

**Detection of oncogenic Human papillomavirus DNA in cervical
swabs from Libyan women, Tripoli, Libya**

*A Thesis Submitted to the School of Science at the Libyan Academy of Graduate
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Science in Microbiology.*

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الكشف عن الحمض النووي لفيروس الورم الحليمي البشري المسرطن في عينات عنق الرحم للنساء الليبيات بطرابلس

مشروع بحث مُقدم لمدرسة العلوم الأساسية بالأكاديمية الليبية للدراسات العليا كجزء
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DEDICATION

To my family

Acknowledgment

I would like to thank Dr. Elfath M Elnifro and Dr. Abdunnabi Ahmed Rayes for their supervision and their guidance in academic writing material. I am especially grateful to Dr. Elfateh M. Elnifro for his supervision in the practical part of the Master. My gratitude also to the Microbiology Department staff at the Academy of Graduate Study.

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الملخص

لا تتوفر معلومات كافية عن مدى انتشار الاصابة بفيروسات الورم الحليمي البشري في عنق الرحم عند النساء الليبيات.

الهدف من الدراسة: اجريت هذه الدراسة لتحديد مدى انتشار الاصابة بفيروسات الورم الحليمي البشري عند النساء الليبيات في مدينة طرابلس.

طريقة الدراسة: كانت هذه الدراسة عبارة عن دراسة سلسلة حالة وصفية والتي اجريت في عيادة النساء والولادة في مستشفى سانت جيمس بطرابلس. تم جمع العينات من عنق الرحم من 53 إمراه متزوجه ومن اعمار مختلفة واخذت الاستشارة الطبية علي مشاكل الولادة وامراض النساء المختلفة. جمعت عينة عنق الرحم من كل امرأة والتي بقت محفوظة في محلول Thin prep عند درجة حرارة (-20°C) حتي تم فحصها واستخدمت هذه العينات للكشف عن المادة الوراثية لفيروس الورم الحليمي البشري.

النتائج: أجريت الدراسة علي المرضى التي تتراوح اعمارهم من 21 الى 59 وكان متوسط الاعداد 36. بينت النتائج وجود الفيروسات في 9 حالات من بين 53 عينة أي بنسبة (16.9 %) من النساء الليبيات اللاتي اجريت عليهن الدراسة، ووجدت اكثر الحالات الايجابية في الفئات العمرية ما بين (20 – 29) بنسبة 66.7 % من الحالات الايجابية بينما وجدت حالة واحدة في الفئات العمرية ما بين (30 – 34) بنسبة 11.1 % وحالتين في الفئات العمرية ما بين (40 – 44) بنسبة 22.2 %. تم فحص العينات علي فئات عمرية مختلفة تتراوح ما بين (21 – 59) سنة. وقورنت هذه النتائج مع دراسات سابقة في دول مختلفة.

الاستنتاج: استنتجت الدراسة ان الاصابة بفيروسات الورم الحليمي البشري يعتبر منتشر بين النساء الليبيات ويحتاج الي دراسات مستقبلية.

ABSTRACT

Introduction: Studies about infection with Human Papilloma Virus (HPV) in women are lacking in Libya and here we plan this small study about this subject.

Objective: The main objective of this study is to detection of oncogenic Human papillomavirus DNA in Libyan women in Tripoli.

Subjects and Methods: the study was a descriptive case series study that was carried out in the Gynecology and Obstetrics Clinic of Saint James Hospital. Fifty three cervical swabs were collected randomly from married women who were having different ages and came for consultation for different gynecologic or obstetric problems. A cervical swab was collected in ThinPrep solution and stored at (-20 °C) until examined. They were subjected for testing for HPV DNA.

Results: The ages of the patients ranged from 21-59 years with a mean of 36 years.

There were 9 (16.98 %) positive cases between 53 samples studied from these women. Most of the cases (6/9) (66,7%) were in the age group 20-29 years old and were more positive cases , while one case (11.1%) in the age group (30-34) and two cases (22,2%) in the age group (40-44).The samples were tested in different age groups of ages ranging from 21-59 years. These results were in accordance with previous studies performed in other countries.

Conclusion: This study we conclude that HPV infection is a fairly common among Libyan women but more studies with larger samples needed.

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LIST OF ABBREVIATIONS

ASCUS	Atypical Squamous Cells of Undetermined Significance
Bp	base pair
BPV	Bovine Papillomavirus
CIN	Cervical intraepithelial neoplasia
COPV	Canine Oral Papillomavirus
CRPV	Cottontail rabbit papillomavirus
CXCA	Cervical cancer
DNA	Deoxyribonucleic acid
DNA	Deoxyribonucleic acid
DPO	Dual Priming Oligonucleotide
DPV	Deer papillomavirus
EDTA	Ethylene diamine tetra acetic acid
EEPV	European elk papillomavirus
EqPV	Equus caballus (horse) papillomavirus
Et Br	Ethidium Bromide
EV	Epidermodysplasiaverruciformis
FcPV	Fringillacoerebs (chaffinch) papillomavirus
FDA	Food and Drug Administration
FdPV	FdPV, Felis domesticus (cat) papillomavirus
FISH	filter in situ hybridization
HaOPV	Hamster oral papillomavirus
HC I	Hybrid Capture I
HC II	Hybrid Capture II
HIV	Human immunodeficiency virus
HPV	Human papillomavirus
HPV cand	HPV candidate
HRC	high-risk Common HPV types

LIST OF ABBREVIATIONS-continues

HSV	Herpes simplex virus
IARC	International Agency for Research on Cancer
ICTV	International Committee on the Taxonomy of Viruses
ISH	In situ hybridization
MnPV	Mastomys natalensis papillomavirus;
mRNA	Messenger RNA
OvPV	Ovine papillomavirus;
PCR	Polymerase chain reaction
PePV	Psittacus erithacus timneh (parrot) papillomavirus
PsPV	Phocoena spinipinnis papillomavirus
RFLP	Restriction Fragment Length Polymorphism
RLU	relative light units
RNA	Ribonucleic acid
ROPV	Rabbit oral papillomavirus
RPV	Reindeer papillomavirus
SCC	Squamous cell carcinoma
TAE	Tris-acetate-EDTA
URR	Upstream regulatory region
UV	Ultra Violet
8-MOP	8-methoxypsoralen

1- INTRODUCTION

Human papillomavirus (HPV) is ubiquitous and have been detected in a wide variety of animals as well as in humans. It's associated with a variety of clinical conditions that range from innocuous lesions to cancer (Table 1) (Bonnezet *al*, 2000)

HPV is known to cause a variety of lesions such as skin warts, mucosal papillomas and lesions in the anogenital area called condylomas (Garland, 2002; Somerset *al*, 1997). The most serious and clinically significant lesions however are the cervical carcinoma and precancerous intraepithelial lesions of the cervix (Holowayet *al*, 1999).

Cervical cancer and its precursor lesions are encountered more commonly in women who have had early sexual intercourse with the male sexual partner acting as the carrier for HPV infection (Franceschiet *al*, 2002). A prerequisite for the development of cervical cancer is infection by HPV, which is a sexually transmitted virus (Garland, 2002 ;Walboomerset *al* ,1999).

The penile skin hosts the virus although it is only rarely involved in carcinoma. Male circumcision may reduce the incidence of infection probably due to better hygiene. During sexual intercourse an infected male may transmit the infection to the female partner (Garland, 2002).

The virus initially infects the cells in the basal layer where the integrins act as receptors. Once inside the cell the virus can exist within the cell in two distinct biological states. The latent form, a small number of viral copies may continue to reside inside the nucleus of the basal cell in a free circular form called an episome.

Table 1. Human papillomavirus types and clinical manifestations.(Bonnezet *al*, 2000)

Clinical manifestations	HPV type
Planter warts	1, 2, 4, 63.
Common warts	2, 1, 7, 4, 26, 27, 29, 41, 57, 65, 77, 3, 11, 28.
Flat warts	3, 10, 26, 27, 28, 38, 41, 49, 75, 76.
Other cutaneous lesions (e.g. epidermoid cysts, laryngeal carcinoma)	6, 11, 16, 30, 33, 36, 37, 38, 41, 48, 60, 72, 73.
Epidermodysplasiaverruciformis	2, 3, 10, 5, 8, 9, 12, 14, 15, 17, 19, 20, 21, 22, 23, 24, 25, 36, 37, 38, 47, 50.
Recurrent respiratory papillomatosis	6, 11.
Focal epithelial hyperplasia de Heck	13, 22.
Conjunctival papillomas /carcinoma	6, 11, 16.
Genital warts (condyloma acuminatum)	6, 11, 30, 42, 43, 45, 51, 54, 55, 70.
Low-risk cervical intraepithelial neoplasia	6, 11, 16, 18, 31, 33, 42, 43, 44, 45, 51, 52, 74.
High-risk cervical intraepithelial neoplasia	6, 11, 16, 18, 31, 34, 33, 35, 39, 42, 44, 45, 51, 52 , 56, 58, 66.
Cervical carcinoma	16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 66, 68, 70.
Other genital carcinoma (vagina, vulva, penis and anus)	16, 18, 31, 45, 33, 35, 39, 51, 52, 56, 58, 66, 68, 70.

The cells with latent HPV infection appear normal under microscope and the detection of virus within these cells requires sensitive molecular techniques.

The second mode of viral infection is the productive form in which viral replication is independent of the host chromosomal Deoxyribonucleic acid (DNA) synthesis, resulting in a large number of virions which presence within the cell results in a cytopathic effect, which is recognizable cytologically and histologically as acanthosis, cytoplasmic vacuolation, koilocytosis, multinucleation and nuclear atypia (Mohammed, 2004).

