Bolis *et al., J. of Virol. Sci.,* Vol. 5: 86- 96, 2019
ISSN: 2636-2937

Journal of Virological Sciences

RESEARCH

Molecular Detection of *Human Papilloma Virus* Genotypes in Children with Focal Epithelial Hyperplasia in Khartoum State

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ABSTRACT

Background: Focal epithelial hyperplasia (FEH) is a common oral infection which is usually missed in the diagnosis because it is symptomless and its lesions can regress by time. On the other hand there is no cell culture for Human papilloma viruses (HPVs).
Objective: Detection of HPV genotypes assocciated with FEH in childern aged three to fourteen years old.
Methods: Fourty seven biopsies and oral smears were collected from Children (3 -14) years old, who attended the pediatric dental clinics of Faculty of Dentistry, University of Khartoum and Khartoum Dental Teaching Hospital, clinically diagnosed by the dental clincian as FEH cases and their guardian agreed to participate. The study used the multiplex polymerase chain reaction (PCR) to detect the most common HPV genotypes associated with FEH in children, beside the Papanicolaou (Pap.) stain to show the cytological features which are usually seen in FEH. Results were analyzed using Statistical Package for Social Science (SPSS) version 16. The Chi-Square test tested the significance difference between the HPV genotypes PCR findings and the Pap. smears. Figures were constructed using Microsoft Exel 2007.
Results: Eleven specimens 23.4% (11/47) were positive for the low risk genotypes. Twenty specimens 42.6% (20/47) were positive for genotypes 13 and 32. None of genotypes 18, 39, 56 and 59 was detected. Twenty seven specimens 57.4% (27/47) were positive for the high risk genotypes. Cytologically twenty nine samples 80.5% (29/36) which were positive for the PCR showed koilocytosis and mitosoid cells. Some specimens were positive for more than one genotype. FEH was more common in females than males. There was a significant difference between the PCR/Pap. findings.
Conclusion: The most frequent genotypes which were detected were HPV 32 followed by HPV 16. HPV genotypes 31, 33, 35, 52, 58 and 66 were detected in this study although they were not mentioned in the studies conducted in FEH. Pap. Stain's and PCR sensitivity was 80.5% while the specificity was 40%.

Keywords: *Human papilloma viruses,* Focal epithelial hyperplasia, Children, polymerase chain reaction, Papanicolaou Stain, Khartoum State.

BACKGROUND

Focal epithelial hyperplasia (FEH) (Heck's disease) is a symptomless benign oral lesions caused by *HPV* specific genotypes (Jayasooriya *et al*., 2004). In 1961 Dr.Heck diagnosed the FEH in a patient from New Mexico and the disease was named after him, then FEH was discovered in 1965 by Dr. Estrada in native Americans (González *et al*., 2005). FEH lesions are multiple, elevated, sessile, soft, with a smooth surface and disappear when they are stretched. FEH shows mitosoid bodies which aren't present in viral warts and papillomas. All age groups are affected but it is more common in young ages. *HPV*- 32 causes FEH in the elderly people while *HPV*- 13 causes FEH in both young and old. Both males and females are affected but it is

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commonly seen in females (Dent and Lombardi, 2008). FEH is found in many populations over the world, but it is common in Africa, Eskimos, South, North and Central America Indians, but it is rare in Asia due to its association with ethnic (genetic) factors beside poverty (Jayasooriya *et al.*, 2004).

The incubation period from the exposure to *HPV* until the appearance of the symptoms varies from weeks to months to years with an average of three months (Anic and Giuliano, 2011). Symptoms either appear as warts or cancer depending on the *HPV* genotype (Cobb, 1990).

Diagnosis of *HPV* infections has many tools: histologically, in situ hybridization, immunohistochemistry, cytologically (Pap. stain), *HPV* E6/E7 seroreactivity, molecular techniques (Fakhry and Gillison, 2006) and Colposcopy (Jeronimo and Schiffman, 2006). The clinical diagnosis is not distinct enough (Chang *et al*., 1991) . Gardasil and Cervarix vaccines are used as *HPV* anticancer vaccines, with targeted age group in both males and females 9- 26 years old (Markowitz *et al*., 2007).

