بسم الله الرحمن الرحيم

Sudan University of Science and Technology

College of Graduate Studies

Detection of Human Papilloma Virus Gentotype -68 Among Cervical Cancer Patients UsingPCR Technique in Khartoum State

الكشف الجزيئي عن فيروس الورم الحليمي البشري نوع-68 لدى مرضى سرطان عنق الرحم بإستخدام تفاعل البلمرة المتسلسل في ولاية الخرطوم

A dissertation submitted for partial fulfillment for the requirement of M.Sc. degree in medical laboratory science(histopathology and cytology).

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الآيـة

بسم الله الرحمن الرحيم

قال تعالى:

فَتَعالى اللّـهُ المَلِكُ الحَقُّ وَلا تَعجَل بِالقُرآنِ مِن قَبلِ أَن يُقضى إِلَيكَ وَحيُهُ وَقُل رَبِّ زِدني عِلمًا ﴿١١٤﴾

صدق الله العظيم

سورة طه الآية 114

Dedication

To the spirit of my father

To the spirit of my precious mother

To my sisters

To my brothers

To my friends

Acknowledgments

First of all, I'm very thankful for Allah the all powerful for giving me everything.

Special thanks to Dr. Ibrahim Bakhit Yousif for his endless support, his patient to follow up and his invaluable guidance and directions.

Also great for all those helpful who proved their loyalty by kind standing and support.

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List of abbreviations

Abbreviation	Full term
CC	Cervical cancer
CDC	Central for disease control and prevention
DNA	Deoxyribonucleic Acid
HPSGs	Heparin Sulfate Proteoglycans
HPV	Human papilloma Virus
HR-HPV	High Risk Human papilloma Virus
IARC	International Agency for Research on Cancer
ICTV	International Committee on Taxonomy of Viruses
PCR	Polymerase Chain Reaction
PV	papilloma Virus
STI	Sexual Transmitted Infection
NALB	Nucleic Acid lysis Buffer

Abstract

This is a descriptive, retrospective case study conducted at research lab inSudan University of Science and Technology,department of histopathology during the period from January to September 2017The study aimed to assess the association between HPV-68 infection and cervical cancer among Sudanese patients through detection viral DNA using PCR technique, there by 40 formalin fixed embed tissue blocks of different cervical cancer type were collected.

The tissue blockswere cut byRotary microtome, prepared for DNA Extraction and then the viral DNA was detecting using PCR and agarose gel electrophoresis, TheData was analysis manually, frequencies and percentage were calculated.

The study includes 40 women their ages ranged between 30 to 70 years with mean age 50, Most of patient were more than 40 years representing 35/40 (88%) and the remaining less than 40 is 5/40 (12%).

The study showed that keratinized cervical cancer represents 87% and non-keratinized is 13%.

The results of the DNA polymerase chain reaction of the human papillomavirus-68 were negativein all cervical tissue samples.

The study concluded that HPV-68 does not play a role in causing cervical cancer.

// مستخلص الدراسة

أجريت هذه الدراسة الوصفية الإسترجاعية بمختبرالأبحاث قسم الأحياء الجزيئية بكلية المختبرات الطبية–جامعة السودان للعلوم والتكنولوجيا خلال الفترة من يناير الى سبتمبر ٢٠١٧م.

هدفت الدراسة إلى تقييم العلاقة بين فيروس الورم الحليمي البشري -٦٨وسرطان عنق الرحم لدى المرضى السودانيين من خلال الكشف عن الحمض النووي الفيروسي باستخدام تقنية تفاعل البلمرة المتسلسل وطريقة فرز الكهربائي ، جمعت أربعون عينة ٤٠ نسيج محفوظة بالفورمالين ومثبتة في قوالب شمع البرافين من مختلف انواع سرطان عنق الرحم قطعت قوالب الأنسجة لشرائح رفيعة بواسطة المشراح الدوار ، أعدت لاستخراج الحمض النووي ومن ثم تم الكشف عن الحمض النووي الفيروسي باستخدام جهاز تدوير تفاعل سلسلة البوليميريز وطريقة الفرز الكهربائي على هلام الأغاروز ، وكان تحليل البيانات يدويا حيث حسبت الترددات والنسبة المئوية.شملت الدراسة أربعين إمراة تراوحت أعمارهن بين ٣٠ و ٧٠ سنة بمتوسط عمر ٥٠ عاما، وكان معظم المرضى من الذين اعمارهن أكثر من ٤٠ سنة و يمثلون ٤٠/٣٥ (٨٨٪) والباقي أقل من ٤٠ سنة ومثلون ٤٠/٥ بنسبة (١٢٪).في هذه الدراسة وجد ان سرطان عنق الرحم الكيراتيني يمثل ٨٧ ٪وغير الكيراتيني يمثل١٣٪ .كانت نتائج تفاعل سلسلة البوليميريزللحمض النووي لفيروس الورم الحليمي البشري نوع -٦٨ سلبية في جميع عينات نسيج عنق الرحم.

خلصت الدراسة إلى أن فيروس الورم الحليمي البشري نوع -٦٨ ليس له دور في تسبيب سرطان عنق الرحم .

Chapter one

1.1. **Introduction:**

Cervical cancer (CC) is the third most common cancer in women worldwide with the burden being highest in developing countries,

Human papillomavirus (HPV) has been implicated with a high frequency in the etiology and pathogenesis of dysplasia and carcinoma of the uterine cervix, as probable high-risk types(Arbyn *et al.*, 2011).

Human papillomavirus (HPV) is the most common sexually transmitted infections among women worldwide. Cervical pre-malignant lesions and cervical carcinoma which is one of the most common cancers among women worldwide is associated with persistent infection caused by high-risk HPV genotypes (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68). In contrast, other genotypes, such as HPV 6, 11, 40, 42, 43, 44, 54, 61, 70, 72, and 81 have been designated as low-risk HPV and rarely, if ever, lead to cancer (Remmink *et al.*, 1995).

Papillomaviruses are ubiquitous and have been detected in a wide variety of animals as well as in humans and are specific for their respective hosts, more than 200 types of HPV have been recognized on the basis of DNA sequence data showing genomic differences,HPV is associated with a variety of clinical conditions that range from innocuous lesions to cancer(Eileen. Burd.2003).

1.2. Justification:

Cervical pre-malignant lesions and cervical carcinoma which is one of the most common cancers among women worldwide is associated with Persistent infection caused by high-risk HPV genotypes (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68), Many Sudanese studies were focused on 16, 18, but other HR types were not holt on study, in this study we attempt to investigate the present of HPV-68 as there is no published data regard this geno type in Sudan.

1.3. Objectives:

1.3.1. General objective:

To detect human papillomavirus type 68 among adult women with cervical cancer using PCR.

Chapter Two

2. literature review

2.1. Background: -

Cancer of the cervix is the third most common cancer in women worldwide, with an estimated incidence of 529,000 cases and 274,000 deaths occurring in 2008 (Ferlay *et al.,* 2010) More than 85% of the cases occur in developed countries such as China. The "two peak" pattern pertained to both rural and urban women. The crude HR-HPV prevalence was seen to peak among urban women aged 15-24 years (18.7%) and among women older than 40 years of age (16.0%). Among rural women, it peaked at age 15-24 years (16.2%) and 35-39 years (18.6%) (Zhao *et al.,* 2012)This pattern differs from that observed in women from Western countries, among whom, HPV prevalence peaked only at their mid-twenties, then steadily decline as age increases (Moscicki *et al.,* 2010). The first peak in China may be due to the fact that younger women are more sexually active and more likely to have multiple partners, especially in urban areas (Zhao, *et al*., 2012).

