regarding children under 15 years of age, no efficacy trials have been performed. There is no strong practical clinical trial that can determine the duration of vaccine efficacy; however Cervarix effectiveness is proven for 6.4-7.4 years while Gardasil is proven for 5 years. (55) Protection after vaccination lasts for almost 5 years. Data are not yet available on the safety and efficacy of HPV vaccines in Africa. (20)

Data published from a meta-analysis by Lu et al. (56) evaluating the efficacy and safety of prophylactic vaccines targeting HPV infection and cervical cancer among women, concluded that prophylactic HPV vaccines were found to be very effective in preventing long standing HPV infection, safe in young female (less than 30 years of age). These vaccines were reported to be simply tolerated. It should be emphasized that, these vaccines prevent infection and cervical disease in a type specific manner. Further studies are needed to evaluate the safety, tolerability of vaccine related side effects and effectiveness of HPV vaccines in older age groups**.**

Earlier systemic reviews addressing evaluation of the efficacy HPV vaccine in preventing persistent cervical HPV infection in women by La Torre et al., (57) and Rambout et al., (58) had documented the protective role of the vaccine in all studies they had reviewed. They stated that the HPV vaccine efficacy in markedly evident in young women aged 15-25 years who had not been infected with HPV yet. We think that in order to evaluate the protective role of HPV in reduction in cervical cancer mortality, long-term follow-up is recommended.

6- Early Diagnosis and cervical cancer prevention:

Initial screening using a Papanicolaou (Pap) test or liquid-based cytology is used to check patients for premalignant or malignant lesions that require further evaluation. Early detection and appropriate treatment of any detected premalignant lesion is essential to prevent cervical cancer development. Pap-smear screening is used to detect any abnormal cytological changes. If any abnormal cytological changes are detected, colposcopy and biopsy of the suspected lesion are to be done. (59)

Cytological examination of cervical smears is mandatory to detect any abnormal squamous cell growth which termed as squamous intraepithelial lesions (SIL), graded low to high according to the degree of cervical epithelium affection and the presence of abnormal cells. Histopathological examination of cervical biopsies can detect and define cervical intraepithelial neoplasia (CIN), a term which means abnormal cells in the cervix that are graded from 1 to 3 according to the thickness of the cervical epithelium. In CIN 3, neoplastic cells invade more than 2/3s of the cervical epithelium. Similar grading exists for vaginal and vulvar lesions. In long standing HPV infection, the virus DNA integrated into the human DNA leads to moderate or severe carcinogenesis classified according to severity into: cervical intra-epithelial neoplasia (CIN 2, CIN 3 or adenocarcinoma in situ (AIS). (60)

Value of molecular testing in diagnosis of cervical cancer:

In developing countries, visual inspection and cytological detection of cervical cancer have been successful in screening large population at risk and provide adequate medical care. However, the necessity was felt for developing more refined and

accurate diagnostic tools. In developed countries, the use of HPV DNA testing have been useful in decreasing mortality rate due to cervical cancer up to 50%. Cuzick et al., [61] stated that, HPV DNA testing is the most beneficial choice for primary screening, but women who are HPV positive follow-up should include cytological examination plus HPV DNA testing.

In a meta-analysis reviewing the results of more than 25 non-randomized studies evaluating the diagnostic accuracy of HPV DNA testing in primary cervical screening Koliopoulos et al., [62] stated that HPV DNA testing is more sensitive but less specific than cytology in diagnosis of CIN2. A combination of cytological examination and HPV DNA has the high sensitivity but decreased specificity. As regard the prognostic value of HPV DNA testing versus cytology, there is no difference in cancer mortality or invasiveness in either cytologically defined or HPV DNA defined cases.

In an earlier study by Zielinski et al., [63] evaluating the use of HPV DNA testing for monitoring women treated for high grade cervical neoplasia, recommended the use of combination of both cytology and HPV DNA testing for better results.

Several studies have shown HPV screening and genotyping to be more sensitive than cytological testing for primary cervical screening. A meta-analysis and systematic review including all randomized controlled trials conducted from 2005 to 2010 was published in 2012 by Murphy et al., [64] who compared the value of HPV testing versus conventional cytological examination in primary cervical screening. They concluded that, HPV testing as the primary screening test for women 30 or 35 years of age and older is the most accurate and reliable. Optimal screening strategies for younger women need to be further evaluated.