HPV seems to successfully evade the immune system of the body (Coleman *et al*, 1994 ; Nakagawa *et al*, 2000). A significant antibody response to infection is only seen in approximately 50% of infected individuals. These antibodies are directed against the capsid proteins of the virus. An existing high titer of neutralizing antibody may successfully limit infection by HPV. This involves a cytotoxic T-cell response encompassing natural killer cells and antigen-specific cytotoxic T cells. The role of cell-mediated immunity is important in the control of established infections. The latter is evident as the prevalence of HPV infection and cervical intraepithelial lesions is increased in immunocompromised patients (Mohammed, 2004).

OBJECTIVE

The main objective of this study is to determine the prevalence of HPV subtypes in Libyan women in Tripoli.

1- LITERATURE REVIEW

1.1. HPV

HPV is one of the most common causes of sexually transmitted diseases both in men and women worldwide. There are more than 120 types of HPV with approximately 40 anogenital types which are usually divided into low- and high-risk groups based on their oncogenic potential. The common types in the low risk group are 6, 11 and occasionally 42, 43, 44 and 54. Most of the benign lesions such condylomas and genital warts are caused by these types. These types may also be involved in low-grade intraepithelial lesions of the cervix. The high-risk group consists of 16, 18, 26, 31, 33, 39, 45, 51, 52, 53, 56, 58, 59, 66, and 68. This group is generally involved in low- and high-grade intraepithelial cervical lesions and invasive carcinoma of the cervix. Type 16 is the most commonly encountered HPV type in invasive carcinoma. (Garland *et al*, 2002; Stewart and Klienhuess, 2003)

1.1.1. Taxonomy

HPV had originally been grouped together with polyomaviruses in one family, the *Papovaviridae*. This was based on similar, non-enveloped capsids and the common circular double-stranded DNA genomes. It was later recognized that the two groups of viruses have different genome sizes, completely different genome organizations and no similarities in major nucleotide or amino acid sequences, they are now officially recognized by the International Committee on the Taxonomy of Viruses (ICTV) as two separate families — *Papillomaviridae* and *Polyomaviridae*. A modified taxonomy and nomenclature has recently been proposed in phylogenetic tree (Figure1).(de Villiers *et al.*, 2004a).

More information about each species and HPV types within a genus is presented in (Table 2), it shows division of the *Papillomaviridae* into genera and species, following the phylogenetic tree each species, the table lists a type species, other papillomavirus types that belong to these species

and biological and pathological properties of each species. (de Villiers *et al.*, 2004a).

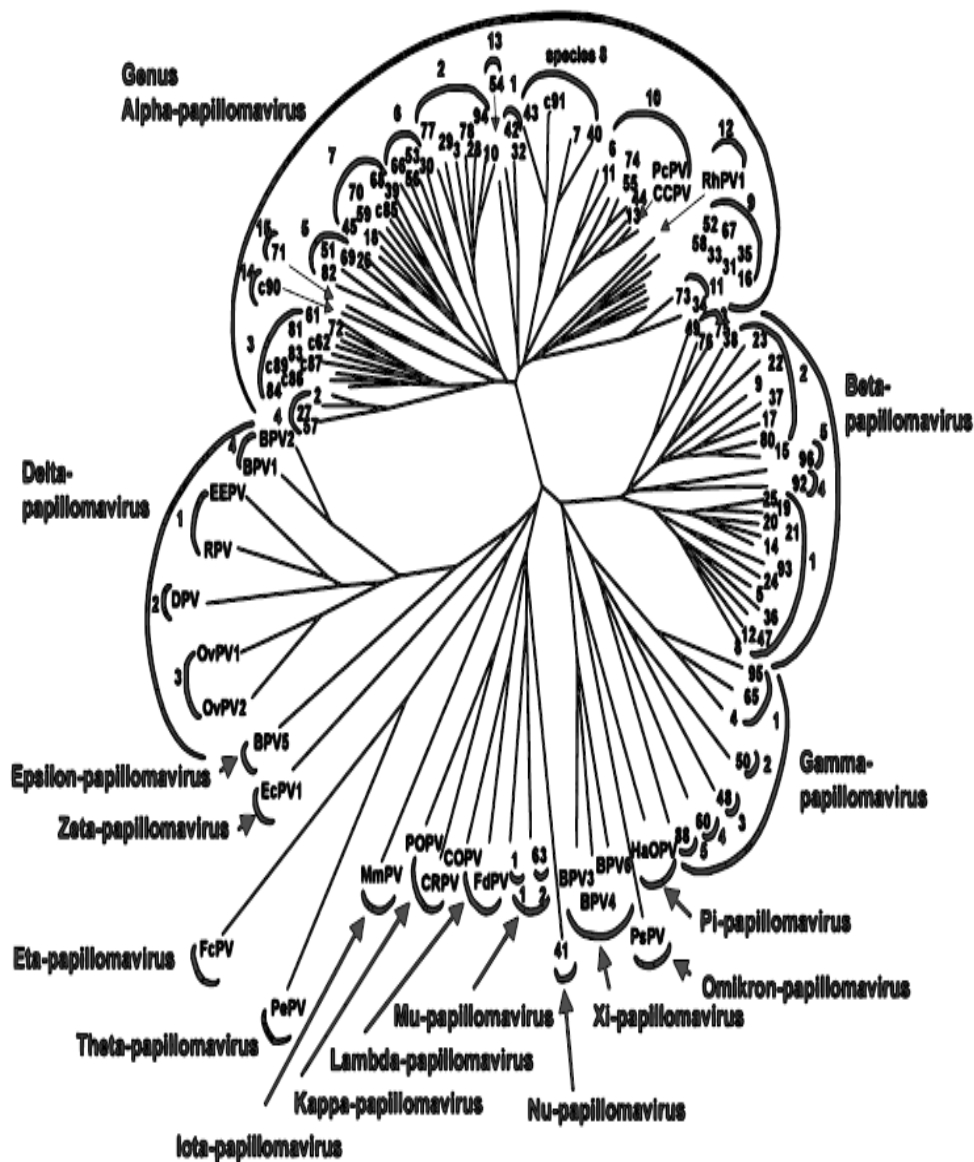


Fig 1. Phylogenetic tree containing the sequences of 118 papillomavirus types (de Villiers *et al.*, 2004a).

Table 2. Characteristics of species within specific genera(de Villiers *et al.*, 2004a).

Genus	Species	Type species	Other papillomavirus types	Comments
Alpha-papillomavirus	1	HPV 32 (X74475)	HPV 42 (M73236)	More frequent in benign lesions (low risk); oral or genital mucosa
	2	HPV 10 (X74465)	HPV 3 (X74462) HPV 28 (U31783) HPV 29 (U31784) HPV 78 HPV 94 (AJ620211)	More frequently cause cutaneous than mucosal lesions; low risk;
	3	HPV 61 (U31793)	HPV 72 (X94164) HPV 81 (AJ620209) HPV 83 (AF151983) HPV 84 (AF293960) candHPV 62 candHPV 86 (AF349909) candHPV 87 (AJ400628) candHPV 89 (AF436128)	Mucosal lesions; low risk
	4	HPV 2 (X55964)	HPV 27 (X73373) HPV 57 (X55965)	Common skin warts; frequent in benign genital lesions in children
	5	HPV 26 (X74472)	HPV 51 (M62877) HPV 69 (AB027020) HPV 82 (AB027021)	High-risk mucosal lesions, also in benign Lesions
	6	HPV 53 (X74482)	HPV 30 (X74474) HPV 56 (X74483) HPV 66 (U31794)	High-risk mucosal, but also in benign lesions
	7	HPV 18 (X05015)	HPV 39 (M62849) HPV 45 (X74479) HPV 59 (X77858) HPV 68 (X67161) HPV 70 (U21941) candHPV85 (AF131950)	High-risk mucosal lesion

Table. 2 (contd)

Genus	Species	Type species	Other papillomavirus types	Comments
	8	HPV 7 (X74463)	HPV 40 (X74478) HPV 43 (AJ620205) candHPV 91 (AF131950)	Low-risk mucosal and cutaneous lesions; HPV 7 also known as butcher's wart virus — often in mucosal and skin lesions in HIV infected patients
	9	HPV 16 (K02718)	HPV 31 (J04353) HPV 33 (M12732) HPV 35 (X74476) HPV 52 (X74481) HPV 58 (D90400) HPV 67 (D21208)	High-risk — malignant mucosal lesions
	10	HPV 6 (X00203)	HPV 11 (M14119) HPV 13 (X62843) HPV 44 (U31788) HPV 74 (U40822) PcPV (X62844)	Mostly associated with benign mucosal lesions; low risk; reports of HPV 6 in verrucous carcinoma
	11	HPV 34 (X74476)	HPV 73 (X94165)	Mucosal lesions — low risk
	12	RhPV 1 (M60184)		Mucosal genital lesions in rhesus monkeys
	13	HPV 54 (U37488)		High-risk mucosal
	14	HPVcand 90 (AY057438)		Low-risk mucosal
	15	HPV 71 (AB040456)		Low-risk mucosal

Table. 2 (contd)