The objectives (aims) of this study is to detect if there are other *HPV* genotypes rather than *HPV* 13 and 32 which are responsible for FEH, beside strengthing the imporatant role of molecular tools eg: polymerase chain reaction (PCR) in the diagnosis of such cases therefore giving chance for better treatment and prognosis.

MATERIALS AND METHODS

Study design, area, time and population:

This descriptive, analytical study was carried out at the Faculty of Dentistry clinics, University of Khartoum and Khartoum Dental Teaching Hospital during the period from February 2012 to August 2014. The population included children with age group between 3-14 years old who were previously diagnosed as FEH patients.

Sample size:

Fourty seven children clinically diagnosed as FEH were included in this study for convenience and limitation of facilities.

Criteria of inclusion:

Children who aged 3 -14 years old, attended the pediatric dental clinics of Faculty of Dentistry, University of Khartoum and Khartoum Dental Teaching Hospital, clinically diagnosed by the dental clincian as FEH cases and their guardian agreed to participate were included in the study.

Criteria of exclusion:

Children who aged 3 -14 years old, attended the pediatric dental clinics of Faculty of Dentistry, University of Khartoum and Khartoum Dental Teaching Hospital, clinically diagnosed by the dental clincian as non FEH cases were excluded. Also adults and FEH cases of children whose guardians refused to participate were excluded from the study.

Sampling method:

Fourty seven biopsies from oral lesions were collected by a surgeon using local anathesia. The biopsy was placed in normal saline for DNA extraction .Secondly a smear was taken from each patient for Pap. stain by rinsing the patient′s mouth with normal saline and got rid of it. Then the lesion was scraped with a clean tongue depresser and smeared onto three clean labeled

glass slides. The wet smears were fixed immediately in 95% ethyl alcohol for overnight (Hassan and Ibrahim, 2014). Finally the smear was stained with Pap. stain to demonstrate the koilocytosis and mitosoid cells .

Sample processing:

DNA extraction from the tissues:

DNA was extracted from biopsies using GF-1Tissue DNA Extraction Kit (Vivantis, Malysia). Extracted DNA was stored at - 20◦C.

DNA quantitification:

DNA was quantified by Spectrophotometric method (eppendrof –biophotmeter) and diluted to a working concentration at 15 µl DNA to 435 µl nuclease free water. The mean 260/280 nm ratio was calculated to assess the purity of the DNA, ranged from1.6-2.0 demonstrating good deproteinization (Vinod, 2004).

Low Risk *HPV*s PCR Typing:

The fourty seven extracted DNA samples were firstly tested for *HPV* low risk genotypes (6/11) using multiplex PCR kit from Sacace Biotechnologies, Italy, following the manufacturing recommendations protocol. PCR-mix 1 tubes were closed and transfered into the thermolcycler (BioRad) with block temperature adjustment only when temperature reached 95°C and started the following program:

Table 1: The PCR protocol of *HPV* low risk genotypes 6 and 11.

Step	t◦C	Time	Cycles
1	95◦C	Pause	
2	95◦C	15 min	1
3	95◦C	1 min	
	65◦C	1 min	42
	72◦C	1min	
4	72◦C	1 min	1
5	10◦C	Storage	

Analysis of PCR results was based on the presence or absence of specific bands of amplified deoxyribonucleic acid (DNA) in 2% agarose gel. The length of specific amplified DNA fragment as base pairs (bp) was: *HPV 6* – 250 bp , *HPV 11* – 425 bp and Internal control – 723bp.

High Risk *HPVs* PCR Typing:

The extracted DNA samples were tested for three pannels of *HPV* high risk genotypes. Pannel one included genotypes (16, 31, 33, 35), pannel two included genotypes (18, 39, 45, 59), pannel three included genotypes (52, 56, 58, 66), using multiplex PCR kit from Sacace Biotechnologies, Italy, following the manufacturing recommendation protocol with negative and positive control for the assessment of the PCR protocol and DNA quality.

Tubes were closed and transfered into the thermocycler (BioRad) only when temperature reached 95◦C and started the following program:

Table 2: The PCR protocol of *HPV* high risk genotypes (16, 18, 31, 33, 35, 39, 45, 52, 56, 58, 59 and 66).