A similar conclusion was drawn in a recent study conducted by Ogilive et al., [65] comparing primary cervical cancer screening with HPV DNA testing to liquid –based cytology. They stated that HPV DNA testing had increased cervical intraepithelial neoplasia detection when compared to liquid based cytology.

The usefulness, specificity, sensitivity and cost effectiveness of HPV DNA testing and genotyping compared to visual inspection methods for cervical cancer screening had been evaluated by Shi et al., [31] in rural China. They concluded that, if the test is to be done once-lifetime, maximum effectiveness had been achieved for women between 35-50 years of age .

7- Various Molecular techniques for detecting HPV:

Since, HPV cannot be cultured in-vitro and serological assays lack sensitivity and specificity, so the detection is entirely based on molecular methods. HPV infection is diagnosed indirectly by detection of its DNA in the cells obtained from a particular anatomic site. [66]

There are three main techniques used for HPV detection and genotyping: Target amplification [amplify a specific DNA sequence from a targeted gene e.g. polymerase chain reaction (PCR)], signal amplification (increase the DNA-proportional signal to detectable levels using branched DNA or hybrid capture technology) and probe amplification (amplify the probe e.g. ligase chain reaction). Other traditional, non-amplified based molecular techniques which include Southern blot hybridization, in situ hybridization (ISH), and dot blot are hardly used nowadays. [33,67]

A-Target Amplification Techniques

Polymerase Chain Reaction

Detection and amplification of HPV DNA is done mainly using polymerase chain reaction (PCR). In order to recognize and amplify a single genotype of HPV type specific PCR is usually done through targeting type-specific DNA sequence. Several repeats of PCR is necessary to determine the exact genotype existing in the sample. [68]

Reverse Line Blot and Linear Array

27 different HPV genotypes could be detected using the reverse line blot assay (Alameda, CA) developed by Roche Molecular Systems. This assay use L1 consensus primer-based PCR with PGMY09/11 primers. It is based on reverse line blot hybridization. The probes are membrane fixed [69] The Linear Array Test is CE-Marked for in vitro diagnostic use in Europe.

The reverse line blot hybridization linear array is the principle of another commercially available INNO-LiPA HPV Genotyping (Innogenetics, Ghent, Belgium), which could indentify 24 low- and high-risk HPV genotypes. This kit also is CE-Marked. The INNO- LiPA test amplifies HPV DNA using SPF10 primers located at the L1 region. The probes are membrane strips in sequence-specific lines and visualized with unaided eye as purple/brown bands. [70]

Amplicor HPV

The Amplicor HPV test developed by Roche Molecular Systems is a PCR-based test. 13 high-risk HPV types could be detected using this assay. PCR is used for amplification of the target DNA which will be followed by nucleic acid hybridization. Amplicor HPV only detects HPV but could not identify HPV specific genotype. [71]

PapilloCheck

PapilloCheck (Greiner Bio-One, Monroe, NC) is a commercial DNA-array based test used for HPV genotyping. Twenty-four genotypes of HPV could be detected using this technique. It depends on the amplification of E1 gene and DNA hybridization to HPV oligoprobes. [72] A laser scanner is needed to

detect the excitation from fluorescently labeled probes which bind to the HPV primers fixed on the PapilloCheck DNA chip containing the immobilized HPV probes.

Multiplex HPV Genotyping Kit

Multiplex Genotyping is a relatively new commercially available test (Multimetrix, Heidelberg, Germany). Multiplex HPV Genotyping kit is PCR-based fluorescent bead array. It could detect up to 24 HPV types with high and low risk genotypes. [73]

Although the Multiplex HPV Genotyping Kit is available for research only , in the near future it might be available for routine diagnostic use due to its high sensitivity. [74]

Real-Time PCR

Real-Time qPCR is a technique that combines the use of fluorescent probe and PCR primers. It is characterized by accurate quantification of HPV in the sample. RT-qPCR is considered a sensitive technique for HPV- DNA identification. [75]

GenoID assay is a commercially available kit using RT-PCR. It can identify 14 high risk and 5 low-risk in about three hours. This kit is also available for three types of RT-PCR platforms: Applied Biosystems 7900 HT, Corbett Rotor-Gene 6600 and Roche LightCycler 2.