Genus	Species	Type species	Other papillomavirus types	Comments
Beta-papillomavirus	1	HPV 5 (M17463)	HPV 8 (M12737) HPV 12 (X74466) HPV 14 (X74467) HPV 19 (X74470) HPV 20 (U31778) HPV 21 (U31779) HPV 25 (U74471) HPV 36 (U31785) HPV 47 (M32305) HPV 93 (AY382778)	Most frequently cause cutaneous lesions, but reports of DNA in mucosa; commonly associated with lesions in EV or immunosuppressed patients; mostly benign lesions, but reported in malignant lesions, also in immunocompetent patients
	2	HPV 9 (X744464)	HPV 15 (X74468) HPV 17 (X74469) HPV 22 (U31780) HPV 23 (U31781) HPV 37 (U31786) HPV 38 (U31787) HPV 80 (Y15176)	Most frequently cause cutaneous lesions, but reports of DNA in mucosa; commonly associated with lesions in EV or immunosuppressed patients; mostly benign lesions, but reported in malignant lesions, also in immunocompetent patients
	3	HPV 49 (X74480)	HPV 75 (Y15173) HPV 76 (Y15174)	Benign cutaneous lesions
	4	HPVcand92 (AF531420)		Pre- and malignant cutaneous lesions

Table. 2 (contd)

Genus	Species	Type species	Other papillomavirus types	Comments
	5	HPVcand96 (AY382779)		Pre- and malignant cutaneous lesions
Gamma-papillomavirus	1	HPV 4 (X70827)	HPV 65 (X70829) HPV 95 (AJ620210)	Cutaneous lesions; histologically distinct homogenous intracytoplasmic inclusion bodies
	2	HPV 48 (U31790) –		Cutaneous lesions
	3	HPV 50 (U31790) –		Cutaneous lesions
	4	HPV 60 (U31792) –		Cutaneous lesions
	5	HPV 88 –		Cutaneous lesions
Delta-papillomavirus	1	EEPV (M15953)	RPV (AF443292)	
	2	DPV (M11910) –		
	3	OvPV-1 (U83594)	OvPV-2 (U83585)	
	4	BPV-1 (X02346)	BPV-2 (M20219)	
Epsilon-papillomavirus	1	BPV-5 (AF457465)	-	
Zeta-papillomavirus	1	EqPV, AF498323	-	
Eta-papillomavirus	1	FcPV, AY957109		
Theta-papillomavirus	1	PePV, AF420235		
Iota-papillomavirus	1	MnPV (U01834)		
Kappa-papillomavirus	1	CRPV (K02708)		
	2	ROPV (AF227240)		Associated with oral lesions

Table. 2 (contd)

Genus	Species	Type species	Other papillomavirus types	Comments
Lambda papillomavirus	1	COPV (L22695)		
	2	FdPV (AF377865)		
Mu-papillomavirus	1	HPV 1 (V01116)		
	2	HPV 63 (X70828) –		
Nu-papillomavirus	1	HPV 41 (X56147)		
Xi-papillomavirus	1	BPV-3 (AF486184)	BPV-4 (X05817) BPV-6 (AJ620208)	
Omikron papillomavirus	1	PsPV (AJ238373)		
Pi-papillomavirus	1	HaOPV (E15110)		

1.1.2. Virology

HPV are members of the Papovaviridae family. It's small, nonenveloped virus, with a diameter of 55nm. that looks like a golf ball (Figure 2).

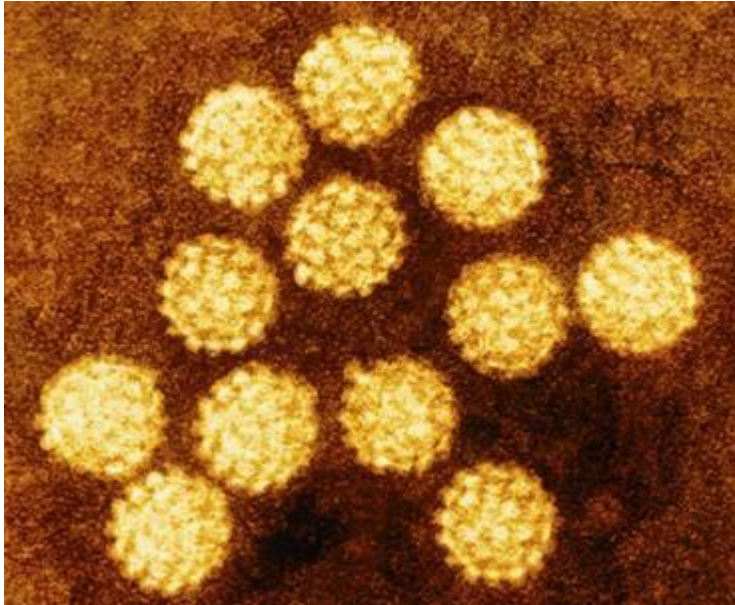


Fig 2. Colorized transmission electron micrograph of the HPV capsid

(<http://web.uct.ac.za/depts/mmi/stannard/papillo.html>)

It has an icosahedral capsid composed of 72 capsomers, which contain at least two capsid proteins, L1 (major) and L2 (minor). Each capsomer is a pentamer of the major capsid protein. Each virion capsid contains several copies (about 12 per virion) of the minor capsid protein. The HPV genome consists of a single molecule of double-stranded, circular DNA containing approximately 7.900 bp associated with histones. (Favre, 1975).

The genome is functionally divided into three regions. The first is a Non-coding upstream regulatory region (URR). This region contains the p97 core promoter along with enhancer and silencer sequences that regulate DNA replication by controlling the transcription of the “early” and “late” regions. The URR region also contains the highest degree of variation in the viral genome. The second is the “early” region, which include the genes E1, E2, E3, E4, E5, E6, E7 and E8. This region is involved in viral replication and oncogenesis. Expression of the early gene products

determines whether an HPV infection is active or latent, or leads to malignant transformation. The third is the “late” region, which encodes the L1 and L2 structural proteins for the viral capsid (Figure 3). HPV gene functions are showed in Table 3 (zurHausen, 1999a).

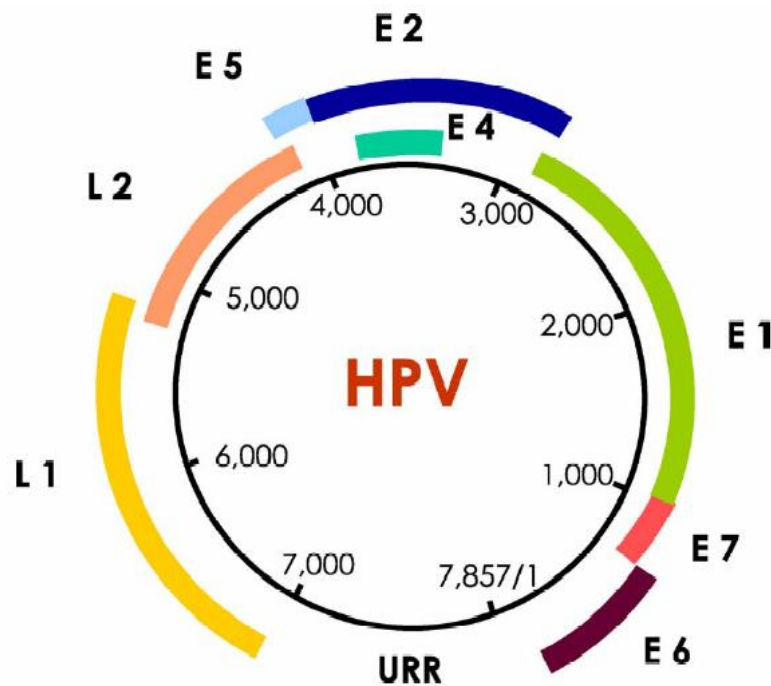


Fig 3. Schematic presentation of the HPV genome showing the arrangement of the early E or nonstructural genes, the capsid genes (L1 and L2) and the upstream regulatory region (de Villiers, 2005)

Table 3. Human papillomavirus gene functions (zurHausen, 1999a).

Gene Category	Gene	Function
Early genes	E 1	Viral replication
	E 2	Modulation of transcription and replication
	E 3	Unknown
	E 4	Productive viral infections
	E 5	Transforming properties
	E 6	Oncoprotein; interaction with p53 protein
	E 7	Oncoprotein; interaction with pRb protein
	E 8	Unknown
Late genes	L 1	Major capsid protein
	L 2	Minor capsid protein

1.1.3. Life cycle

HPV are specifically epitheliotropic and their life cycle takes place within stratified squamous epithelia.

1.1.3.1. Entry

It is assumed that HPV initiate infection by penetrating through microtraumas in the epithelia to reach the basal cells, which are believed to be the target cells for HPV infection. The mechanism for virus entry into the basal cells is not entirely understood. Subsequent steps in the life cycle of the virus can be divided into three stages: establishment, maintenance, and production.

1.1.3.2. Establishment of the non-productive infectious state

Once an HPV particle enters the host cell, it must rely primarily on the cellular machinery to replicate its DNA. In infected basal cells, the HPV genome becomes established as a low copy number nuclear plasmid. Within these cells, only early viral gene products are expressed, and this is consequently referred to as the ‘non-productive’ stage of infection.

1.1.3.3. Maintenance of the non-productive infectious state

A hallmark of HPV infection is its long-term persistence over many years, which, in the case of high-risk types, is a prerequisite for the development of cancer. This requires that the viral genome be maintained over multiple cell divisions; how this is achieved is still unclear.

1.1.3.4. Productive stage

This begins when the daughter cells derived from the infected basal cells start to differentiate. The virus delays the terminal differentiation programme of the cell, and redirects the cell's DNA replicative capacity. This then allows amplification of the viral genome and expression of the late viral genes necessary for the production of progeny virus, and subsequent viral release (IARC, 2007).