2.2. Epidemiology of HPV infection: -

HPV transmission are the most common sexually transmitted infections in the United States. About 14 million new genital HPV infections occur each year. (Satterwhite *et al*., 2013) In fact, the Centers for Disease Control and Prevention (CDC) estimates that more than 90 percent and 80 percent, respectively, of sexually active men and women will be infected with at least one type of HPV at some point in their lives. (Chesson *et al*., 2014).

High-risk HPVs cause several types of cancer, virtually all cases of cervical cancer are caused by HPV, and just two HPV types, 16 and 18, are responsible for about 70 percent of all cases, (Winer *et al*., 2006)

Fifty-eight biopsies of SCC from Algerian women were analyzed to assess the presence of low and high-risk HPV strains. PCR and Hybrid Capture 2 tests revealed that out the 58 studied samples 51 (88%) were positive to HR-HPV strains and 7 (12%) were HPV infection free. (Abdel Halim *et al.*, 2013).

Prevalence of HPV and cervical lesions byISH using pan HPV probe tested on 217 Egyptian women, was positiveamong 66%, negative in 29% and non-significant in5% of cases. (Howayda *et al.*, 2007)

2.3. Papillomaviruses structures: -

HPV is a small (52–55 nm), non-enveloped virus with a circular, double-stranded DNA. more than 100 different HPV genotypes have been identified based on differences in DNA sequence. These HPV types can be classified according to various criteria, e.g. their tissue tropism, oncogenic potential and phylogenetic classification. More than 30 types are commonly found in the genital tract. These include several HPV genotypes, such as HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73 and 82 that are known as high-risk HPV, capable of causing progression to cancer in the uterine cervix, and further three genotypes (HPV 26, 53, and 66) Papillomaviruses (PV) are small viruses, belonging to the Papillomaviridae family. They are widespread in nature, and have been identified in many animal species (Arbyn *et al.,* 2011) PV are small, non-enveloped DNA (deoxyribonucleic acid) viruses that measure 55 nanometers (nm) in diameter and comprise an icosahedral capsid composed of 72 pentameric capsomers of the major capsid protein L1, partly associated with the minor capsid protein L2. Enclosed within the capsid is the viral genomic DNA which is packaged as a minichromosome. (Globocan *et al.,* 2008).

2.4. Papillomavirus taxonomy: -

PV phylogenetic classification is based on the nucleotide sequence of the open reading frame encoding the major structural protein L1, as specified by the International Committee on Taxonomy of Viruses(ICTV). PV of different genera share less than 60% identical L1 nucleotide sequences, PV within a genus share 60 to 70% identity, while an identity between 70% and 90% defines a species. PV subtypes show 90 to 98% and variants more than 98% L1 nucleotide sequence identity. The Papillomaviridae family presently consists of 189 PV types spread over 29 genera. The Human Papillomavirus (HPV) types are grouped in 5 genera; the alpha (α) genus contains the HPV types most frequently associated with human diseases. Based on their tropism, HPV can be separated into cutaneous (skin) and mucosal (genital) types. Mucosal HPV are mainly sexually transmitted, and the risk of infection increases by increasing number of sexual partners. Genital HPV infections are the most common sexually transmitted infections Mucosal HPV types are also classified on the basis of their oncogenic potential; according to the last evaluation by the International Agency for Research on Cancer (IARC) expert group, types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 are carcinogenic to humans (Group 1A) and designated “high-risk” types; the types 26, 53, 66, 67, 68, 70, 73 and 82 are defined as probable possibly carcinogenic (Groups 2A and 2B) (Niccolai *et al.,* 2013).

2.5. HPV life cycle: -

Papillomaviruses display an unusual life cycle. Unlike most viruses that infect a target cell and produce progeny virus from that same cell, in papillomavirus infections the initially infected basal cell must undergo mitosis and differentiate to produce new virions. Moreover, they do not encode polymerases or other enzymes necessary for viral replication, relying

on the host cell replication proteins to mediate viral DNA synthesis. Two modes of viral DNA replication are recognized (Shafti *et al*., 2003).

the reproductive replication, where the virus enters the lower portion of the epidermis and the plasmid is maintained by low level replication in synchrony with the cell and the vegetative replication, which occurs in the more differentiated epithelia cells in the absence of cellular DNA synthesis.

The mechanisms regulating the switch from plasmid maintenance to vegetative viral DNA replication are not known, and may involve changes in cellular and/or viral factors occurring in differentiating keratinocytes (Shafti *et al*., 2003).

Infection by HPV requires the virus particles to gain access to and enter in the cells of the epithelial basal layers. Binding and entry of the virus are mediated through the viral coat proteins (L1 and L2) and heparin sulfate proteoglycans (HPSGs) and/or α6β4 integrin's (way not conserved among the HPV types. Virus uptake mostly occurs by clathrin-coated endocytosis (not conserved among all HPV types, (Globocan., 2008)**.**

disassembling of the viral particle in the late endosome/lysosome and transfer of the viral DNA to the cell nucleus (facilitated by L2 protein).

Expression of the early proteins E1 and E2 establishes the viral genomes an episome and leads to the expression of the other early proteins (E4, E5, E6 and E7). This phase is characterized by a low HPV copy number (approximately 10 to 200 viral copies per cell) Upon cellular differentiation, a late viral promoter is activated and drives expression of late proteins' (McLeod *et al,* 2010)Expression of L1 and L2 is restricted to cells of the granular layer with viral particle assembly taking place in the cornified layer. (Arbyn *et al*,.2011).

2.6.Human papilloma virus transmission:-

Papillomaviruses are a diverse family of viruses found in most mammals and birds, capable of causing epithelial tumors in humans and some related species. The name derives from the Latin word *Pailla* - a nipple-like projection and the Greek word *oma* - a swelling or tumor. The types found in humans are termed human papillomavirus (HPV) and constitute a large family in the human host; over 100 HPV types have been identified, of which 40 infect the genital tract. Many of these types have been shown to be ubiquitously distributed around the globe (deVilliers *et al,*. 2004**).**
and some appear to have become more common in the population (Laukkanen *et al*,. 2003).

HPV transmission occurs primarily during sexual activity, and as such HPV infection is the world's most common sexually transmitted infection (STI). The prevalence in women is most common before age 30, but there is a second incidence peak later on in life. In men, however, the prevalence is more stable across all ages (Moscicki *et al*,. 2012).

Most infections are asymptomatic with most women (75-80%) infected with HPV at some time during their life, but longitudinal studies show that 90% of infections are cleared within one-two years, it is unclear whether HPV is sufficiently infectious to be transmissible during the entire duration of the infection t a (Tota *et al*,.2011).

Oral HPV is mainly transmitted through oral sex or open-mouth kissing, whereas genital HPV is transmitted through skin-to skin contact during sexual activity, and not the exchange of body fluids like in other (bacterial) STI's such as Chlamydia and gonorrhea. This means that protection conferred by condoms exists, but is not complete. Other transmission routes

known for HPV include vertical transmission from mother to infant, a risk which is greater after vaginal than caesarean birth (Tseng *et al*,.1998).

HPV has a particular affinity to so-called squamocolumnar junctions, where there is a transition between squamous and glandular cells in an epithelium. Such junctions are found in the cervix, anus and tonsils and HPV is also capable of residing on skin tissue, e.g. on the penis. In addition to this, HPV infections appear capable of site-to-site transmission (or auto-inoculation), meaning that a woman who is cervicovaginally positive may well become anally positive for the same type within a certain time interval and indeed, anal HPV infections are common in young women (Castro *et al*,.2012).