Step	t◦C	Time	Cycles
1	95◦C	Pause	
2	95◦C	15 min	1
3	95◦C	30 sec	
	63◦C	40 sec	42
	72◦C	50 sec	
4	72◦C	1 min	1
5	10◦C	Storage	

Analysis of PCR results was based on the presence or absence of specific bands of amplified DNA in 2% agarose gel. In the new tube the contents of the 4 tubes with amplified DNA of the 4 controls (*HPV 16, 31, 33* and *35*) were mixed. The same procedure for the controls of PCR-mix-1 "18-59" and PCR-mix-1 "52-66" was repeated. A volume of 15 µl of amplified products was added on the agarose gel. The length of specific amplified DNA fragments was:

Table 3: The length of specific amplified DNA fragments for *HPV* high risk genotypes.

PCR-mix-1(16-35)			PCR-mix-1(18-59)			PCR-mix-1(52-66)		
Type	Length	IC	Type	Length	IC	Type	Length	IC
HPV 16	325 bp		*HPV 18*	425 bp		*HPV 52*	360 bp	
HPV 31	520 bp		*HPV 39*	340 bp		*HPV 56*	325 bp	
HPV 33	227 bp	723 bp	*HPV 45*	475 bp	723 bp	*HPV 58*	240 bp	723 bp
HPV 35	280 bp		*HPV 59*	395 bp		*HPV 66*	304bp	

***HPV 13* PCR typing:**

The presence of *HPV 13* DNA in the samples was identified by PCR with specific primers from Eurofins Genomics Company, Germany. *HPV 13* forward primer sequence: 5′-AAA TCC CAG CAG AAT TAT AT-3′ / reverse primer sequence: 5′AAA GAG ATG ATG TAG TGG C-3′ that amplified a 240 bp fragment of L1 gene (Cuberos *et al*., 2006). The PCR was performed in 50 µl reaction mix containing 1 X PCR buffer, 3 mM $MgCl_2$, 200 µm dNTPs, 50 pmol of each primer, and 1.0 unit of Taq polymerase. Thermal cycling conditions were: 94◦C for four minutes, followed by 38 cycles of 94◦C for 30 seconds, 50◦C for 30 seconds and 72◦C for 30 seconds, final extension of 72◦C for 10 minutes and 15◦C forever. Finally the PCR products were visulized in 2% agarose gel (González-Losa *et al*., 2011).

***HPV 32* PCR typing:**

The presence of *HPV 32* DNA in the samples was identified by PCR with specific primers from Eurofins Genomics Company, Germany. *HPV 32* forward primer sequence: 5′-TAT AAC GGA CGG CAT TTC AGA TTC -3′ / reverse primer sequence: 5′GTC ACT CCA CGC AGG CAC AC -3′ that amplified a 382 bp fragment of E6/E7genes. The PCR was performed in 25µl reaction mix containing 1 X PCR buffer, 2 mM $MgCl_2$, 200 µm dNTPs, 0.5 µm of each primer, and 0.625 u of Taq polymerase. Thermal cycling conditions were: 95◦C for nine minutes, followed by 40 cycles of 95◦C for one minute, 58◦C for 10 seconds, 75◦C for 30 seconds, final extension of 75◦C for 5 minutes and 15◦C forever. Finally the PCR products were visulized in 2% agarose gel (Herrel *et al*., 2009).

Gel electrophoresis:

All PCR products were visualized on 2% agarose gel in 1x Tris boric EDTA (TBE) to specify the *HPV* genotype depending on the length of specific amplified DNA fragments and documented by the Gel documentation system (Uvitec, Cambridge,UK) (Vinod, 2004).