1.1.4. Prevalence, geographic distribution

Most sexually active individuals will acquire at least one genotype of anogenital HPV infection at some time during their lifetime. The most comprehensive data on cervical HPV prevalence in women with normal cytology (the great majority of infections do not produce concurrently diagnosed cytological abnormalities) is provided by a meta-analysis including over 150000 women (Castellsague *et al*, 2007; de Sanjose *et al*, 2007).

After adjusting to the extent possible for study design, age, and HPV DNA detection assays, the estimated worldwide HPV DNA point prevalence was approximately 10%. The highest estimates were found in Africa and Latin America (20–30%), and the lowest in southern Europe and South East Asia (6–7%).

The eight most common HPV types (HPV 16, 18, 31, 33, 35, 45, 52, and 58) by geographic region. HPV 16 is the most common type in all regions with levels of prevalence ranging from ~3–4% in North America to 2% in Europe. HPV 18 is the second most common type worldwide (Figure 4).

Generally, similar results for the regional estimates of point prevalence of HPV DNA were observed in an IARC population-based prevalence survey conducted in 15613 women aged 15–74 years from 11 countries around the world (Clifford *et al*, 2005a).

The age-specific prevalence curve showed a clear peak in women up to 25 years of age with subsequent decline until an age range of 35–44 years, and an increase again in all regions included in the meta-analysis except Asia (de Sanjose *et al*, 2007). In the IARC population-based survey, a first peak was observed in women under 25 years of age, and a second peak after 45 years of age in most Latin American populations, but the HPV prevalence was high across all age groups in a few places in Asia and in Nigeria (Franceschiet *al*, 2006). In this survey, the prevalence of high-risk HPV correlates well with cervical cancer incidence, and the strength of the correlation steadily increases with age (Maucort-Boulchet *al*, 2008).

Data on HPV DNA prevalence and natural history of genital HPV infection in men is scant, and difficult to evaluate. There is great variation in the prevalence depending on anatomical sites sampled, sampling methods, and HPV DNA detection assays. In general, the overall HPV prevalence is over 50%, and the proportion of low-risk types is higher in men than in women (Giuliano *et al*, 2008). However, the biological or clinical meaning of the HPV DNA detected in the superficial layers of genital skin is not yet clear. Unlike what has been observed in women, no clear age pattern is detected in HPV prevalence rates in men (Giuliano *et al*, 2008). HPV prevalence is lower in the oral cavity than in other anogenital sites. Among women who practiced prostitution, HPV DNA prevalences for specimens from the cervix, vagina, and oral cavity have been observed to be 27.8%, 26.1%, and 15%, respectively (Canadas *et al*, 2004). HPV infections of the skin are extremely common, but the type distribution is different (beta and gamma genera predominating) than the

mucosal types in the alpha genus that commonly infect the anogenital tract and the oral cavity.



Fig 4. HPV DNA crude prevalence and HR-HPV type-specific prevalence among women with normal cytology by world region: meta-analysis including 157.879 women from 36 countries (IARC, 2007).

1.1.5. Relationship to Cervical cancer (CXCA)

CXCA and Cervical intraepithelial neoplasia (CIN) are essentially caused linked to persistent HPV infection transmitted through genital-to-genital contact. Certain strains of HPV are associated with different risk levels of the transformation into cervical tumors. The majority of invasive cervical cancer contains HPV DNA from high-risk HPV strains 16 or 18 (Munoz, 2000).

1.1.6. Diagnosis

Until recently, the only means of diagnosing HPV infection was direct or enhanced visual inspection, a specific but not particularly sensitive method. Applying acetic acid increases the sensitivity of visual inspection for all HPV infections, but does not distinguish between benign and precancerous lesions (Johnson, 1994).

The past decades have witnessed the introduction of increasingly effective molecular diagnostic techniques to detect HPV infection.

Southern Blot method, developed in 1975, is the bases for all other specific HPV detection methods. In this method, DNA is extracted and enzymatically digested. The resulting fragments are then separated by electrophoresis according to their size. The isolated fragments are denatured and identified with the help of single-strand fragments of complementary DNA or Ribonucleic acid (RNA) labeled with radioactive or calorimetric molecules. This method requires relatively large quantities of DNA, is tedious and is impossible to automate (Cuzick, 1999).

Dot Blot method (ViraPap, Viratype) is a simplified version of the Southern Blot that avoids the enzymatic digestion and electrophoresis stages. It may be partially automated but requires large amounts of DNA and has lower sensitivity and specificity (Cuzick, 1999).

FISH method (*filter in situ hybridisation*) is also derived from the Southern Blot but skips the DNA extraction step. The target cells are applied directly to a solid support and treated to DNA denaturation and identification.

While the method is simple, it does not perform adequately in clinical use (Cuzick, 1999).

In situ hybridization (ISH) is used directly for analyzing biological tissues fixed to microscope slides. The permeability of cellular membranes is enhanced and nucleic DNA is denatured using either alkaline solutions or heat. The steps that followed are similar to those of the Southern Blot.

Recent variants of this method have allowed for typing of all of HPVs and have made automation possible (Cuzick, 1999). ISH detects HPV only in the presence of active synthesis of viral DNA, as opposed to other more sensitive methods (Nuovo, 1998). It's useful for doubtful biopsy results and for diagnosing HPV laryngeal papillomatosis wherein the viral replication is high (Coutlée, 2002).

Hybrid Capture is based on hybridization in a homogenous solution of unmarked single-strand RNA probes that complement the targeted sequences. The kinetics of re-association between the nucleic acid strands work best in a liquid solution, as opposed to the previous tests, which rely on heterogeneous reaction background. The cellular DNA is denatured and hybridized with complete specific RNA probes. The resulting molecular hybrids are then captured in a microplate well or in a tube by universal capture antibodies specific for the DNA-RNA hybrids. After washing, the captured DNA-RNA molecules are detected using multiple monoclonal antibodies conjugated with alkaline phosphatase. The addition of chemiluminescent dioxetane causes the emission of light that can be measured by a luminometer (Coutlée, 2002). This reaction is expressed in relative light units (RLU) corresponding to a 1.0 pg/ml concentration of DNA (Cuzick, 1999). The threshold value recommended in the United States is 1 pg/ml. In other countries, the value varies between 0.5 and 5 pg/ml, depending on the prevalence of HPV infection (Terry, 2001).

The first-generation Hybrid Capture I (**HC I**) was difficult to automate and had a less than optimum sensitivity and specificity. The method was able to detect the presence of one of 9 high-risk HPV genotypes (16, 18, 31, 33, 35, 45, 51, 52 and 56) and one of 5 low-risk types (Cuzick, 1999a), without identifying the specific type of the present HPV (Franco, 2002).

Hybrid Capture II (**HC II**) detects more HPV genotypes (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 68) and its sensitivity is similar to PCR. The format of the test is conducive to automation and large-scale use. The HC II is an effective test that requires less expertise than cytology (Terry, 2001). Currently, the HC II test is the only HPV test available for commercial use (Cuzick, 1999). The method is also simple to learn and allows for easy quality control (Coutlée, 2002).

The Polymerase chain reaction (**PCR**) allows the amplification of the sample DNA, by using a repetitive technique of denaturation, hybridization with complementary primers and DNA synthesis. The method requires minimal amounts of DNA and has a high sensitivity and specificity. The PCR for HPV infection use consensus primers that are capable of amplifying a large number of HPV genotypes. The most used consensus PCR protocols are MY09/MY11, GP5+/GP6+, SPF and PGMY. The MY09/MY11 PCR is less sensitive for HPV 35 and there are occasional variations between batches. A new variant of the method, PGMY09/11, is more sensitive, particularly for HPV 35, 52 and 56 (Coutlée, 2002). The GP5+/GP6+ is better for detecting HPV 43, 35 and 44 but is less sensitive for HPV 53 and 61, as well as for multiple infections. The PGMY and SPF PCR have a better reproducibility because they do not require the use of degenerated primers and are integrated in a nonisotopic typing format (line dot and line probe) according to a standardized protocol and using standardized reagents (Van Duin, 2002). Intralaboratory and interlaboratory reproducibility, along with the precision of these PCRs are excellent for HPV detection and typing (Alary, 2002). Although they are more analytically sensitive than HC II, PGMY and SPF PCRs are possibly less specific (Coutlée, 2002). The detection of the amplimers can be automated, allowing quick analysis of an important number of samples (Cuzick, 1999).

A new method for HPV detection and genotyping is the **DNA Chip**. This method, still under evaluation, uses PCR amplification products and able to identify 22 HPV genotypes. Compared to the HC II, the sensitivity and specificity of the DNA Chip are 100% and 98% respectively, with a correspondence of 98.6% . Other future possibilities are the quantification of high-risk HPV using real-time PCR, the detection of integrated HPV and the use of real-time PCR to measure the transcriptional activity level Messenger RNA (mRNA) of high-risk HPV (Coutlée, 2002).

1.1.7. Treatment

There is no medical treatment for HPV, but as most HPV infections are cleared rapidly by the immune system, in most cases it's unnecessary to treat a virus which may indeed cause no problems (Louise, 2006).

1.1.8. Prevention and vaccination

Condoms offer a degree of protection against the initial transmission of HPV infections. However, as HPV has a field effect and is present over all the genitalia, this protection is not comprehensive or complete.

