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Determination of HR-HPV Genotypes in HPV Infected Women in Khartoum State

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Abstract: ***Humanpapiloma virus is the main causes of cervical cancer, nearly all cases are attributed to HPV infection, particularly high risk HPV (HR-HPV).Detection of HR-HPV genotypes can facilitate the developing of HPV vaccines which will eliminate the cancer and reduce cervical cancer morbidity and mortality. This study was aimed to determine the HR- HPV genotypes in HPV infected women with its relation to their cytological investigation in Khartoum state. Seventy six cervical samples positive for HPV previously collected from women attended to different hospitals and clinic center in Khartoum state. The specimens were processed and examined with PCR for HPV DNA detection and HR-HPV genotyping, then correlate to corresponding cytological result (previously processed with conventional Papanicolaou stain). HR-HPV were detected in 67 (88.2%) cases, majority were multiple infection 44 (47.4%) cases. Therewith abnormal cervical smear were diagnosed in 13 (17.1%)cases which classified as follows, LSIL in 7 (9.2%), LSIL+ HPV infection in3 (3.9%), HSIL in 3 (3.9%). The Study concluded that the HR- HPV genotypes were detected in most cases (88.2%), with high multiple infection. And the most frequent HR- HPV genotypes were HPV 16, 39, 35, 59, 56, 58 and 18. Therefore involvement of vaccination at this high risk HPV genotype is necessary.***

1. Introduction

Cervical cancer may be a greatest universal public pathological state poignant socio economically disadvantaged populations (Sankaranarayanan et al., 2009). It's hierarchical the seventh most endemic cancer and therefore the second virtually cancer in women after breast cancer globally (Pisani et al., 2002). Cancer of cervix uteri is that the second notable issue of cancer death in women worldwide with over 270000 deaths reported every year. More than eightieth of those of these deaths occur in developing countries (Muchiri et al., 2006; Kent, 2010).

Studies have incontestable that, with the probable exception of a really rare kind of extremely differentiated epithelial cell malignant neoplastic disease, Human Papilloma virus (HPV) is responsible of 99.7% of all nosy carcinomas of the cervix (Walboomers et al., 1999; Sonc et al., 2001).

HPV is a highly infectious virus transmitted through oral, anal, or genital sexual contact, as well as through non-penetrative sex involving skin-to-skin contact (Garland et al., 2009; Yanofsky et al., 2012). Of the 120 HPV genotypes identified, 40 are capable of infecting the anogenital tract and are categorized into low and high risk on the basis of their capacity to induce malignancy (Gross and Pfister, 2004; Yanofsky et al., 2012).

Fifteen are classified as high-risk types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73, and 82), three as probable high-risk (26, 53, and 66), and twelve as low-risk (6, 11, 40, 42, 43, 44, 54, 61, 70, 72, 81, and CP6108) (Muñoz et al., 2003), however even those might cause cancer. HPV 16 and 18 are commonly acknowledged causing concerning 70%of cervical cancer cases in conjunction with HPV 31, they're the prime risk factors for cervical cancer (Walboomers et al., 1999).

Knowledge of HPV standing is turning into progressively vital as sorting screen once detection of abnormal cells of undetermined significance (Bollmann, et al. 2003b) and as a primary screen for cervical cancer detection (Cuzick, 2000). HPV typewriting has an essential prognostic or restorative worth, since it will recognize HPV sorts of high and low oncogenic dangers. Recognizable proof of terrible HPV genotypes may permit decision of those patients UN agency area unit at accumulated risk for unwellness and should thus offer further clinical worth. An essential interest for this methodology is that HPV testing and distinguishing proof of awful HPV type's sorts should be sensitive and specific (Speich, et al. 2004).