Papanicolaou stain:

Each oral smear was stained with the following Papanicolaou stain procedure to demonstrate the koilocytes and mitosoid cells.The wet smear was fixed in 95% ethyl alcohol overnight. Transfered to 70% ethyl alcohol for 1 min. Washed in distilled water (D.W) for 2 min. Stained in Harris' haematoxylin for 8 min. Washed in D.W for 1 min. Differentiated in 1% acid alcohol (just rinse). Bluing was done in running tap water for 10 min. Transfered to 70% ethyl alcohol for 1 min. Transfered to 95% ethyl alcohol for 1 min. Stained in orange G 6 (OG6) for 2 min. Rinsed in 95% ethyl alcohol (twice). Stained in eosin azure 50 (EA50) for 3 min. Rinsed in 95% ethyl alcohol. Dehydrated in absolute alcohol. Dried by air. Cleared in xylene for 2 min. Mounted in distyrene plasticizer xylene (DPX) and examined under the light microscope using times 40 objective lense (Bancroft and Gamble, 2002).

Ethical consideration:

The ethical clearance for the study was obtained by the research board of the faculty of dentistry, University of Khartoum. The consent of the children's guardian was taken before any process. All the details and objectives of the study were well explained to the children's guardian.

Analysis:

Results were analyzed using Statistical Package for Social Science (SPSS) version 16 (SPSS Inc. 233 South Wacker Drive, 11th Floor Chicago, IL 60606-6412). The Chi-Square test was used to test whether there was a significant difference or not between the *HPV* genotypes PCR findings and the Pap. smears. There was a significant difference between them (P values were more than 0.05). Figures were constructed using Microsoft Exel 2007.

RESULTS

Eleven specimens 23.4% (11/47) were positive for the low risk genotypes 6 and 11 (Fig**.** 1).

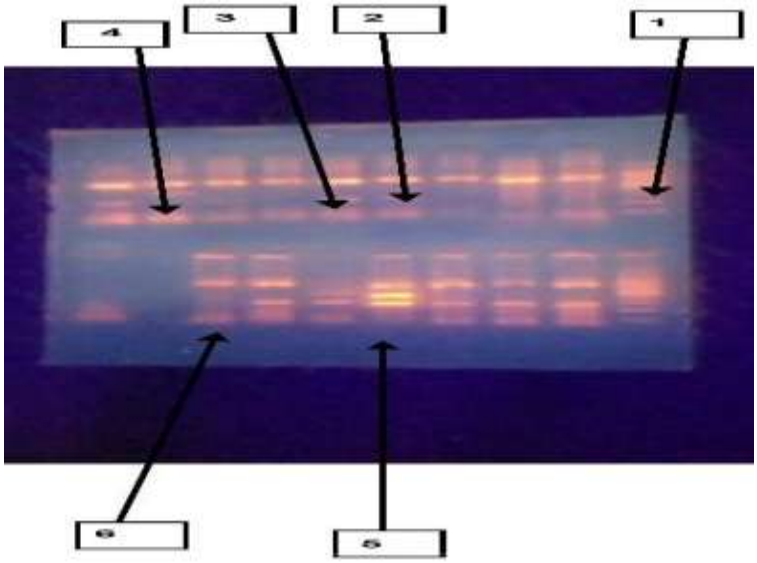

Fig. 1: PCR products of *HPV* low risk genotypes in biopsies from clinically diagnosed children as FEH patients. 1: DNA marker (ladder 100 bp). 2: Internal control (723 bp) / *HPV*6 (250 bp) / *HPV*11(425 bp, faint band). 3: Internal control (723 bp) / *HPV*6 (250 bp). 4: Internal control (723 bp) / *HPV*11(425 bp). 5: Positive control, internal control (723 bp) / *HPV*6 (250 bp) / *HPV*11(425 bp). 6: Negative sample, only internal control (723 bp).

Twenty specimens 42.6% (20/47) were positive for genotypes 13 and 32 (Fig. 2). None of genotypes 18, 39, 56 and 59 was detected (Fig. 2).