Two prophylactic vaccines have been developed; Gardasil (Merck) and Cervarix (GlaxoSmithKline). It is hoped that these will avert 70% or more of squamous cell carcinoma (SCC) by preventing many HPV infections. Gardasil is a quadrivalent vaccine, protecting against four types of HPV – 16,18, 6 and 11. Cervarix is bivalent and protects against HPV types 16 and 18. In September 2006 Gardasil was granted a licence by the European Commission for use in children and adolescents aged 9 to 15 years, and in adult females aged 16 to 26 years. (Louise, 2006).

1.2. Cervical cancer (CXCA)

The assumption that CXCA was caused by a sexually transmitted agent was not considered until 40 years ago, when studies by ZurHausen .

demonstrated a potential role of HPV in the development of cervical tumors (zurHausen *et al*, 1974a; zurHausen *et al*, 1974b).

CXCA occurs in a wide range of ages, mainly from mean age of 40 to 50 years. Cervical intraepithelial neoplasia (CIN) on the other hand, which is a precursor of CXCA, is classified histologically into three classes (CIN I- CIN III), based on its severity. CIN III is often called cervical carcinoma in situ. The peak incidence of cervical cancer in situ occurs at younger age as compared to invasive type, in situ cancer is most common around age thirty (Magnusson, 2000).

Both CXCA and precursor lesions have now been firmly associated with high-risk HPV infection (Sincock *et al*, 1992; Wallin *et al*, 1999; zurHausen, 2000).

Currently, the most widely used method to detect HPV DNA is by obtaining a specimen from the cervix. However, some studies have shown that HPV detection by self-collected vaginal swab is comparable to virus detection in swabs obtained directly from the cervix (Sellorset *al*, 2000, Gravittet *al*, 2001). The self-collected vaginal samples also offer advantages over endocervical swabs in terms of acceptability to clients and in reducing the need for trained personnel and clinical materials required for a cervical sample collection (Sellorset *al*, 2000; Dzubaet *al*, 2002). The self-collected sample for HPV detection seems to offer a potential opportunity for more widely accessible cancer prevention and for population based molecular epidemiologic research.

1.2.1. Epidemiology

CXCA is the second most common cancer among women after breast cancer (Mohammed, 2004). Worldwide approximately 466,000 new cases are diagnosed every year and around 1.4 million women are living with invasive cervical cancer, most of whom are in developing countries. CXCA is the leading cause of death among women in these countries. In the

developed countries, the precancerous intraepithelial lesion is recognized early due to mass screening programs, and this has led to a dramatic decrease in the prevalence of invasive cervical cancer (Stewart and Klienhuess, 2003)

CXCA is a particularly significant problem in developing countries, where about 80% of new CXCA cases occur every year (Figure 5) (Parkin, 2000).

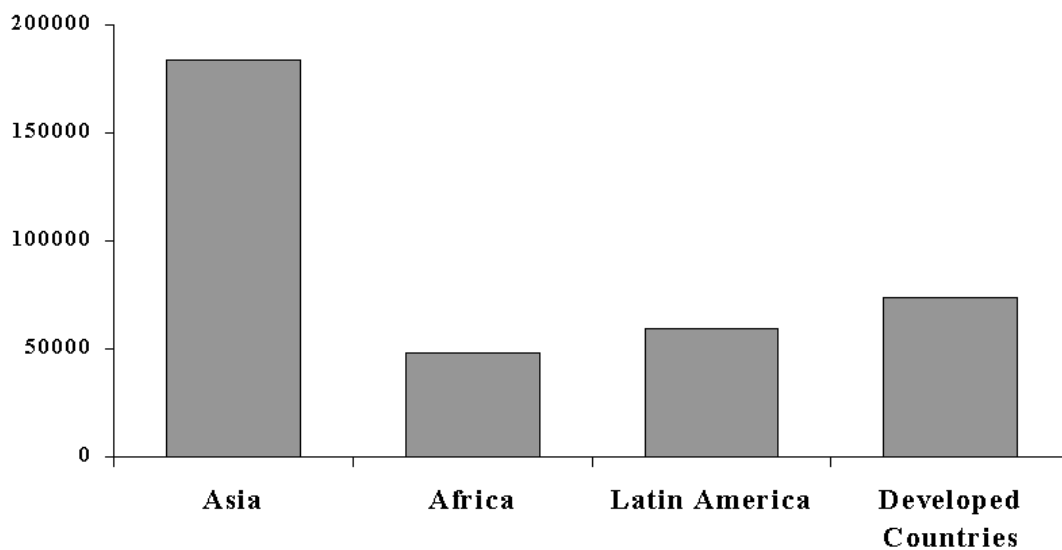


Fig 5. Estimated Number of New Cervical Cancer Cases per Year(Parkin, 2000).

In Riyadh, Saudi Arabia, among 120 samples used in the study, 38 (31.6%) were found positive using PCR for HPV-16 or HPV-18. Of the 38 positive specimens, 16 (42.1%) were positive for HPV-16, 4 (10.5%) were positive for HPV-18, and 18 (47.4%) were positive for both (Table 4). The result of this study showed HPV-16 was 13.3% (16/120), HPV-18 was 3.3% (4/120), and of both HPV-16 and HPV-18 was 15% (18/120). (Al-Muammar *et al*, 2007).

Table 4. Prevalence of HPV-16 and HPV-18 in 120 cervical specimens

		HPV-18		Total
		Positive	Negative	
HPV-16	Positive	18	16	34
	Negative	4	82	86
Total		22	98	120

Positive for both HPV types, 38/120 = 31.6%

In another study in Chiang Mai, Thailand, that included 531 women who were asked to do self-collected vaginal samples. Their ages ranged from 20 to 65 years. Of the 531 tested women, 61 were positive for HPV DNA with an overall HPV prevalence of 11.5% (Table 5). (Wongworapatet *et al*, 2008).

Table 5. Prevalence of HPV infection in the Thailandez study (Wongworapat *et al*, 2008).

Types of HPV	No. Positive sample	Percentage (Total = 531)
All types of positive HPV	61	11.5
High-risk type HPV	27	5.1
Low-risk type HPV	40	7.5
Multiple infection	14	2.6

In Tunisia a study concluded to characterize HPV cervical infection in 106 Tunisian married women and 51 legal prostitutes. Epidemiological data were collected and correlated with HPV molecular genotyping. There was a higher relative frequency of HPV-DNA in prostitutes (39%) than in

married women (14%)($p=0.001$). Molecular analysis of HPV types showed that the most prevalent type in prostitutes was HPV-16, a high-risk oncogenic type. In married women, the most prevalent type was HPV-6 which is associated with a low risk for cervical cancer. HPV-DNA detection was markedly increased in young adult women and in those having recent sexual experience (Hassenet *et al*, 2003).

In a similar study in Algeria a hospital-based case-control study included a total of 198 CXCA cases (including 15 adeno- and adenosquamous carcinomas) and 202 age-matched control women. HPV DNA in cervical cells was evaluated using a PCR technique. HPV infection was detected in 97.7% of CXCA cases and 12.4% of control women (OR= 635). Nineteen different HPV types were found. HPV 16 was the most common type in both CXCA cases and control women, followed by HPV 18 and 45.

Twelve types (HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 66 and 73) were found as single infections in CXCA cases. It is concluded that distribution of HPV types in CXCA cases and control women in Algeria is more similar to the one found in Europe than the one in sub-Saharan Africa, where HPV 16 is less prevalent (Doudja *et al*, 2005).

In Sharkia Governorate, Egypt where a study was concluded with 42 patients with CIN II and III, 30 patients with invasive CXCA, and 45 control women who had undergone hysterectomy for any cause other than CXCA. HPV detection and genotyping in cervical biopsies by PCR and Restriction Fragment Length Polymorphism (RFLP). HPV DNA was detected in 85.7% (36/42) patients with CIN II and III. HPV genotypes were arranged in order of decreasing frequency as follows: HPV 16 50.0% (21/42), HPV 45 in 14.3% (6/42) HPV 33 in 11.9% (5/42), HPV 18 in 9.5% (4/42) and HPV 31 in 7.1% (3/42). In patients with invasive CXCA, 93.3% (28/30) were positive for HPV DNA. In order of decreasing

frequency, HPV genotypes were: HPV 16 in 66.7% (20/30), HPV 18 in 16.7% (5/30), HPV 33 in 10.0% (3 /30) and both HPV 31 and HPV 45 in 6.7% (2/30). About 13.3% of invasive CXCA and 7.1% CIN II & III specimens exhibited multiple infections without significant difference ($P > 0.05$). HPV 16 and HPV 33 infections show a higher risk for development of advanced stages of invasive CXCA but without a statistically significant difference (AbdEl-Azim, 2011).

1.2.2. Pathogenesis

The pathogenesis of cervical cancer is initiated by HPV infection of the cervical epithelium during sexual intercourse (Figure 6). Even though a high percentage of sexually active young women are exposed to HPV infections, only a very small percentage go on to develop cervical cancer (Elfgren, 2000). Several studies have suggested that most women successfully clear the HPV infection, presumably through the action of a competent immune system. Approximately, 90% of lesions regress spontaneously within 12 to 36 months (Chua and Hjerpe, 1996). Other factors such as genetic predisposition, frequency of reinfection, intratypic genetic variation within HPV type, coinfection with more than one HPV type and hormone levels may also influence the ability to clear an HPV infection.