HPV types are traditionally divided into low-oncogenic-risk types and high-oncogenic risk types referred to as low-risk (LR-HPV), and high-risk (HRHPV), respectively. This since the different types exhibit different disease-causing characteristics. The most noted low-risk types, where cancer risk is negligible, are HPV6 and HPV11, responsible for 90 percent of genital warts, and causative of the rare disease recurrent respiratory papillomatosis (Munoz N *et al* ,.2006).

Around 13-15 HR-HPV types have been identified, the most noted of which are HPV16 and HPV18, together held accountable for 70% of cervical cancer tumor cases and are as such the primary targets for globally intended vaccines (see further below). HPV16, which has been described as "the major player" (Gillison *et al*,.2012).

occupies a particularly important place in the HPV-related disease pantheon due to its strong carcinogenicity, Apart from viral genotype, it is now understood that persistence of infection is a main determinant of cancer risk in HPV-infected women, although only 10-30% of infections will persist

beyond 2 years ,Persistence of HPV infection is defined as infection detected a number of years (such as 4-12 years) after incident infection, and it appears that long-term persistence of HR-HPV without development of cervical disease is less common than previously thought, The potential for latency, re-activation and/or re-infections with HPV is still however not fully understood (Trottier *et al,*. 2010), although repeated infections appear common in young women (Moscicki *et al*,. 2010).

It is well established that persistent infection with high-risk (HR) human papillomavirus (HPV) is the most important cause of CC, causing the vast majority of cases (Burchell.*et a*l, 2006).

Fifteen HPV types have been epidemiologically established as oncogenic or HR: 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73 and 82. A further twelve types have been classified as low-risk: 6, 11, 40, 42, 43, 44, 54, 61, 70, 72, 81, and CP6108 (D'S-ouza *et al*,. 2009).

HR-HPV types 16 and 18 account for around 70% of all CC cases (Winer *et al*, 2006).

2.7.HPV infection: -

HPV infections are the most common sexually transmitted infections in the United States. About 14 million new genital HPV infections occur each year. (Satterwhite *et al*, 2013**)** In fact, the Centers for Disease Control and Prevention (CDC) estimates that more than 90 percent and 80 percent, respectively, of sexually active men and women will be infected with at least one type of HPV at some point in their lives(Chesson *et al*,. 2014). Around one-half of these infections are with a high-risk HPV typeand Most high-risk HPV infections occur without any symptoms, go away within 1 to 2 years, and do not cause cancer, (Hariri et al., 20011).

28.Cervical cancer:

About 70% of cervical cancers occur in developing countries, representing 13% of female cancers. (Ferlay et al., 2010) In low-income countries, it is the most common cause of cancer death(In developed countries, the widespread use of cervical screening programs has dramatically reduced rates of cervical cancer.(Canavan and Doshi, 2000)

2.9Cervical cancer subtypes: -

Histologic subtypes of invasive cervical carcinoma include the following, though squamous cell carcinoma is the cervical cancer with the most incidences. (Kumar et al., 2007) Squamous cell carcinoma (about 80-85%), adenocarcinoma (about 15% of cervical cancers in the UK, (Cancer Research UK website, 2009) .

2. 10.Cervical cancer in Sudan: -

In Sudan hospitals in 2000, cancer was the third leading cause of death after malaria and viral pneumonia, accounting for 5% of all deaths. In women, breast, cervical and ovarian cancer remained the three most common cancers. Breast and Cervical Cancer account for about 50% of all cancers in Sudanese women. (Hamad, 2006).

According to hospital-based statistical data from the Radiation and Isotopes Centre of Khartoum and the National Cancer Institute of the University of Gezira in Wad Medani, Gezira State, cervical cancer is the second most common cancer type among women in Sudan, breast Cancer 29 –34.5% Cervical Cancer 12 –15.5%. (Hamad, 2006).

In Sudanthere are 923 new cases of cervical cancer representing 4.5/100,000In Sudan, invasive cervical cancer is leading cause of cancer death among women. (Hassan and Khirelseed, 2009).

Chapter Three

Material and Methods

3.1. Study design:

This study was a descriptive retrospective study

3.2 Study area:

The study was done in Omdurman Military Teaching hospital, from the period January to September 2017, to investigate the presence of HPV -68 in cervix cancer in Khartoum State.

3.3. Study duration:

This study was conducted in the period from January to September 2017.

3.4. Study Sample:

Populations subjected in this study were paraffin tissue blocks already diagnosed with cervical squamous cell carcinoma

3.5. Exclusion criteria

Women without Cervical cancer were excluded from this study.

3.6. Ethical consideration: -

Permission to carry out this study was taken from the college of graduates studies, Sudan University for Sciences and Technology.

3.7. Sample size

Sample size was forty samples with cervical squamous cell carcinoma.

3.8. Data collection tools and variables

Master sheets were used to record all patients and sample data; age, presenting symptom, type of SCC, residence and residence. Master sheets were also used to record all PCR result.

3.9. Data presentation: -

Data was presented in a form of tables.

3.11. Data analysis: -

The collected data was recorded and analyzed manual analysis, frequency and mean were calculated.

3.1.1 Sample processing:

Onesection from each block measures four micrometers was cut using Leica microtome (Leica Microsystems, Nussioch Gmbll, model: RM 2125RT, ser NO. 8843/04-2005-China),Another one section measured ten microns was placed in Eppendorf tube for PCR method.

3.13. Methods of detection: -

PCR technique (PCR machine Model Lab Ltd politer thermal cycle - Germany) Cycler,40 Senso Quest GmbH D-37085, Hannah-Vogt-Germany) was used to detect HR- HPV, Geno type 68 infections in paraffin sections using specific primers.

3.14.DNA extraction from histological paraffin sections:-

3.14.1.Removal of paraffin: -

Ten microns' sections from each tissue block were cut with a Rotary microtome and then placed in a labeled Eppendorf tube. An empty paraffin block was sectioned in between tissue blocks to avoid cross-contamination.

Then 1ml xylene was added in each labeled Eppendorf tube for 10 min and then centrifuged at 14000 rpm for 10 min, then supernatant was discharged.

This step was repeated at once, 1 ml of absolute ethanol was added and the tubes were centrifuged for 10 minutes' supernatant was discharged

Then 80% ethanol 1 ml was added for 10 min and then was centrifuged at 14000 rpm for 10 min then supernatant was discharged and repeat this step.

Then 50% ethanol 1 ml was added for 10 min and then was centrifuged at 14000 rpm for 10 min then supernatant was discharged and repeat this step at once time, Then 1 ml H_2O was added and incubate at 4C for overnight.

3.14.2.Digestion of protein: -

Samples were centrifuged at 14000 rpm for 10 min then supernatant was discharged, and 700ul of nucleic acid lysis buffer was added, then 50ul of proteinase k was added, then incubate for 24hrs at 65°C, and additional 50ul proteinase-k was added and incubate for 24hrs at 65°C.

3.14.3.Precipitation and Isolation of DNA: -

Saturated NACL 250ul was added, the tubeswere stand at room temp for 10 min, and then was centrifuged at 14000 rpm for 10 min then supernatant was aspirated to clean micro centrifuge tube, and 1 ml of ice-col 100% ethanol was added and mixed and placed at -20°C for 20min ,and centrifuged at 14000 rpm for 10 min, then supernatant was discharged, and wash with 1.5 ml of 70% ethanol, was add then centrifuged at 14000 rpm for 10 minutes, and supernatant was discharged, allow to air dry for 10 min , and then 60ul of TE buffer was added

3.15. HR-HPV PCR detection:

E1, genes regions of HR-HPV type (68*)*, was detected PCRwas done using Maxime PCR Premix kit (iNtRON, Korea) and using specific primers from (Macrogen, Korea), ready mix, the primers sequences were.