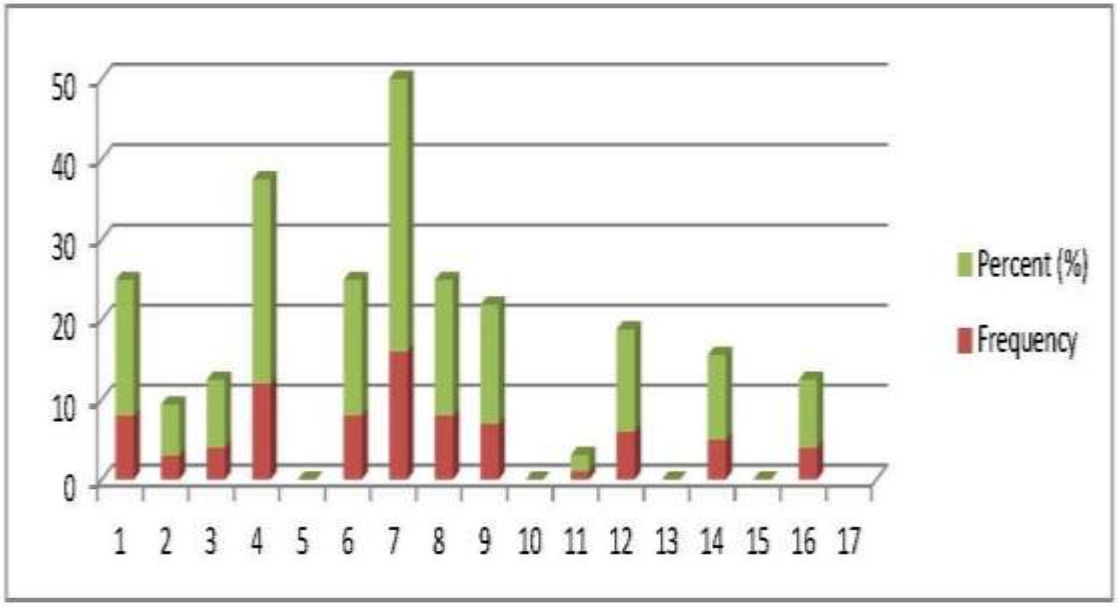

Fig. 2: Frequency and Percentage of *HPV* genotypes found in the biopsies taken from clinically diagnosed children as FEH patients. 1: *HPV6.* 2: *HPV11.* 3: *HPV13.* 4: *HPV16.* 5: *HPV18.* 6: *HPV31.* 7: *HPV32.* 8: *HPV33.* 9: *HPV35.* 10: *HPV39.* 11: *HPV45.* 12: *HPV52.* 13: *HPV56.* 14: *HPV58.* 15: *HPV59.* 16: *HPV66.*

Twenty seven specimens 57.4% (27/47) were positive for the high risk genotypes 16, 31, 33, 35, 45, 52, 58 and 66.

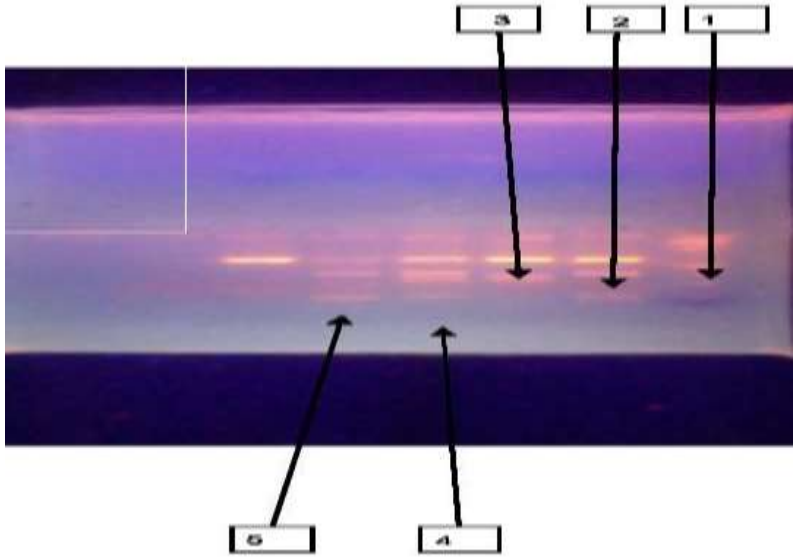

Fig. 3: PCR products of *HPV* high risk genotypes found in biopsies from clinically diagnosed children as FEH patients. **1:** DNA marker (ladder 100 bp). 2: Internal control (723 bp) / *HPV* 16 (325bp). 3: Internal control (723 bp) / *HPV* 33(227bp) / *HPV* 35 (280bp). 4: Internal control (723 bp) / *HPV* 16(325bp) / *HPV* 33 (227bp). 5: Internal control (723 bp) / *HPV* 16 (325bp).