2. Materials and Methods

Seventy six DNA positive for HPV previously isolated from cervical samples which scraped previously from women attended to different hospitals and clinic center in Khartoum state after being given their informed consent. Samples were examined cytological with conventional pap stain and molecular for HPV detection. In This study the HPV positive (76) samples were subjected to PCR to detect HR HPV genotype according to the instruction of the manufacturer (Sacace Biotechnologies) as follows:

Amplification: for high risk (12 types) HPV detection. Four PCR mix were used (PCR mix-1 "16-35", PCR mix-1 "18-59", and PCR mix-1 "52-66", 2.5x buffer, and TaqF polymerase.

All required quantity of the tubes for samples and controls were prepared. Reaction mix for each PCR mix-1, was prepared by adding for each one new tube, 5* (N+1)µL of PCR-mix-1, 10* (N+1) of 2.5x buffer, and 0.5* (N+1) of TaqF polymerase (N= sample account). From these reaction

mix, 15 µL were added into each labeled tubes (samples and controls), then10 µL of DNA sample was added to appropriate tube, while controls were prepared as follows:

Negative control: 10 µL of DNA buffer (instead of DNA sample) was added to the labeled tube.

Positives controls:

- Of PCR-mix-1 "16-35": 10 µL of HPV 16, 31, 33, and 35 DNA were added to the 4 labeled tubes.
- Of PCR-mix-1 "18-59" 10 µL of HPV 18, 39, 45, and 59 DNA were added to the 4 labeled tubes.
- Of PCR-mix-1 "52-66" 10 µL of HPV 52, 56, 58, and 66 DNA were added to the 4 labeled tubes.

All tubes were shut and moved into a thermocycler at 95°C; A 15-minutes denaturation step at 95°C was trailed by 42 cycles of amplification with a PCR processor (PE9600; Perkin-Elmer). Each cycle incorporated a denaturation venture at 95°C for 30 second, a primer annealing step at 63°C for 30 second, and a chain elongation at72°C for 40 second. The last elongation phase was prolonged by one minute to guarantee a complete extension of the amplified DNA (the storage was at 10°C).

The positive controls of the 4 tubes with amplified DNA for each PCR-mix-1 were mixed in new tube, and then 10-15 µL of amplified products were added on the agarose gel.

Amplification products were electrophoresed, visualized using, and compared with 100 bp ladder DNA marker as before in HPV detection. (The length of specific amplified DNA fragments is: HPV 16 – 325 bp, 31- 520, 33- 227, 35- 280, 18- 425, 39- 340, 45- 475, 59- 395, 52- 360, 65- 325, 58- 240, 66- 304 bp).

3. Results

Seventy six cervical samples previously examined cytologically and diagnosed as positive for HPV; were assessed in the present study for HPV genotyping .The age of study group are ranged from 15-83 with 36.79±11.14, most of them are between 20 to 49.

The descriptions of cytological feature compared to age in study population are shown in table (1) the precancer cells were diagnosed in 13 (17.1%) cases, most of them were diagnosed with low-grade squamous intraepithelial lesion (LSIL), 7 (9.2 %) while LSIL+HPV infection detected in 3 (3.9%) cases, majority of these were observed in ages less than50 years old, however two (2.6%) out of three cases represented high-grade squamous intraepithelial lesion (HSIL) were at age more than 59 and other one (1.3%) at age group less than 20.These results are highly significant (P value .0)

The 12 HR-HPV genotype were detected in 88.2% (67/76) cases, while11.8 (9/76) cases as negative (positive for HPV other than these 12 types).

Determination of the different HPV genotypes was done by examination of the presence of high-risk HPV genotype including (16, 18, 31, 33, 35, 39, 45, 52, 56, 58, 59, 66). In 76 positive HPV cases, single HPV infection were detected in 23 (30.3%) cases, multiple infection (infection by more than one types) observed in44 (57.9 %), 36 (47.4%) of them were free for SIL, while positive HPV for other un-known genotype (other than the 12 types) in 9 (11.8%) cases, 9.2% (7/76) cases of these were seen in patients with normal pap smear and other 2.6% (2/76)cases were represented LSIL+HPV (Table 2).