Reversed: 5-AAGGCACARGGACACAACA-3

Forward: 5-GCGTCCTAAWGGRAAYTGGTC-3

To each sample in the new sterile tube 5 µl of PCR-mix-1, 6 µl of 2, 5 x buffer and 2 µl of TaqF Polymerase were added to prepare Reaction Mix. Then 10 µl of Reaction Mix was added to each sample tube, then to appropriate tube 2 µL of qualified DNA sample was added.

(Amplicon (bp) was 250-325 bp).

For HPV an initial denaturation at an initial denaturation step was 94°C for two minutes followed by 40 cycles of 30 seconds for secondary denaturation

(94°C), 20 seconds for annealing (63°C), 40 seconds for first extension (72°C), with three minutes to additional extension step at 72°C after the last cycle.

3.15.1. Gel electrophoresis: -

1% agarose gel in 1 X TBE was prepared first, thereafter five µl of the PCR product was placed in well of gel electrophoresis (S/N 0706.11016 Biometra GmbH, Rudolf-Wissell-Straße 30, D-37079 Göttingen-Germany) for 20 min (V 59, A 34). After that gel electrophoresis was estimated under ultra violet using Gel documentation (S/N BUV025080832/1504-586, Biometra GmbH, Rudolf Wissell-Straße 30, D-37079 Göttingen- Germany) to detect presence or absence of band for HPV-68.

Chapter four

Result: -

A total of 40 cases (patients with histopathological confirmed cervical squamous cell carcinoma) was included in this study. The age of patients was ranged from 30-70 years with average mean of 50 years, most of patients were above forty, and the seventh decant reported to be the highest frequency as showed in table 4.1,In table.4.2. observe of frequency histological type of SCC, keratinize type is more frequency (87%) than non-keratinize (13%), and result PCR test was negative for HPV-68 in all cases was show in table 4.3.

Table 4.1. the age frequency among study population

Age group	Frequency	Percent
30-40	5	12%
41-50	10	25%
51-60	10	25%
61-70	15	38%

Table 4.2. The frequency of histological types

Histological type	Frequency	Percent
Keratinized	35	87%
Non keratinized	5	13%
Total	40	100%

Table 4.3. DNA testing of HPV -68 among cases

Negative	Positive	Total
40	0	40

Figure (1) Agarose gel electrophoresis of convention PCR products -1 ladder(100bp) 2: positive *control* 3,4,5: negative samples.

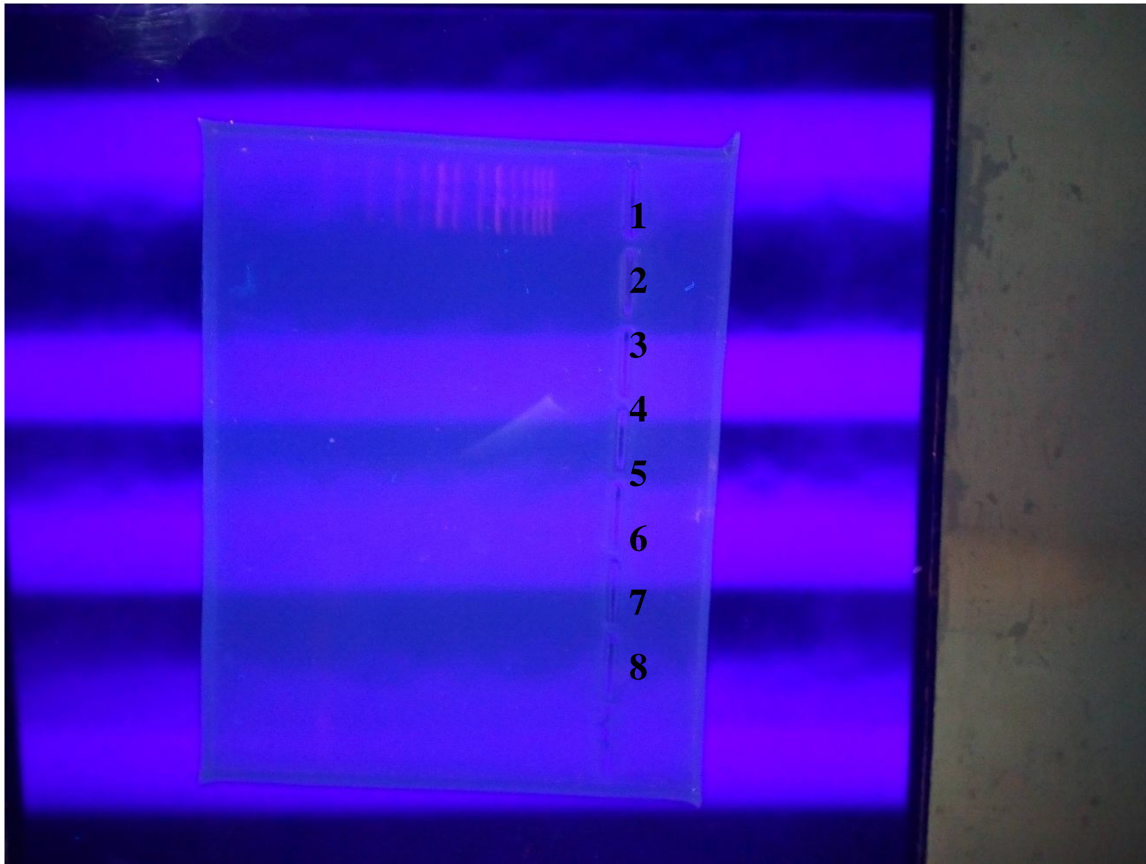

Figure (2) Agarose gel electrophoresis of Convention PCR products 1= ladder(50bp): 2,3,4,5,6,7,8: negative samples.

Chapter five

Discussion: -

It was well recognized that the association of HPV and cervical carcinogenesis was based on the presence of HPV DNA sequence, (Molijn, A., Kleter, B., Quint, W. & van Doorn, 2005).

Persistent infection by oncogenic types of HPV is considered as etiological factor for cervical carcinoma development. Although HPV is not a sufficient factor for developing the cancer, several other co factors were identified, such as: infection by other sexually transmitted diseases (STI) (HIV, Chlamydia trachomatis Cytomegalovirus, (Missaoui *et al*., 2010) Chattopadhyay, 2011) and Herpes Simplex Virus (HSV-2). (Di Luca*et al*., 1989) .

The Present study aimed to investigate the HR-HPVs geno type 68 by using PCR and method among cervical squamous cell carcinoma, as the first time in Sudan Concerning HPV-68 infection among cervical cancer.

The current study showed that most of cervical cancer patients were in the higher age, this is in keeping with the natural history of HPV infection.

The mean age at presentation was 50 years, which were higher compared to studies conducted byReimers *et al*., 2009, but similar to studies done byKrishnamurthy *et al*., 1997,Herbert *et al.,* 2001 and Patel *et al*., 2009.Also the same age was obtained in Iran by Zarchi and his team, their study concluded that, the average age group incidence of 50 years (Zarchi *et al*., 2010). Our findings were consistent with global reports (Pagliusi and Garland, 2007) and national study byHusain *et al*, they concluded that; the commonest age group affected was patients grouped between 61- 70 years

(38%) followed by 51-60 years (25%) and 41-50 years (25%) and lowest age group was affected between 30-40years (12%),(Husain *et al*., 2011)

Our study showed that keratinized SCC was more frequent than non-keratinize among Sudanese women with SCC as the keratinized one represents (87%) compared with non-keratinized SCC which comprised (13%).