Twenty seven specimens 57.4% (27/47) were positive for more than one genotype: genotypes 6 and 11, genotypes 16 and 33, genotypes 16/ 32 and 52, genotypes 16/ 31/ 32 and 35 (Table 4).

Seven samples 19.4% (7/36) did not reveal koilocytosis or mitosoid cells although they were positive in the PCR, six samples 60% (6/10) revealed koilocytosis or mitosoid cells although they were negative in the PCR. Four samples were negative for all *HPV* genotypes by PCR and negative also for the Pap.stain at the same time.

The frequency and percentage of the Pap. positive smears were thirty six samples 76.6% (36/47), the negative ones were eleven samples 23.4% (11/47).

Ten specimens 21.3% (10/47) were negative for all *HPV* genotypes. One specimen 2.1% (1/47) was invalid for the genotypes 6 and 11. Two specimens 4.3% (2/47) were invalid for the high risk genotypes. The percentage of the infected females was higher than the infected males 44.7% and 32% (21/47 and 15/47) respectively. The remaining 23.4% (11/47) of both sexes were negative for *HPV* genotypes.

Table 4: Samples which were positive for more than one *HPV* genotype.

Sample no.	***HPV* genotype detected**
1, 2	16 – 32 - 52
6	6 - 11
10	11 -13 - 32 – 45
11	6 - 13
15	6 – 31 - 52
16	6 – 31 - 33
18	33 - 58
20	13 - 58
21	16 – 31 -32 – 52 - 58
24	31 - 32
25	16 – 31 – 32 - 35
26	32 - 35
27	32 - 33
29	16 - 31
30	16 - 66
31	16 - 35
32	11 - 35
35	13 – 16 – 31 – 32 – 52 - 58
36, 39	16 - 66
37	33 - 35
38	16 – 33 - 58
42	32 – 33 - 35
44	16 – 32 – 35 - 66
46	32 - 33

Cytologically twenty nine samples 80.5% (29/36) which were positive for the PCR showed koilocytosis and mitosoid cells (Fig. 4).

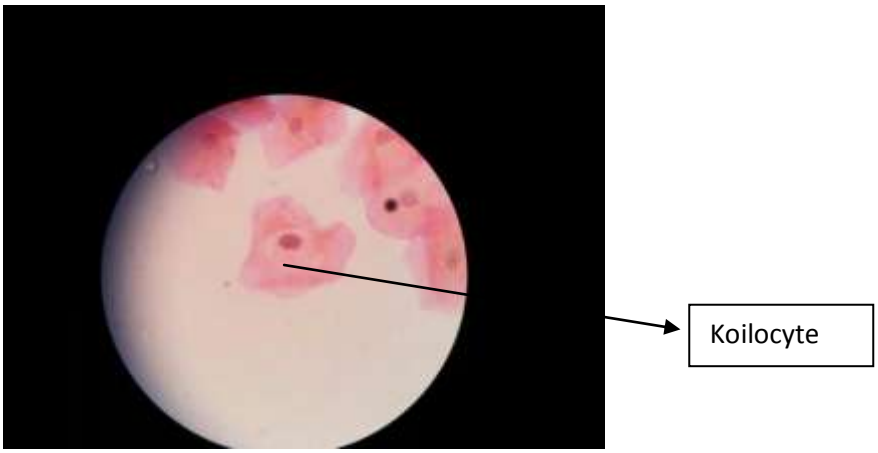

Fig. 4: Pap. smear of the oral smears which were taken from the children who were clinically diagnosed as FEH showing koilocyte with perinuclear hallow (empty area arround the nucleus).