The high risk HPV subtypes encountered, HPV 16 was the highest one, it was detected in 28 (36.8%) cases, followed by HPV 39 in 23 (31.6%), then HPV 35 in 15 (19.7%), HPV 59 in 14 (18.4%), HPV 56 in 11 (14.5%), HPV 58 in 10 (13.2%), HPV 18 in 7 (9.2%), HPV 66 in 6 (7.9%), HPV 52 in 4 (3.5%), HPV 45 in 4 (3.5%), HPV 33and 31 in 2 (2.6%)cases for each. The most frequent HPV genotypes identified in normal cytology were HPV 16 and 39 (34.9%, 22/76) each, followed by HPV59 (20.6%, 13/63), HPV35 (19.0%, 12/63), HPV56 & 58 (12.7%, 8/63)each, HPV of unknown genotype (11.1, 7/613), HPV66 (9.5%, 6/63), HPV18& 52 (6.3%, 4/63) each, HPV31 & 45 (4.8%, 3/63), HPV33 (3.2%, 2/63) of cytological normal cases.

The HPV subtypes detected in association with cytological changes, as in **table (3**) include, HPV 16, (38.5%, 5/13), followed by HPV39, 35, 56 (30.7%, 4/13), HPVHPV18, 58 and patients of other types (other than 12 types) (15.4%, 2/13) each and HPV 59, 45 (7.7%1/13) each of cervical precancerous cases. However 84.6.3% (11/13) has multiple infection.

Table 1

Age group		Cytological results			
	Negative	LSIL	HSIL	LSIL+HPV	Total
15-19	0	1	1	0	2
20-29	17	3	0	0	20
30-39	24	1	0	2	27
40-49	18	2	0	0	20
50-59	4	0	0	1	5
More than59	0	0	2	0	2
Total	63	7	3	3	76

Table 2

HR-HPV results	Cytology result				Total
	Negative	LSIL	HSIL	LSIL+HPV	
Single positive	20	2	0	1	23
Multiple positive	36	5	3	0	44
Non for 12HR-HPV	7	0	0	2	9
Total	63	7	3	3	76

Table 3

HPV genotype	Cytological changes			
	LSIL	HSIL	LSIL+HPV	Total
18	1	0	0	1
16, 18	1	1	0	2
58	1	0	0	1
35.45.56	1	0	0	1
16, 58	0	1	0	1
35	0	0	1	1
16, 39, 56	1	0	0	1
16, 35	0	1	0	1
16, 39	1	0	0	1
39, 59, 56	1	0	0	1
None of 12 types	0	0	2	2
Total	7	3	3	13