The present study reveals that HPV-68 subtype was not detected, that neglect the role of this sub genotype in the causation of cervical cancer in Sudan, the prevalence of this virus was variable according to the geographic area for example it was (12.2%). In Sudia Arabia as reported by (Al-Muammar *et al*, 2007).It was (7.4%) in Madrid, Spain as reported by (Benjamín Garcia-Espinosa *et al.,* 2012). In Poland as reported by Slawa Szostek et al (the prevalence of HPV among women was 1%) (Slawa Szostek et al., 2008).

5.2 Conclusion

- The infection with HR-HPV subtypes 68 is not observed among Sudanese women with cervical cancer in Sudan.
- keratinizing SCC was predominant type over the non-keratinizing SCC.

Recommendation: -

On the basis of the obtained results we recommended: -

- Further studies with large sample size could be done to highlight this area

References:

AbdelhalimKhenchouche, Nabila Sadouki, Arab Boudriche, Karim Houali, Abdelaziz Graba, Tadamasa Ooka and Abdelmadjid Bouguermouh (2013).Human Papillomavirus and Epstein-Barr virus co-infection in Cervical Carcinoma in Algerian women. *Virology Journal*, **10**: 340.

Al-Muammar T, Al-Ahdal MN, Hassan A, Kessie G, Dela Cruz DM, Mohamed GE, (2007)**.** Human papilloma virus-16/18 cervical infection among women attending a family medical clinic in Riyadh. *Ann Saudi Med;***27**(1):15- 25.

Arbyn M, Castellsagué X, de Sanjosé S, Bruni L, Saraiya M, Bray F, Ferlay J: (2011)**.**Worldwide burden of cervical cancer, *Ann Oncol*, **22**(12):2675–2686.

Benjamín García-Espinosa, Ernesto Moro-Rodríguez,and Emilio Álvarez-Fernández, (2012).Genotype distribution of human papillomavirus (HPV) in histological sections of cervical intraepithelial neoplasia and invasive cervical carcinoma, *JBioMed Central***12**(533):1471-2407.

Burchell AN, Winer RL, de Sanjose S, Franco EL (2006).Epidemiology and transmission dynamics of genital HPV infection. *Vaccine*. (**24**) Suppl 3:S3/52-61.

Canavan TP, Doshi NR (2000).Cervical cancer. *Am Fam Physician*. **61** (5): 1369–76.

Castellsague, X., F. X. Bosch, and N. Munoz (2002).Environmental co-factors in HPV carcinogenesis. *Virus Res*. **89:** 191–199.

Castro FA, Quint W, Gonzalez P, Katki HA, Herrero R, van Doorn LJ (2012). Prevalence of and risk factors for anal huma papillomavirus infection among young healthy women in Costa Rica. *Journal Infection Disease*, **206(**10):1103.

Chattopadhyay K. A (2011).Comprehensive review on host genetic susceptibility to human papillomavirus infection and progression to cervical cancer. Indian J Hum Genet. **17**: 132-44.

Chesson HW, Dunne EF, Hariri S, Markowitz LE, (2014).The estimated lifetime probability of acquiring human papillomavirus in the United States. *Sexual transmit diseases* journal,**41** (11): 660-664.

de Villiers EM, Fauquet C, Broker TR, Bernard HU, zur Hausen H,.(2004**.** Classification of papillomaviruses. *Virology*; **324**:17-27.

Di Luca, D., S. Costa, A. Rotola, S. Pilotti, P. Monini, E. Caselli, F. Rilke, and E. Cassai (1987).Simultaneous presence of herpes simplex and human papillomavirus sequences in human genital tumors. *Int. J. Cancer,***40**: 763–768.

D'Souza G, Agrawal Y, Halpern J, Bodison S, Gillison ML (2009). Oral sexual behaviors associated with prevalent oral human papillomavirus infection*. J Infect Dis,* **9***:* 199:1263.

Eileen M. Burd (2003). Human Papillomavirus and Cervical Cancer, *clinical microbiology reviews*,**16**(1):1–17.

Ferlay J, Shin HR, Bray F, Forman D, Mathers C, Parkin DM,(2010). Estimates of worldwide burden of cancer,*J cancer base*, **127** (2) :893-917**.**

Gillison ML, Alemany L, Snijders PJ, Chaturvedi A, Steinberg BM, Schwartz S (2012).Human papillomavirus and diseases of the upper airway: head and neck cancer and respiratory papillomatosis. *Vaccine,***5** (30): F34-54.

Globocan (2008). Cancer Incidence and Mortality Worldwide: IARC Cancer Base No. 10 [Internet]. [http://globocan.iarc.fr].

Hamad H, M. A (2006). Cancer initiatives in Sudan. *A symposium article Annals of Oncology*. **17** (8): viii32–viii36.

Hariri S, Unger ER, Sternberg M, (2011). "Prevalence of genital human papillomavirus among females in the United States, the National Health and Nutrition Examination Survey, 2003–2006". *J, of Infectious Diseases*; **204** (4): 566–573.

Hassan FM, Khirelseed M. (2009).Cervical Cancer Screening among Sudanese Women*Gulf J Oncology,* **6** (4): 28–34.

Herbert A, Singh N, Smith JA, (2001). Adenocarcinoma of the Uterine Cervix Compared with Squamous Cell Carcinoma. *Cytopathology*, **12** (1): 26-36.

Howayda S Abd El All, Amany Refaat and Khadiga Dandash. (2007). Prevalence of cervical neoplastic lesions and Human Papilloma Virus infection in Egypt *National Cervical Cancer Screening Projec. Infectious Agents and Cancerm,* **2**: 12.

Husain N, Helali T, Domi M, BedriZ S. (2011).Cervical cancer in women diagnosed at the National Health Laboratory. *Sudan: A call for screening*, **6**(3): 183–190.

Krishnamurthy S, Yecole BB, Jussawalla DJ (1997). Uterine Cervical Adenocarcinomas and Squamous Carcinomas in Bombay. J *Obstet Gynaecol Res*,**23**(6): 521-7.

Kumar V, Abbas AK, Fausto N, Mitchell RN. (2007).Robbins Basic Pathology*, Saunders Elsevier*, **15** (8): 718–721.

Laukkanen P, Koskela P, Pukkala E, Dillner J, Laara E, Knekt P, (2003). Time trends in incidence and prevalence of human papillomavirus type 6, 11 and 16 infections in Finland. *J Gen Virol*, **84(**9):210.

Lewkowitz AK, Hu SY, Chen F, Li LY, Zhang QM, Wu RF, Li CQ, Wei LH, Xu AD, Zhang WH, Pan QJ, Zhang X, Belinson JL, Sellors JW, Smith

JS, Qiao YL, Franceschi S (2006). Prevalence of human papillomavirus and cervical intraepithelial neoplasia in China:a pooled analysis of 17 population-based studies. *Int J Cancer*, **131** (29) :29-38.

Lipeng Jing, Xingming Zhong, Weihuang Huang, Yang Liu, Man Wang, Zhulin Miao, Xiaoping Zhang,Jing Zou, Baowen Zheng, Congde Chen, Xiaoman Liang, Guang Yang, Chunxia Jing,and Xiangcai Wei,(2014).HPV genotypes and associated cervical cytological abnormalities, *JBMC,* **14**(388),1471-2334.