B-Signal Amplification Techniques

Hybrid Capture Assay

In this assay fluorescent or chemiluminescent signal is amplified to assist detection, rather than the target DNA amplification. This assay is FDA approved. It is only useful for HPV detection not for genotyping. The principle is that specimens containing HPV DNA are hybridized with a HPV-specific RNA probe, this will produce a DNA:RNA hybrid molecule. A microplate-well coated with the first antibody targeting the DNA-RNA hybrids. Alkaline phosphatase-conjugated secondary antibody targeting the first antigen-antibody molecule then a signal is detected after the addition of a chemi- luminescent substrate. Signal amplification is due to several alkaline phosphatase molecules which have been conjugated to each antibody, and multiple conjugated antibodies can bind each captured hybrid. [76]

CareHPV

CareHPV is based on Hybrid Capture Assay (HC2) principle and is considered a HC2 spin-off (developed by Qiagen). It is the most common type of tests used for cervical screening in under developed countries. It can detect HPV but not genotypes. It is less expensive and almost about 90% accurate. [77]

Cervista HPV HR and Cervista HPV 16/18

This is the only test for HPV screening and genotyping which is FDA approved since 2009. The Cervista HPV HR which detect 14 high risk HPV genotypes and Cervista HPV16/18 which detect only high risk genotypes 16 and 18 are commercially available supplied by Third Wave Technologies, now Hologic, Bedford, MA.

Cervista test is based on a unique signal amplification technique called the Invader technology which uses 2 simultaneous isothermal reactions. Simply, the target DNA is denatured, a DNA probe including a sequence-specific region binds to the HPV DNA molecule. Then a specific Cervista test probe called the Invader which associates also with the target sequence, resulting in a 1-base overlapping. A specific Cervista test enzymes cleave the probe, releasing a 5' oligo flap. Multiple flaps are generated from each target DNA molecule. That flap then associates with a florescence resonance energy transfer (FRET) probe, resulting in cleavage at the indicated site. The fluorophore, is released from the quencher, causing a fluorescent signal which is directly proportional to the amount of target DNA. This results in a detectable fluorescent signal. [78]

The genome of HPV (ds-DNA molecule) is composed of eight types of Open Reading Frame (ORF) proteins responsible for replication, of them E6 and E7 has important role in cell proliferation and closely linked with HPV-associated carcinogenesis. [12-13] It could be of diagnostic interest if we were be able to measure the expression of HPV E6 and E7 genes, as over-expression of these genes will denote ongoing HPV-related carcinogensis. This could be achieved using the reverse-transcriptase (RT)-CR to detect HPV mRNA. Using RT-PCR for sensitive quantification of gene expression (protein products) of these genes (E6-E7) might give an indication on HPV virulence. Monitoring the levels of expression of these genes might be useful for screening and monitoring of cancer progression. [79] The only limitation is that RT-PCR still needs to be optimized for use in large scale HPV screening to ensure clinical sensitivity and specificity. [80]

Clinical efficacy of any HPV screening method should depend on the usefulness of the test to predict cervical cancer, not only HPV virus detection.. This is very important especially for young women, since virus detection has no clinical value because HPV infections in young females are almost temporary, with little or no long-term significance. HPV in these women mostly self- resolves and never develops into cancer. [81]

Before approval of any appropriate technique for use in clinical laboratory many aspects should be clarified such as the cost of the test, population to whom this test is directed, specificity and sensitivity

of the test. In underdeveloped countries, although our goal is early and accurate detection of cervical cancer, we might have to compromise for specificity and sensitivity in order to get rapid accurate , less expensive and easy to use test. Searching for molecular technique that is sensitive and specific for early and accurate diagnosis of cervical cancer must be a priority for researchers working in this field.

Corresponding author
Hammoudah SAF
-Center of Genetics and Inherited Diseases(CGID), Taibah University. Al Madinah AL Moonwarrah, Saudi Arabia. 009664243248
-Clinical and Chemical Pathology Department, Tanta University, Faculty of Medicine, Tanta, Egypt.
Saharfathi2011@yahoo.com
00201001709456,00201000030773, 009664243248

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