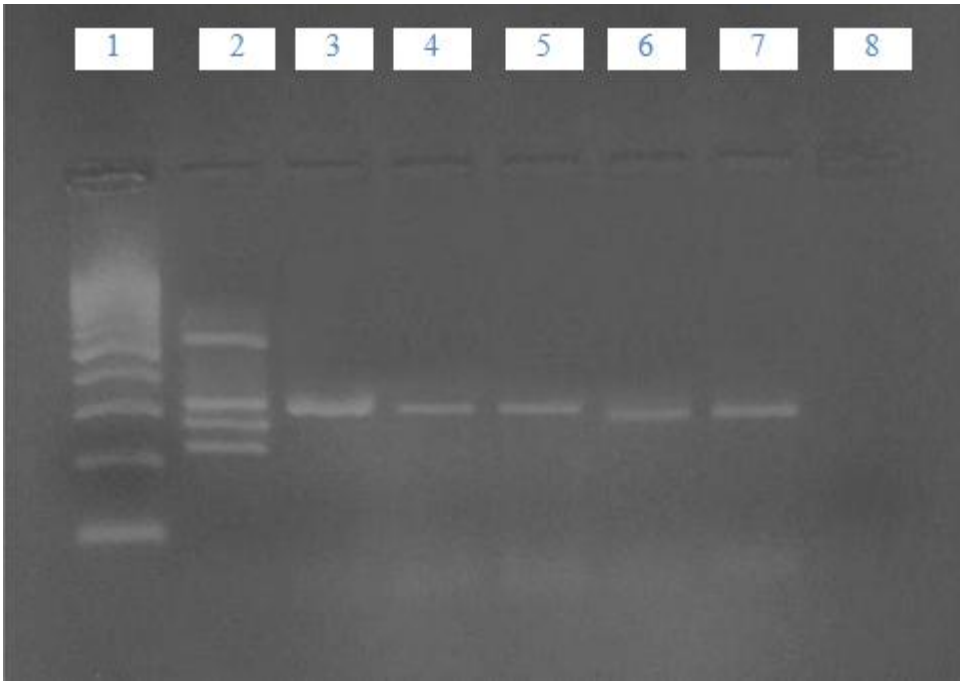

Plate 1: Detection of high Risk HPV genotypes group I (16, 31, 33 and35)on ultra violet trans illuminator:
Lane 1: 100 bp molecular marker; 2= positive control; 3, 4 and 5= Genotype 16 (325 bp); 6 and 7= Genotype 35 (235 bp); 8= negative control

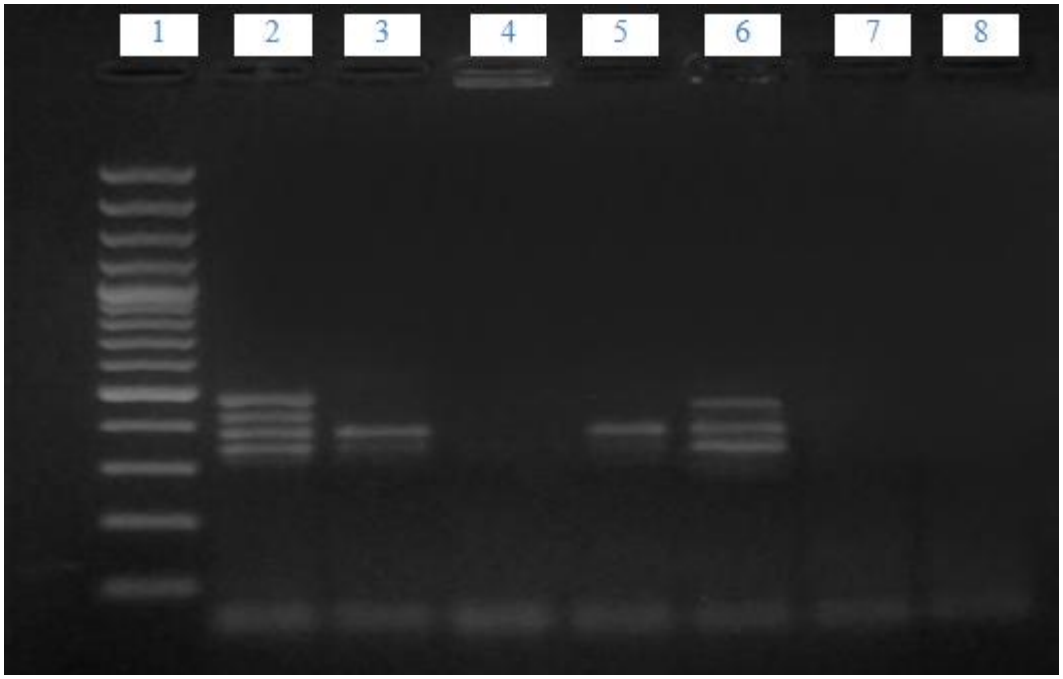

Plate 2: Detection of high Risk HPV genotypes group II (18, 39, 45, and 59)on ultra violet transilluminator:
Lane 1=100 bp molecular marker; 2=positive control; 3= Genotypes 39 and 59 (340and 395 bp); 4, 7= negative; 5=Genotype 59 (395 bp); 6= Genotype 39, 59 and 18 (340, 395 and 425bp); lane 8=negative control.