McCredie MR, Sharples KJ, Paul C. *et al*. (2008).Natural history of cervical neoplasia and risk of invasive cancer in women with cervical intraepithelial neoplasia 3: A retrospective cohort study. *Lancet Oncology***9**(5): 425-434.

McLeod M, Harris R, Purdie G, Cormack D, Robson B, Sykes P, Crengle S, Iupati D, Walker N, (2010): Improving survival disparities in cervical cancer between Maori and non-Maori women in New Zealand: *a national retrospective cohort study Aust N Z J Public Health,* **34**(2):193–199.

Missaoui N, Hmissa S, Trabelsi A, Frappart L, Mokni M, Korbi S. (2010)."Cervixcancer in Tunisia: clinical and pathological study. *Asian Pac J CancerPrev*. **11(**8): 235.

Molijn A, Kleter B, Quint, W. & van Doorn, L. J, (2005). Molecular diagnosis of human papillomavirus (HPV) infections. *J Clin Virol*,**32**(1): S43–51.

Moscicki AB, Ma Y, Jonte J, Miller-Benningfield S, Hanson E, Jay J, (2010). The role of sexual behavior and human papillomavirus persistence in predicting repeated infections with new human papillomavirus types. *Cancer Epidemiol Biomarkers Prev,***19**(20):55-65.

Moscicki AB, Schiffman M, Kjaer S, Villa LL9, (2012). Updating the natural history of HPV and anogenital cancer. *Vaccine*,**31**(5): 42-51.

Munoz N, Castellsague X, de Gonzalez AB, Gissmann L, (2006). Chapter 1: HPV in the etiology of human cancer. *Vaccine*, **24**(3): S31-10.

Niccolai LM, Russ C, Julian PJ, Hariri S, Sinard J, Meek JI, McBride V, Markowitz LE, Unger ER, Hadler JL, Sosa LE, (2013). Individual and geographic disparities in human papillomavirus types 16/18 in high-grade cervical lesions: *associations with race, ethnicity, and poverty Cancer*, *JBM*, **119**(16):3052–3058.

Pagliusi SR, Garland SM (2007). "International standard reagents for HPV detection". Dis Markers, 23 (**2**):83-96.

Patel NR, Rollison DE, Barnholtz-Sloan J. (2009). Racial and Ethnic Disparities in the Incidence of Invasive Cervical Cancer in Florida. *Cancer,* 115(**17**): 3991-4000.

Reimers LL, Anderson WF, Rosenberg PS, (2009).Etiologic Heterogeneity for Cervical Carcinoma by Histologic Type, Using Comparative Age-period-cohort Models. *Cancer Epidemiol Biomarkers Prev*, **18**(3): 792-800.

Remmink AJ, Walboomers JMM, Helmerhorts TJM, Voorhort FJ, Roozendaal L, Risse EKJ, Meijer CJLM, Kenemans P, (1995). The presence of persistent high-risk HPV genotypes in dysplastic cervical lesions is associated with progressive disease natural history up to 36 months. *Int J Cancer*, **61**: 306-311.

Satterwhite CL, Torrone E, Meites E, (2013).Sexually transmitted infections among US women and men: Prevalence and incidence estimates, *Sexually Transmitted Diseases*,**40**(3): 187-193.

Shafti-Keramat, S, Handisurya A, Kriehuber E, Meneguzzi G, Slupetzky K, Kirnbauer R, (2003). Different heparin sulfate proteoglycans serve as cellular receptors for human papillomaviruses. *J Virol*,**77**(24): 13125-35.

Slawa Szostek, Malgorzata Klimek, Barbara Zawilinska and Magdalena Kosz-Vnenchak,(2008). Genotype-specific human papillomavirus detection in cervical smears,*ABP*, **4**(55):687–692.

Epidemiology and burden of HPV infection and related diseases: implications for prevention strategies. *Prev Med*m **53** Suppl; 1: S12-21.

Trottier H, Ferreira S, Thomann P, Costa MC, Sobrinho JS, Prado JC, (2010). Human papillomavirus infection and reinfection in adult women: the role of sexual activity and natural immunity*, Cancer Res*,**70**(85):69-77.

Tseng CJ, Liang CC, Soong YK, Pao CC, (1998). Perinatal transmission of human papillomavirus in infants: relationship between infection rate and mode of delivery *Obstet Gyne col,***9**(19): 2-6.

Winer RL, Hughes JP, Feng Q, O'Reilly S, Kiviat NB, Holmes KK, (2006).Condom use and the risk of genital human papillomavirus infection in young women. *New England Journal of Medicine*,**354**(25): 2645–2654.

Zarchi MK, Akhavan A, Fallahzadeh H. (2010). Outcome of Cervical Cancer in Iranian Patients According to Tumor Histology, Stage of Disease and Therapy. *Asian Pacific J Cancer Prev*; **11**: 1289-1291.

Zhao FH, Tiggelaar SM, Hu SY, Xu LN, Hong Y, Niyazi M, Gao XH, Ju LR, Zhang LQ, Feng XX, Duan XZ, Song XL, Wang J, Yang Y, Li CQ, Liu JH, Liu JH, Lu YB, Li L, Zhou Q, Liu JF, Zhao N, Schmidt JE, Qiao YL,. (2012). A multi-center survey of age of sexual debut and sexual behavior in Chinese women: suggestions for optimal age of human papillomavirus vaccination in China,*Cancer Epidemiol*, **36** (3):84-90.