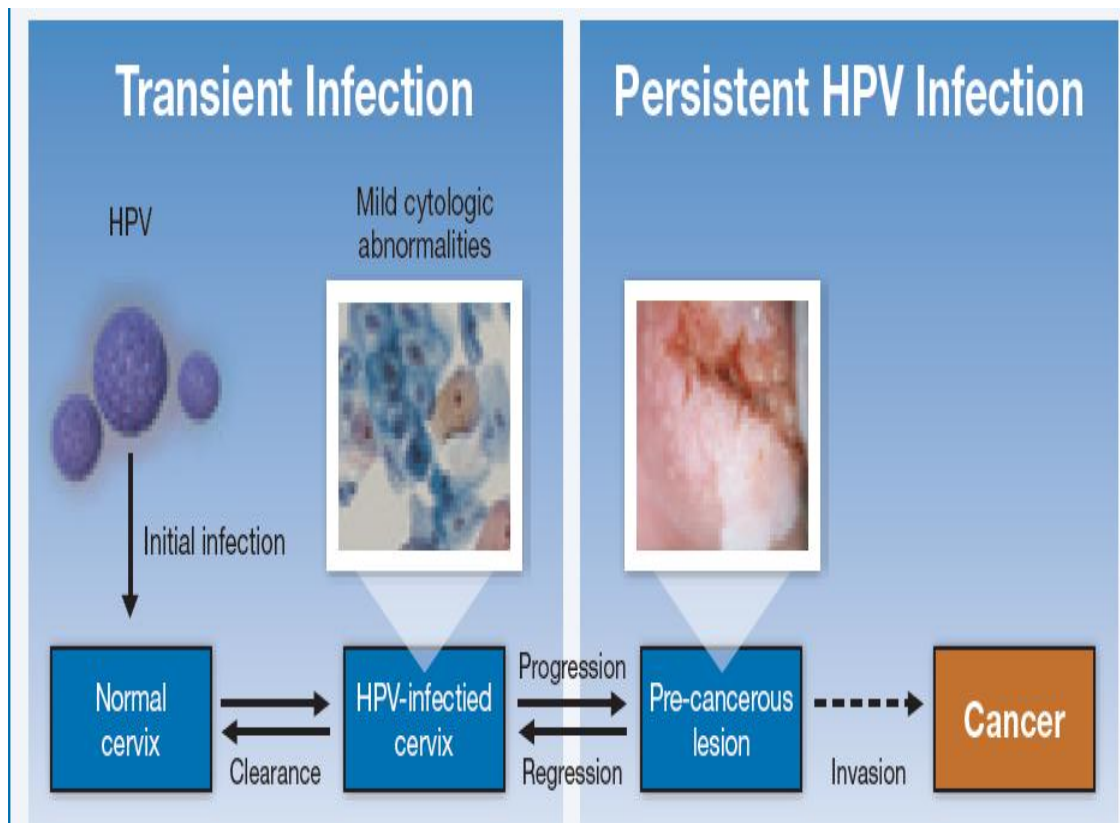


Fig 6. Steps of cervical carcinogenesis (Wright TC and Schiffman M, 2003)

1.2.3. Mortality

Worldwide there are more than 273,000 deaths from cervical cancer each year and it accounts for 9% of female cancer deaths (Yang *et al*, 2004).

2. MATERIALS AND METHODS

2.1. Study design

This study is a case series descriptive study done in Clinic of Saint James Hospital. Fifty three cervical swabs taken randomly from married women of different ages and investigated for the presence of DNA for HPV.

2.2. Study site

Sample collection as well as the detection of HPV DNA were done in the laboratory of Saint James Hospital.

2.3. Sample collection

A total of 53 cervical samples were collected from married women, doing follow up in the Gynecology Clinic of Saint James Hospital using sterile cotton swabs. Cotton tips containing cells were cut off and stored in closed containers with ThinPrep solution at (-20 °C); every container was given a number. HPV DNA was detected using PCR. Ethical approval was obtained from the Department of Biological Science at the Libyan academy. Questionnaires were completed for each case including personal data (name, address, age, nationality).

2.4. Materials Requirement

- Disposable latex gloves (powder free)
- Pipettes (adjustable)
- Sterile pipette tips
- 1.5 ml tube
- PCR tube (0.2 ml)
- Nucleic Acid isolation kit (SPEEDTOOLS DNA EXTRACTION Kit) BIOTOOLS B&M Labs, S.A. Valle de Tobalina - 52 - Nave 39. 28021 Madrid, Spain.
- Seeplex® HPV4A ACE Screening Kit. Seegene Germany: Ludwig-Erhard Str. 30-34, D 65760, Eschborn, Germany.

- Desktop centrifuge
- Vortex mixer
- Gel electrophoresis system (Gel XL Plus) and UV transilluminator (DyNA light Dual Intensity) Labnet, USA.
- PCR machine (GeneAmp PCR System 9700 of Applied Biosystems)
- Biological cabinet
- Refrigerator and freezer.
- Micro tube rack
- Spatulas .
- Biohazard bags .
- Biohazard waste container .

2.5. DNA extraction Kit

The SPEEDTOOLS DNA EXTRACTION Kit is designed for the rapid isolation of highly pure genomic DNA from:

- cultured cells - whole blood – Serum – Plasma .

2.5.1. Kit Reagents

- Buffer BB1
- Reagent BB2
- Buffer BB5 (concentrated)
- Buffer BBW
- Buffer BBE
- Proteinase K (lyophilized)
- Proteinase Buffer

2. 5.2. Preparation of the Reagents

- Proteinase K: was prepared by adding 1.35 ml of Proteinase Buffer to dissolve lyophilized Proteinase K. This solution is stable

at +4°C for up to 6 months, and it's to store of –20°C is recommended if the solution will not be used up during this period.

- Buffer BB3: was prepared by transferring Buffer BB1 to Reagent BB2 completely and mix well. The resulting solution (a label is provided) Buffer BB3 is stable for up to one year at room temperature (20-25°C).
- Buffer BB5: was prepared by adding 28 ml of ethanol (96-100%) to buffer BB5 concentrate. Store Buffer BB5 at room temperature (20-25°C).

2.5.3. Purification of DNA

Purification of the DNA according to following table (Table 6):

Table 6. Purification of DNA.

Step	Description		
1	SAMPLE LYSIS • 25 µl Proteinase K and up to 200 µl of sample (equilibrated to room temperature) were pipetted into 1.5 ml microcentrifuge tube. • 200 µl lysis Buffer BB3 was added to the samples and vortex the mixture vigorously (10-20 s). Incubate samples at 70°C for 10 – 15 min.		200 µl SAMPLE + 25 µl PROTEINASE K + 200 µl BUFFER BB3 mix 70°C 10-15 min
2	ADJUST DNA BINDING CONDITIONS 210 µl ethanol (96-100%) was added to each sample and vortex again.		+ 210 µl ETHANOL mix

Table 6 (contd)

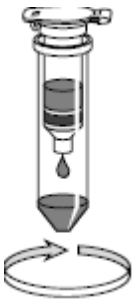
Step	Description		
3	BIND DNA For each preparation, take one column placed in a 2 ml centrifuge tube and load the sample. Centrifuge 1 min at 11,000 RPM. If the samples are not drawn through the matrix completely, repeat the centrifugation at higher <i>g</i>-force (<15,000). collecting tube was discarded with flow through.		Load lysate into a column 1 min, 11,000 RPM
4	WASH SILICA MEMBRANE 1st Wash The column was placed into a new 2 ml collecting tube and it was added 500 µl Buffer BBW. Centrifuge 1 min at 11,000RPM. collecting tube was discarded with flow through and was reused the tube. 2nd Wash The column back was placed into the latter collecting tube and it was added 600 µl Buffer BB5. Centrifuge 1 min at 11,000 RPM collecting tube was discarded with flow through.		+ 500 µl BUFFER BBW 1 min, 11,000 + 600 µl BUFFER BB5 1 min, 11,000 RPM

Table 6 (contd)

Step	Description		
5	DRY SILICA MEMBRANE The column was placed into 1.5 ml microcentrifuge tube and was centrifuged 1 min at 11,000 RPM. Residual ethanol is removed during this step.		1 min, 11,000 RPM
6	ELUTE HIGHLY PURE DNA The column was placed in a new 1.5 ml microcentrifuge tube and was added 100 µl prewarmed elution Buffer BBE (70°C). Buffer was dispensed directly onto the silica membrane. Buffer was incubated at room temperature for 1 min. Buffer was centrifuged 1 min at 11,000 RPM . The eluate was contained your pure DNA sample.		+ 100 µl BUFFER BBE (70°C) Incubate 1 min 1 min, 11,000 RPM

2.6. PCR DNA Amplification

The Seeplex® HPV4A ACE Screening is a qualitative in vitro test for the identification of HPV-16 and 18 and screening of 16 high-risk HPV types (HRC) (26, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66,68, 73, 82) and HPV-6/11 types in liquid based cervical cytology specimens and cervical swabs according to following table (Table 7):.

Table 7. Identification of HPV.

HPV	Detection
HPV-16	Identification
HPV-18	Identification
HRC*	Screening
HPV-6/11	Screening

* HRC (16 high-risk HPV types): 26, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66,68, 73, 82.

2.6.1. Principles

PCR DNA amplification technique is highly sensitive and specific.

Seegene holds a new concept oligo technology, “Dual Priming Oligonucleotide (DPO) technology, which provides freedom in primer design and PCR optimization and maximizes PCR specificity and sensitivity by fundamentally blocking non-specific priming.

The Seeplex® HPV4A ACE Screening is based on three major processes: nucleic acid isolation ; PCR amplification of target DNA using DPO primers; detection on agarose gel electrophoresis.