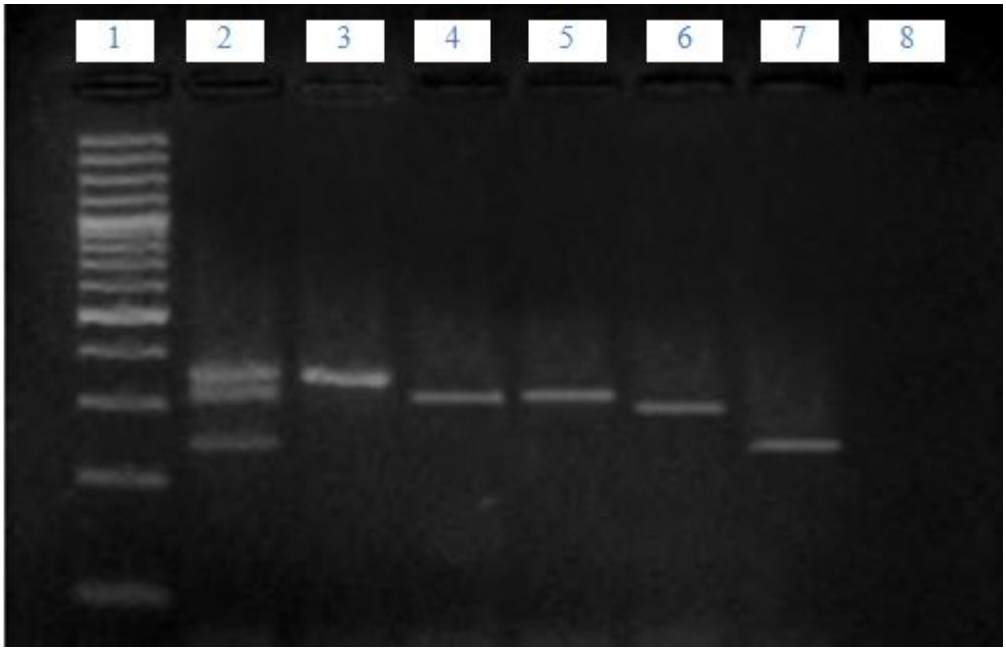

Plate 3: Detection of high Risk HPV genotypes group III (52, 56, 58 and66))on ultra violet trans illuminator:
Lane 1: 100 bp molecular marker; 2=positive control; 3= Genotype 52 (360 bp); 4, 5= Genotype 56 (325 bp); 6= Genotype 66 (304 bp); 7= Genotype 58 (240 bp); 8=negative control

4. Discussion

The cytological investigation showed that the prevalence of premalignant cell is 17.1% (13/76) HPV infected cases with age ranging from 16-83years, although 82.9% (63/76) of patients were represented normal pap smear, 36 (47.4%) of them were having multiple (two or more) infection and 9.2% (7/76) infected with HPV other than these 12HR-HPV types. This indicates that not all infected women will develop cancer and may clear the virus by immune system. This result supported by Eileen (2003), who noticed that identification of high-hazard HPV is vital however may not be adequate for the advancement of cervical malignant growth. Studies propose that whether a lady will create cervical malignancy relies upon an assortment of extra factors that in concert with disease related HPV types in the process that prompts cervical malignant growth (Eileen, 2003). These other factor such as smoking, immune suppression, human immunodeficiency virus (HIV) infection and multi partners.

Also may be conventional pap missed the abnormal change (false negative), so the uses of LBC instead of conventional pap in screening in adjacent molecular diagnosis to detect women at risk. Other suggestion that may be at early infection and may progress to premalignant and malignant lesions, especially they have high multiple infection (47.4%) which increase the risk for developing the disease. However it constitute (57.9 %) of all HPV positive cases. This incidence is variable according to different geographical region.