Appendix

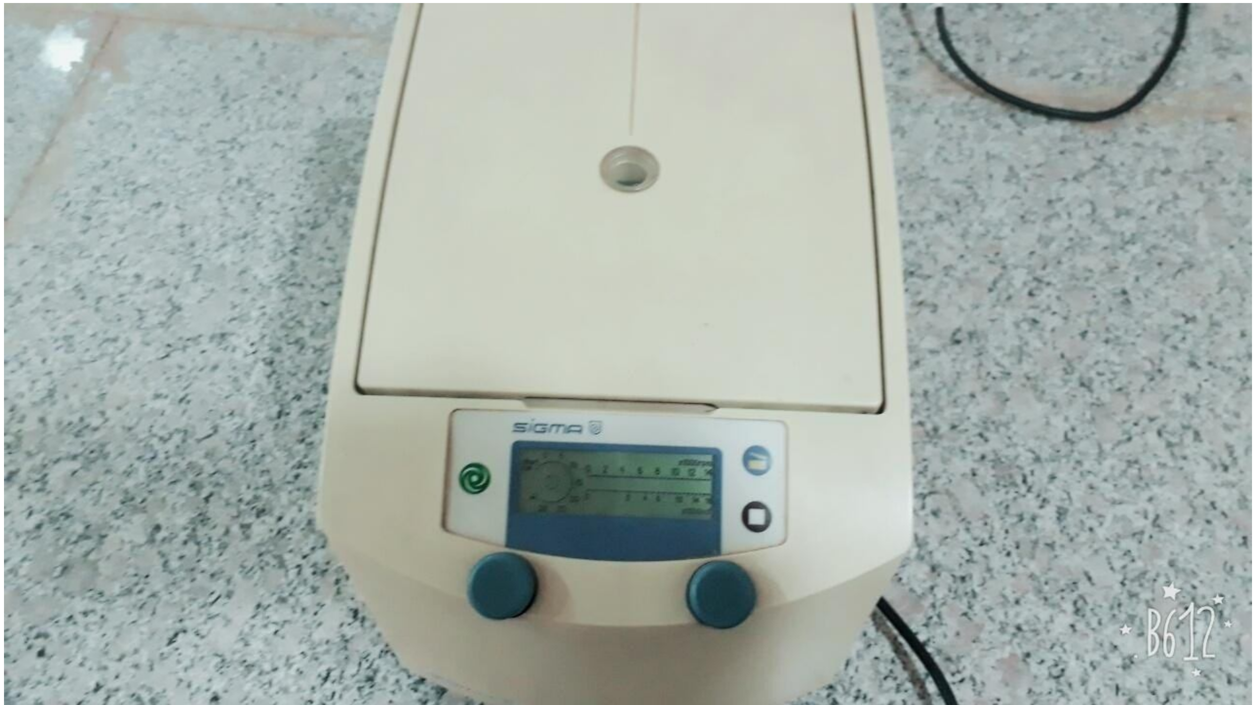

Figure (3) Microcenterfuge device

Figure (4) Thermal cycle device

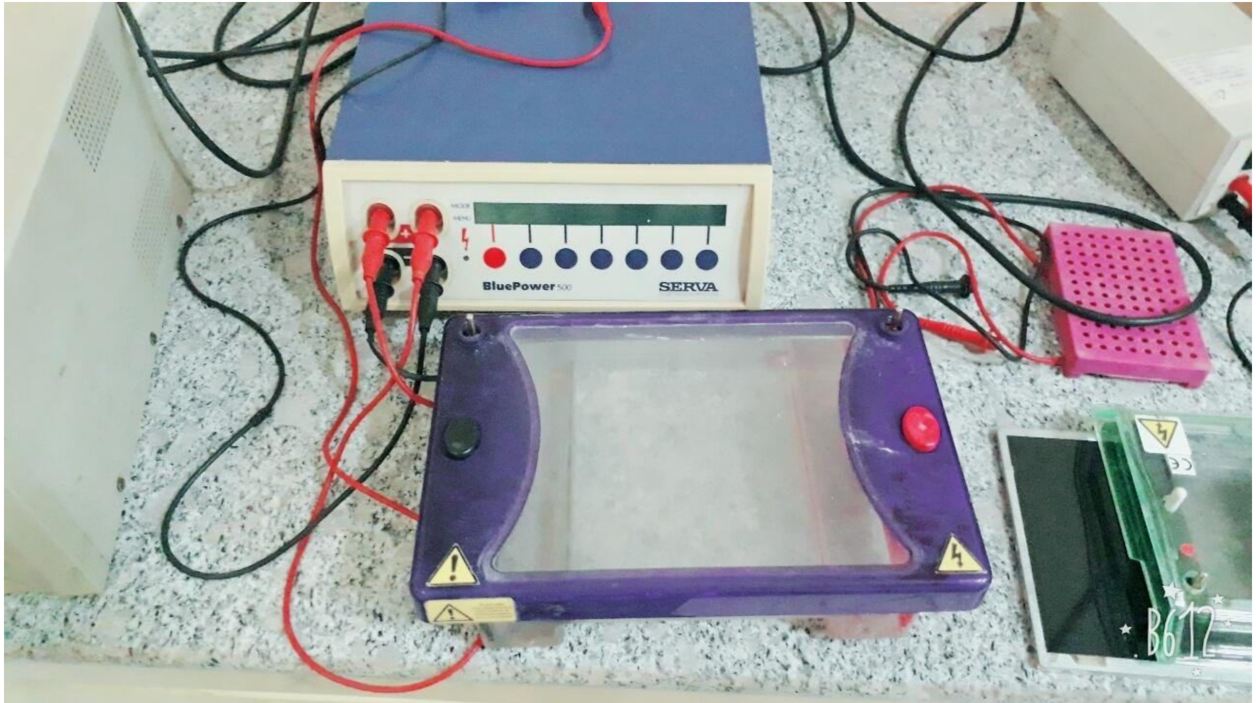

Figure (5) gel electrophoresis and power supply device

Figure (6) UV Light transilluminater **device**

Certificate of Analysis

The Certificate is issued only for the users who purchase the following product from iNtRON

iNtRON Biotechnology, Inc.
1801, 137, Sagimakgol-ro, Jungwon-gu,
Seongnam-si, Gyeonggi-do
13202 Korea

Section A : Product Information

Product	Maxime™ PCR PreMix Kit (i-Taq)		
Catalog Number	25025	**Lot Number**	32310250

Section B : Validity of Production Process / Quality Control

1. Equipment Compliance

The suitability of all facilities put into manufacturing process is maintained and managed under controlled stage. (ISO9001, ISO13485 standards).

Process	Pipettor	ALHP	VDO system	Inspection	Packaging
Passed	✓	✓	✓	✓	✓

2. Dispensing accuracy of manufactured batch

The precision of dispensing process of production has been monitored at real-time. The sampling from dispensing process of production was accomplished from pre/mid/final dispensing process. The accuracy of dispensing was estimated amount of included tracking dye within PCR reagents by observation through spectral diffraction photometry assay.
Permission range of error 100±4% to maintain 100% PCR efficiency from scope. When the CV value (coefficient of variation, %) is under 3%, the manufactured product was guaranteed well production maintained the accuracy without lot variation. The dispensing accuracy of this batch product is estimated below, respectively

No.	Fields	Results
1	Accuracy	100 %
2	Error rate (Max / min)	+ 3.7 % / - 3.7 %
3	CV %	1.49%
4	No. of Outliers	0
5	CALL	■ Pass ☐ Fail
6	Acceptance criteria	Within100 ± 4%

[Precision of Dispensing Process (Accuracy, %)]

97.4%	96.3%	99.0%	97.3%	100.1%	97.1%	97.5%	97.5%	99.3%	97.0%	100.5%	98.5%
100.0%	99.8%	100.7%	101.5%	99.3%	99.6%	99.6%	99.6%	99.5%	98.1%	98.3%	99.3%
100.2%	99.5%	102.4%	99.5%	101.3%	98.3%	100.9%	98.2%	102.0%	99.5%	100.6%	100.2%
99.4%	99.9%	100.5%	100.6%	102.1%	101.4%	97.1%	98.3%	98.9%	100.2%	102.0%	103.7%
100.8%	100.8%	101.9%	101.3%	99.8%	99.1%	99.9%	100.6%	100.5%	101.8%	101.2%	102.7%
101.8%	102.2%	101.1%	102.0%	100.8%	97.5%	100.1%	100.1%	99.3%	99.3%	100.4%	102.5%
99.3%	98.6%	100.1%	100.3%	100.7%	98.8%	99.1%	98.2%	100.0%	99.3%	101.5%	102.4%
99.6%	99.1%	100.7%	102.3%	98.5%	98.7%	99.8%	99.5%	99.7%	100.3%	99.9%	102.0%

- Acceptance criteria has been established through previous research, the performance of the product in the range of error of less than 4% to maintain the level of 100%.
- Accuracy : average of 96 wells
- Error rate (Max / Min) : Maximum - average / minimum - average
- CV% : standard deviation / average x 100 %
- Wavelength of tracking dye : BLUE - 616nm, Orange - 485nm

3. End point PCR

The results was confirmed by end-point-PCR to estimate the performance suitability of final product, the quality stability of this manufactured batch compared to the previous batch to ensure equality. The end-point PCR performance of the products are as follows:

Condition 1 (Normal PCR) - Lot to lot Test	CALL	Condition 2 (Normal PCR) - Lot to lot Test	CALL
Control / Test: 1 2 3 4 5 6 7 N M 1 2 3 4 5 6 7 N	■ Pass ☐ Fail	Control / Test: 1 2 3 4 5 6 7 N M 1 2 3 4 5 6 7 N	■ Pass ☐ Fail