The Seeplex® HPV4A ACE Screening is designed to simultaneously genotype (HPV-16 and 18) and screening for 16 high-risk HPV types (26, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66,68, 73, 82) / HPV-6/11. The 2X Multiplex Master Mix contains dNTP and enzyme for the specific

amplification of the virus genome and 5X HPV4A ACE PM is primer mixture for ignition of PCR.

The Internal Control has been added to the Seeplex® HPV4A ACE Screening to identify processed specimens containing substances that may interfere with PCR amplification. The Internal Control is a DNA plasmid. These features were selected to ensure equivalent amplification of the Internal Control and HPV-16, HRC, HPV-6 /11 and HPV-18 target DNA. The Internal Control is introduced into each amplification reaction and is co-amplified with target DNA from the clinical specimen.

In PCR, efficiency can be reduced by inhibitors that may be present in the clinical specimen. In addition, the 8-methoxypsoralen (8-MOP) system is used to extinguish the template activity of contaminated DNA. 8-MOP is known to intercalate into double stranded nucleic acids and form a covalent interstrand cross link after photo activation with incident light of wavelength of 320 – 400 nm. Open the PCR tube after UV irradiation (365 nm) for 20 min on amplified PCR products to prevent carry-over contamination.

2.6.2. Reagents

The reagents contain according to following table (Table 8):

Table 8: Reagents contain.

Symbols	Contents	Volume	Description
PRIMER	5X HPV4AACE PM	200 ul	A Primer Mixture (PM) : • primer pairs for HPV • primer pair for internal control • template of internal control
PREMIX	2X Multiplex Master Mix	500 ul	• DNA polymerase • Buffer containing dNTPs (dATP, dCTP, dGTP, dTTP) • MgCl₂ and stabilizers
MOP	8-MOP solution	150 ul	• System to prevent carry-over Contamination
MARKER	HPV 4A ACE Marker	50 ul	Amplicon size markers : • HPV4A Marker is used to identify the approximate size of target product run on a agarose gel electrophoresis
CONTROL+	HPV 4A ACE PC	15 ul	Positive control (PC) : • Mixture of 4 HPV type clones
CONTROL -	HPV 4A ACE NC	15 ul	Negative control (NC) : • Sterilized water as Negative control

2.6.3. Kit Storage

The components of the Seeplex® HPV4A ACE Screening should be stored at -20°C and are stable until the expiry date stated on the label. Repeated thawing and freezing should be avoided, as this may reduce the sensitivity. If the reagents are to be used only intermittently, they should be frozen in aliquots.

2.6.4. Amplification

A- Prepare the reaction Master mix as follows:-

4 μl	5X HPV4AACE PM
3 μl	8-MOP solution
10 μl	2X Multiplex Master Mix
17 μl	Total volume of reaction Master mix

Note: Calculate the amount of each reagent needed based on the number of reactions (samples + controls).

B- Mix by inverting over 5 times or quick vortexing, and centrifuge briefly.

C- Aliquot 17 μl of the PCR Master mix in 0.2 ml PCR tubes and close the tubes.

D- Add 3 μl of each template.

17 μl	PCR Master mix
3 μl	Template
20 μl	Total volume of reaction

- Use a new pipette tip for each sample.
- For the negative control reaction, use 3 μl of the HPV4A ACE NC instead of sample's nucleic acid.
- For the positive control reaction, use 3 μl of the HPV4A ACE PC instead of sample's nucleic acid.

- please be careful not to cross contaminate the reaction Master mix and samples from positive control.

E- Place the tube in a preheated (94°C) thermal cycler.

F- Immediately start the PCR reaction using the following program.

Segment	No. of cycles	Temperature	Duration
1	1	94°C	15 min
2	40	94 °C	0.5 min
		60 °C	1.5 min
		72 °C	1.5 min
3	1	72 °C	10 min

2.7. Agarose gel electrophoresis

2.7.1. Preparing agarose gel

Agarose gels were prepared by adding 1.5 gram of agarose to 5ml of TAE buffer and 45ml of distilled water, and dissolved using Microwave for 2 minutes. The agarose was poured in casting stand with combs of electrophoresis trough and kept at room temperature until solidify. The electrophoresis tank was filled with TAE buffer. 5 µl of Gene Ruler™ 50bp DNA ladder was pipetted into the first hole. 5 µl of Gene Ruler™ loading dye solution was mixed with 5µl of amplified product and pipette into next hole. Electrophoresis unit was covered with lid and the run was started with voltage 100 for 45 minutes. After the run was completed, the gel was incubated into ethidium bromide (EtBr) tank for 3 minutes and transferred to Ultra Violet (UV) light to check for bands with 409 bp. In length for outer amplified product and with 342 base pair(bp) in length for nested amplified product.

2.7.2. Reference value of kit

- Reading the results according to following table (Table 9) (figure 7)

Table 9: Reading the results

HPV4A ACE Screening	Size (bp)
Internal control	1000
HPV-16	588
HRC*	465
HPV-6/11	302
HPV-18	230

* HRC (16 high-risk HPV types) : 26, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66,68, 73, 82.



Fig 7. PCR result using the Seeplex® HPV4A ACE Screening

- **Interpretation of Results**

Interpretation of Results according to following table

(Table 10)

Table 10 : Interpretation of Results

	Internal control	Target	Interpretation
Case 1	+	+	Target pathogen detected. Specimen is positive.
Case 2	+	-	Pathogen not detected. Specimen is a presumptive negative for HPV. A negative result does not preclude HPV infection because results depend on adequate specimen collection, absence of inhibitors, and sufficient DNA to be detected.
Case 3	-	+	Target pathogen detected. Specimen is presumptive positive. These results should be confirmed by retesting.
Case 4	-	-	Inhibitory Specimen. HPV DNA, if present, would not be detectable. Process another aliquot of the original specimen and repeat the test. Inhibitors are often labile and specimens initially inhibitory may not be inhibited when repeated. If the original specimen is not available, a new specimen must be collected.

2.8. Statistical analysis

The data were computerized and analyzed using the statistical package for social science (SPSS) version 16. Statistical analysis was performed using frequency distribution, percentage, Chi2 square and Fisher's Exact Tests. The Fisher's Exact Test was used to evaluate association of age and clinical diagnosis and to evaluate association of age and type of HPV load.

Frequency Distributions

Frequency distributions were used to determine the frequency or number of people that fall into a certain category.

Fisher's exact test

Fisher's exact test is a statistical significance test used for small sample sizes. It is one of a number of tests used to analyze contingency tables, which display the interaction of two or more variables. Fisher's exact test was invented by English scientist Ronald Fisher, and it is called exact because it calculates statistical significance exactly, rather than by using an approximation.

3. RESULTS

During the study, 53 samples were collected from 53 women by sterile cotton swabs and analyzed by PCR for the detection of HPV DNA.

The age range of the study group was between 21-59 years old, with mean age of 36 years old.

3.1. Age groups for individual sample search

The age distribution of the study sample is shown in table (11).

Table 11: Distribution of patients age

Class	Frequency	Percent %
20-24	5	9.4
25-29	6	11.32
30-34	16	30.19
35-39	9	17
40-44	8	15.09
45-49	6	11.32
50-54	2	3.77
55-59	1	1.89
Total	53	100

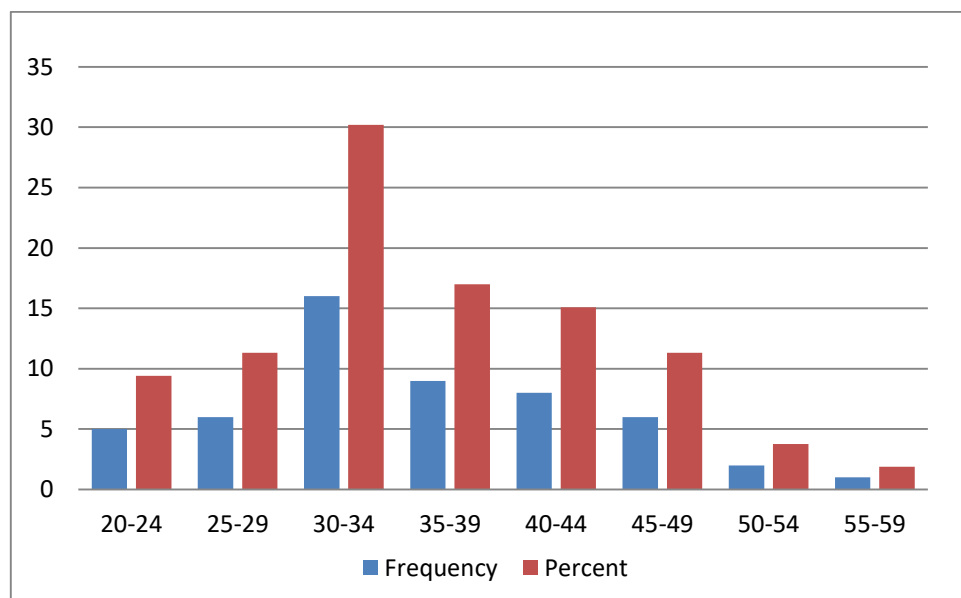


Fig 8: Distribution of patients age

3.2. Diagnosis:

A PCR assay was carried out for 53 sample included in this the results showed 9 samples were positive for HPV (16.98%) (table 12).