Studies indicate that infection with numerous HPV types can happen (*Jacobs et al.*, 1997, Kleter *et al.*, 1999, Quint et al., 2001). Up to 39% of Multiple-infection can been seen. The existence of multiple HPV genotypes genotypes would in general increment with the seriousness of cervical illness (Kleter et al., 1999). Study in Ethiopia and Sudan showed that multiple infection were identified in 59% and 49% of samples positive for HPV genotypes from Ethiopia and Sudan respectively (Ebba Abate *et al.*, 2012).

As indicated by the International Association for Research in Cancer (IARC), the most common high hazard HPV genotypes which taint the cervix are: HPV16 (53%), HPV18 (15%), HPV45 (9%), HPV31 (6%), and HPV33 (3%) Muñoz, 2000). The widely recognized HPV genotypes detected in HSIL were HPV16 (59.9%), HPV31 (18.1%), HPV 52 (14.8%), HPV51 (14.0%), and HPV18 (13.2%) Castle et al., 2010).

HPV 16 was found to be the most frequent HPV genotype in Ethiopia and the Sudan accounting for 91% and 82.5 % respectively of positive HPV cervical cancer and precancerous patients. Followed by HPV 52, 58 and 18 sequentially common HPV genotypes identified in Ethiopia. On the other hand, HPV 18, 45 and 52 consecutive were the other common HPV genotypes next to HPV 16 identified in samples collected from Sudan (Ebba Abate *et al.*, 2012).

Other somewhat similar result by Dawit Wolday, et al (2018), who consider HPV genotype dissemination among ladies with normal and abnormal cervical cytology displaying in a tertiary gynecology referral Clinic in Ethiopia, they reported that HR-HPV subtypes encountered among ladies with normal cytology were HPV16 (20.3%), trailed by HPV35 (8.7%), HPV56 and HPV58 (each 5.8%), HPV18, HPV31 and HPV39 (each 4.4%), HPV45 (2.9%) and HPV59 and HPV68 (each 1.5%), the most well-known HR-HPV infections in ladies with atypical cytology included HPV16 (62%), trailed by HPV45 (8.7%), HPV 31, HPV35 and HPV59 (each 4.4%), and HPV18, HPV52 and HPV56 (each 2.2%).

These studies are not far away from the present study, that the most frequent HR-HPV infections in ladies free for SIL were HPV 16, 39, 35, 59, 56, 58, 18, 66, 52, 45, 33and 31from higher prevalence encountered HPV 16 (36.8%,) to the lowest HPV 33 and 31 (2.6%)each. However the most common identified HPV genotypes in abnormal cytology were HPV 16, 39, 35and56 followed by HPV18and 58.

The little difference may be due to small sample size for our study. Majority of cases were at age less than 50 years and the peak at age group 30-39 then decreased to only 5 cases at age group50-59, this may be due to clear of virus by immune system, while two cases at age older than 59 were develop HSIL.

The most important observation that LSIL and HSIL detected in a two patients less than 20 years are associated mainly with HPV 16, and also patients more than 59 years represented HSIL is linked to HPV 16. This result somewhat similar to Castle *et al* (2010), who concluded that, HPV16-positive HSIL was all the more ordinarily analyzed in more youthful ladies. Further agreement on previous study noted that the ratio of HSIL (CIN2 and CIN3) linked to HPV16 was greater in younger women than in older women (Porras *et al*., 2009).

5. Conclusion

Our study indicate that the most frequent HR- HPV genotypes infected Sudanese ladies, either with normal and abnormal cytology were 16, 39, 35, 59, 56, 58 and 18 with high multiple infection. Although most of cases were with normal cytological smear. The introduction of vaccination to these HR –HPV type is necessary. Also study suggest involvement of large size of sample to detect the pandemic HPV genotype in Sudan.

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