4. Sensitivity and Specificity

The sensitivity and specificity of final product is able to be checked through the amplication results of true or false positive/negative against known positive/negative samples. The pass criteria is above 96% of sensitivity and specificity. The sensitivity and specificity of this manufactuered products are as follows:

True positive	True negative	False positive	False negative	Sensitivity	Specificity
24	8	0	0	100 %	100 %

- Sensitivity (%) : True positive / (True positive + False negative) x 100 %
- Sensitivity (%) : True negative / (True negative + False positive) x 100 %

Section C : Additional Information

1. Manufacturing Conditions

Process	Mixing	Liquid Handling	Inspection	Packaging	Quality Control
Temperature	24 ± 2℃	24 ± 2℃	24 ± 2℃	24 ± 2℃	24 ± 2℃
Humidity	24 ± 3 %	24 ± 3 %	24 ± 3 %	24 ± 3 %	24 ± 3 %

2. Storage Conditions

The Maxime PCR PreMix Kit (i-Taq) is should be kept under -22 ~ -18℃

Issue Date: 14-Feb-17
Doc. No.: IS2017.02.TC01

All Products are for research use only.
Caution : Not for human or animal diagnostic or therapeutic uses. If you have any further questions about this Certificate of Analysis, please contact Technical Service at 82-505-550-5600

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Maxime PCR PreMix Series

Research Use Only

ISO 9001/14001 Certified Company

Maxime PCR PreMix Kit (*i*-Taq)

for 20μl rxn / 50μl rxn

Cat. No. 25025(for 20μl rxn, 96 tubes) **Cat. No. 25026**(for 20μl rxn, 480 tubes)
Cat. No. 25035(for 50μl rxn, 96 tubes)

DESCRIPTION

iNtRON's *Maxime* PCR PreMix Kit has not only various kinds of PreMix Kit according to experience purpose, but also a 2X Master mix solution. *Maxime* PCR PreMix Kit (*i*-Taq) is the product what is mixed every component: *i*-Taq™ DNA Polymerase, dNTP mixture, reaction buffer, and so on- in one tube for 1 rxn PCR. This is the product that can get the best result with the most convenience system. The first reason is that it has every components for PCR, so we can do PCR just add a template DNA, primer set, and D.W. The second reason is that it has Gel loading buffer to do electrophoresis, so we can do gel loading without any treatment. In addition, each batches are checked by a thorough Q.C., so its reappearance is high. It is suitable for various sample's experience by fast and simple using method.

STORAGE

Store at -20°C; under this condition, it is stable for at least a year.

CHARACTERISTICS

- High efficiency of the amplification
- Ready to use: only template and primers are needed
- Stable for over 1 year at -20°C
- Time-saving and cost-effective

CONTENTS

- *Maxime* PCR PreMix (*i*-Taq, for 20μl rxn) 96 (480) tubes
- *Maxime* PCR PreMix (*i*-Taq, for 50μl rxn) 96 tubes

Component in	20 μl reaction	50 μl reaction
i-Taq™ DNA Polymerase(5U/μl)	2.5U	5U
dNTPs	2.5mM each	2.5mM each
Reaction Buffer(10x)	1x	1x
Gel Loading buffer	1x	1x

Note : The PCR process is covered by patents issued and applicable in certain countries. iNtRON Biotechnology does not encourage or support the unauthorized or Unlicensed use of the PCR process. Use of this product is recommended for persons That either have a license to perform PCR or are not required to obtain a license.

PROTOCOL

1. Add template DNA and primers into *Maxime* PCR PreMix tubes (*i*-Taq).
   Note 1 : Recommended volume of template and primer : 3μl~9μl
   Appropriate amounts of DNA template samples
   - cDNA : 0.5-10% of first RT reaction volume
   - Plasmid DNA : 10pg-100ng
   - Genomic DNA : 0.1-1ug for single copy

   Note 2 : Appropriate amounts of primers
   - Primer : 5-20pmol/μl each (sense and anti-sense)
2. Add distilled water into the tubes to a total volume of 20μl or 50μl.
   Do not calculate the dried components

Example Total 20μl or 50μl reaction volume

PCR reaction mixture	Add	Add
Template DNA	1 ~ 2μl	2 ~ 4μl
Primer (F : 10pmol/μl)	1μl	2 ~ 2.5μl
Primer (R : 10pmol/μl)	1μl	2 ~ 2.5μl
Distilled Water	16 ~ 17μl	44 ~ 41μl
Total reaction volume	20 μl	50 μl

Note : This example serves as a guideline for PCR amplification. Optimal reaction conditions such as amount of template DNA and amount of primer, may vary and must be individually determined.

3. Dissolve the blue pellet by pipetting.
   Note : If the mixture lets stand at RT for 1-2min after adding water, the pellet is easily dissolved.
4. (Option) Add mineral oil.
   Note : This step is unnecessary when using a thermal cycler that employs a top heating method(general methods).
5. Perform PCR of samples.
6. Load samples on agarose gel without adding a loading-dye buffer and perform electrophoresis.

SUGGESTED CYCLING PARAMETERS

PCR cycle		Temp.	PCR product size		
			100-500bp	500-1000bp	1Kb-5Kb
Initial denaturation		94°C	2min	2min	2min
30-40 Cycles	Denaturation	94°C	20sec	20sec	20sec
	Annealing	50-65°C	10sec	10sec	20sec
	Extension	65-72°C	20-30sec	40-50sec	1min/Kb
Final extension		72°C	Optional. Normally, 2-5min		

EXPERIMENTAL INFORMATION

- *Comparison with different company kit*

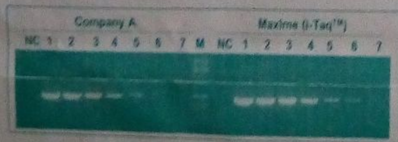

Fig.1. Comparison of *Maxime* PCR PreMix (*i*-Taq) and Company A's PreMix system by amplifying 1 Kb DNA fragment.
After diluting the λDNA as indicates, the PCR reaction was performed with *Maxime* PCR PreMix (*i*-Taq) and company's A product.
Lane M, SiZer-1000 DNA Marker; **lane 1**, undiluted λDNA; **lane 2**, 200 ng λDNA; **lane 3**, 40 ng λDNA; **lane 4**, 8 ng λDNA; **lane 5**, 1.6 ng λDNA; ; **lane 6**, 320 pg λDNA; **lane 7**, 64 pg λDNA; **lane NC**, Negative control

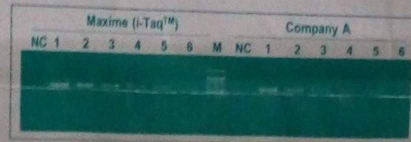

Fig.2. Comparison of *Maxime* PCR PreMix (*i*-Taq) and Company A's PreMix system by amplifying 570 bp DNA fragment (GAPDH).
Total RNA was purified from SNU-1 using easy-BLUE™ Total RNA Extraction Kit (Cat. No. 17061). And then, the first strand of cDNA was synthesized using Power cDNA Synthesis Kit (Cat. No. 25011). After diluting the cDNA mixture as indicates, the RT-PCR reaction was performed.
lane M, SiZer-100 DNA Marker; **lane 1**, undiluted cDNA; **lane 2**, 1/2 diluted cDNA; **lane 3**, 1/4 diluted cDNA; **lane 4**, 1/8 diluted cDNA; **lane 5**, 1/16 diluted cDNA; **lane 6**, 1/32 diluted cDNA; **lane NC**, Negative control