Table12:The frequency of HPV in the study group

PCR results	Frequency	Percent %
Negative	44	83.02
Positive	9	16.98
Total	53	100

3.3. Relationship between HPV infection and age :

To find out whether there is a relationship between infection of the virus and age we used Chi² test. Since 13 cells (81.3%) have expected count less than 5 , so we used the Fisher's exact test to verify this relationship.

Table 13: Chi² test to measure the relationship between virus infection and age

Chi-Square Tests			
	Value	df	P value. Sig. (2-sided)
Pearson Chi-Square	11.592 ^a	7	0.115
Likelihood Ratio	11.449	7	0.120
Linear-by-Linear Association	4.298	1	0.038
N of Valid Cases	53		
a. 13 cells (81.3%) have expected count less than 0.5. The minimum expected count is 0.17.			

Table (14) There was significant relationship between age and type of HPV (Fisher's Exact Test is 0.016).

Table14: Fisher's Exact Test to check the relationship between virus infection and age

	Value	Df	P value. (2-sided)	Exact Sig. (2- sided)	Exact Sig. (1- sided)	Point Probability
Pearson Chi-Square	16.557(a)	7	.020	.019		
Likelihood Ratio	16.766	7	.019	.019		
Fisher's Exact Test	13.502			.016		
Linear-by-Linear Association	6.548(b)	1	.011	.011	.005	.003
N of Valid Cases	53					

a 13 cells (81.3%) have expected count less than 5. The minimum expected count is .17.

b The standardized statistic is -2.559.

Table (15) shows the Distribution of patients by age groups

Table 15: Distribution of patients by age groups

age * clinical diagnosis Cross tabulation			clinical diagnosis		Total
			negative	positive	
Age	20-24	Count	2	3	5
		% within age	40.0%	60.0%	100.0%
		% within clinical diagnosis	4.5%	33.3%	9.4%
		% of Total	3.8%	5.7%	9.4%
	25-29	Count	3	3	6
		% within age	50.0%	50.0%	100.0%
		% within clinical diagnosis	6.8%	33.3%	11.3%
		% of Total	5.7%	5.7%	11.3%
	30-34	Count	15	1	16
		% within age	93.8%	6.3%	100.0%
		% within clinical diagnosis	34.1%	11.1%	30.2%
		% of Total	28.3%	1.9%	30.2%
	35-39	Count	9	0	9
		% within age	100.0%	.0%	100.0%
		% within clinical diagnosis	20.5%	.0%	17.0%
		% of Total	17.0%	.0%	17.0%
	40-44	Count	6	2	8
		% within age	75.0%	25.0%	100.0%
		% within clinical diagnosis	13.6%	22.2%	15.1%
		% of Total	11.3%	3.8%	15.1%
	45-49	Count	6	0	6
		% within age	100.0%	.0%	100.0%
		% within clinical diagnosis	13.6%	.0%	11.3%
		% of Total	11.3%	.0%	11.3%
	50-54	Count	2	0	2
		% within age	100.0%	.0%	100.0%
		% within clinical diagnosis	4.5%	.0%	3.8%
		% of Total	3.8%	.0%	3.8%
	55-59	Count	1	0	1
		% within age	100.0%	.0%	100.0%
		% within clinical diagnosis	2.3%	.0%	1.9%
		% of Total	1.9%	.0%	1.9%
Total		Count	44	9	53
		% within age	83.0%	17.0%	100.0%
		% within clinical diagnosis	100.0%	100.0%	100.0%
		% of Total	83.0%	17.0%	100.0%

3.4. Types of human papillomavirus (HPV):

Seven patients (77.8%) were infected with HRC types , one patient (11.1%) was having HPV (type6) and one patient (11.1%) infected by HPV (type 16).

Table 16: Distribution of patients by type virus

type of HPV					
		Frequency	Percent	Valid Percent	Cumulative Percent
Valid	Positive (type 6)	1	11.1	11.1	11.1
	Positive(HRC)	7	77.8	77.8	88.9
	Positive (type 16)	1	11.1	11.1	100.0
	Total	9	100.0	100.0	

Table (17) showed that three of the infected patients (33.3%) were in the age group 20 – 24 years , 3 patients (33.3%) were in the age group 25-29, one patient (11.1%) was in the age group 30-34 years, 2 patients (22.1%) were in the age group 40 – 44 years. No cases were found in the older age groups.

Table 17: Distribution of infection according to age group

Age					
		Frequency	Percent	Valid Percent	Cumulative Percent
Valid	20-24	3	33.3	33.3	3.33
	25-29	3	33.3	33.3	66.6
	30-34	1	11.1	11.1	67.7
	40-44	2	22.2	22.2	100.0
	Total	9	100.0	100.0	

Table (18) showed that one patient had HPV 6(100%), 2 patients (28.6%) had HRC virus were aged 20 – 24 years, 3 patients (42.9%) had HRC virus were aged 25-29, one patient (11.1%) had HRC virus was aged 30-34, one patient (22.1%) had HRC virus was aged 40 – 44 years , one patient (100%) had HPV type 16 virus was aged 40 – 44 years.

Table 18:Distribution of HPV serotype according to age group.

age * type of HPV Crosstabulation						
			type of HPV			
			Positive (type 6)	Positive(HRC)	Positive (type 16)	Total
age	20-24	Count	1	2	0	3
		% within type of HPV	100.0%	28.6%	.0%	33.3%
	25-29	Count	0	3	0	3
		% within type of HPV	.0%	42.9%	.0%	33.3%
	30-34	Count	0	1	0	1
		% within type of HPV	.0%	14.3%	.0%	11.1%
	40-44	Count	0	1	1	2
		% within type of HPV	.0%	14.3%	100.0%	22.2%
	Total	Count	1	7	1	9
		% within type of HPV	100.0%	100.0%	100.0%	100.0%

Table (19) showed that there were 12 cells (100%) have expected count less than 5, therefore we must use Fisher's exact test to verify this relationship.

Table 19: Chi² test to measure the relationship between type of virus and age

Chi-Square Tests			
	6.000(a)	Df	P value. (2-sided)
Pearson Chi-Square	5.716	6	.423
Likelihood Ratio	3.165	6	.456
Linear-by-Linear Association	9	1	.075
N of Valid Cases	6.000(a)		
a. 12 cells (100.0%) have expected count less than 5. The minimum expected count is .11.			

Table 20: Fisher's Exact Test to measure the relationship between type of virus and age.

	Value	df	P value. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)	Point Probability
Pearson Chi-Square	6.000(a)	6	.423	.583		
Likelihood Ratio	5.716	6	.456	0.750		
Fisher's Exact Test	6.494			0.750		
Linear-by-Linear Association	3.165(b)	1	.075	.167	.083	.083
N of Valid Cases	9					

There was no significant relationship between age and serotype of HPV as shown in tables 19 and 20.

4. DISCUSSION

HPV is considered as one of the viruses that cause health problems including its role in the pathogenesis of skin warts, mucosal papillomas and lesions in the anogenital area called condylomas. By far the most serious and clinically significant lesions however are the cervical cancer and precancerous intraepithelial lesions of the cervix mainly caused by infection with some genetic types of the virus, such as 3,6,11,16,18,31,33,41,45,51,53. Infection with HPV can occur at any age group of both sexes.

We undertook this study to identify the genetic distribution of the HPV in the cervix in Libyan women.

Nine from the 53 cases (16.98 %) were positive for HPV. Most of the cases (66.7%) were in the age group (20-29), one case (11.1%) in the age group (30-34) and two cases (22.2%) in the age group (40-44).

Comparing our results with the results of other studies done in neighboring countries like in Riyadh, Saudi Arabia, HPV DNA was detected in 31.6% of 120 samples almost twice the prevalence among our Libyan patients. Serotype 16 was found in 42.1% compared to 11% among our patients and serotype 18 in 10.5% of the Saudi patients but we could not find it among our Libyan patients. Serotypes 16 and 18 were found together in 47.4% of the Saudi patients but we did not find such double infection among our patients. These important differences could be attributed to real differences or to differences in the technique used by the researchers in Saudi Arabia.

In the study done in Tunisia, that included a study was conducted on 106 married women and 51 legal prostitutes (total of 157 cases), there was a higher relative frequency of HPV-DNA among the prostitutes (39%) than

among the married women (14%) ($p=0.001$) the later is almost equal to the prevalence among the Libyan women in our study. Molecular analysis of HPV types showed that the most prevalent type in prostitutes was HPV-16, a high-risk oncogenic type. In married women, the most prevalent type was HPV-6 which is associated with a low risk for cervical cancer. HPV-DNA.

In Algeria, a study was conducted on 198 women with cervical cancer and 202 women who used as standard samples for the comparison, and the results were as follow (97.7%) of the cases of women with cervical cancer and (12.4%) of women who used as standard samples for the comparison. This number is also near to the prevalence of HPV among the Libyan women in our study. The most commonly observed serotype was HPV16 in 19 cases in both categories.

Finally, we found that the prevalence of HPV among the Libyan women in this study is quite similar to its prevalence in neighboring countries the fact that gives force to the methodology and results of our study.

5.1. CONCLUSIONS

This study showed a strong relationship between age and the extent of the spread of the virus since most of the cases were younger than 40 years.

The prevalence of HPV among the Libyan women is low and is similar to its prevalence in neighboring countries.

5.2. RECOMMENDATIONS

- Further studies to include more cities Libya and a larger sample size are required.
- Work on the establishment specialized centers for epidemiological surveillance of such diseases.
- Educational campaigns for women and young girls explaining the importance of early and periodic gynecologic checkups.
- The use of vaccine against HPV in young girls to reduce the risk of HPV infection and hence cervical cancer.

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