DISCUSSION:

In this study the age group of the patients ranged from three to fourteen years old, this is in agreement with the literature review which indicated that the younger age groups are more affected (Jayasooriya *et al*., 2004, González *et al*., 2005, Dent and Lombardi, 2008, Markowitz *et al*., 2007, Borborema-Santos *et al*., 2006, Pfister *et al*., 1983, Castro and Bussoloti Filho, 2006, Mosannen-Mozaffari *et al*., 2010, Flaitz, 2000, Pinheiro *et al*., 2012, Nartey *et al*., 2003, Ponte *et al*., 2010, Bassioukas *et al*., 2000, Liu *et al*., 2012, Ledesma-Montes *et al*., 2005, Ficarra *et al*., 1991). The females represented 44.7%, the males 32% of the patients, the remaining 23.4% of both sexes were negative for *HPV* genotypes and this agrees with the reports of many studies which revealed that FEH is more common in females than in males (Markowitz *et al*., 2007, Borborema-Santos *et al*., 2006, Mosannen-Mozaffari *et al*., 2010, Nartey *et al*., 2003, Ponte *et al*., 2010, Bassioukas *et al*., 2000, Ghandour, 1989). The frequency and percentage of *HPV* genotypes were variable in the PCR findings (0% - 44.4%). There was a significant difference between the PCR and the Pap. stain findings (P value > 0.05). Some patients were positive for the PCR and negative for the Pap. stain and vice versa. However, this is in contrast to literature report from studies showed that there was no significant difference between the PCR and Pap. stain (Pinheiro *et al*., 2012, Nartey *et al*., 2003, Ponte *et al*., 2010, Bassioukas *et al*., 2000, Ledesma-Montes *et al*., 2005, Ficarra *et al*., 1991). The frequency of the positive Pap. smears was 76.6% and the negative smears 23.4%.

The study′s findings agree with studies which proved that *HPV* genotypes rather than *13* and *32* are assocciated with focal epithelial hyperplasia such as *HPV 1, 6, 11, 16, 18* and *55* (Borborema-Santos *et al*., 2006, Pinheiro *et al*., 2012, Nartey *et al*., 2003, Bassioukas *et al*., 2000, Ficarra *et al*., 1991, Ledesma-Montes *et al*., 2007). It disagrees with the studies which reported that only *HPV 13* and *32* are the main caustive agents for FEH (Jayasooriya *et al*., 2004, González *et al*., 2005, Dent and Lombardi, 2008, Markowitz *et al*., 2007, Pfister *et al*., 1983, Castro and Bussoloti Filho, 2006, Flaitz, 2000, Honarmand, 2010) . Cytological findings are in agreement with studies′s findings where koilocytosis and mitosoid cells are important features of

FEH (Jayasooriya *et al*., 2004, Castro and Bussoloti Filho, 2006, Pinheiro *et al*., 2012, Nartey *et al*., 2003, Bassioukas *et al*., 2000, Ledesma-Montes *et al*., 2005, Ficarra *et al*., 1991).

Because as it was revealed in this study that other *HPV* genotypes rather than *HPV* 13 and 32 associate FEH, some of which were high risk *HPV* genotypes. So upon the presence of this important fact the diagnosis and treatment of FEH depending only on *HPV* 13 and 32 as the only causative agents may be uneffiecient. However, this may progress to development of oral cancer.

CONCLUSION

1- In this descriptive, analytical study it was found that the frequency and percentage of *HPV* genotypes associated with FEH cannot be ignored, since most of the cases showed severe oral lesions in different parts of the oral cavity with difficulty in eating, drinking, swallowing, talking or even having a normal appearance among the other childern which may affect the physcological side of the child.
2- The percentage of infection with *HPV* for FEH in this study ranged between 2.7%-44.4%.
3- The percentage of Pap. smears which showed koilocytosis and mitosoid cells is 76.6%
4- There was a significant difference between PCR and Pap. stain findings (P value > 0.05).
5- The most frequent genotypes which were detected were *HPV 32* (16/36 samples were positive, 44.4%) followed by *HPV 16* (12/36 samples were positive, 33.3%).
6- *HPV* genotypes 31, 33, 35, 52, 58 and 66 were detected in this study although they were not mentioned in the studies conducted in FEH.
7- Pap. Stain's and PCR sensitivity was 80.5% while the specificity was 40%.

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RECEIVED: Oct. 2018; **ACCEPTED**: Dec. 2018; **Published:** Jan. 2019

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Cite this article as:
Bolis *et al.*, (2019): Molecular Detection of Human Papilloma Virus Genotypes in Children with Focal Epithelial Hyperplasia in Khartoum State, Vol. 5: 86- 96.