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ORIGINAL ARTICLE

Genotyping of Human Papillomavirus (HPV) from Patients with Cutaneous Warts

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ABSTRACT

Objectives: To study the prevalence of genotyping of cutaneous human papillomaviruses in patients presented with skin warts in the Basrah and Missan provinces.

Methods: A total of 152 cutaneous samples were collected from patients presented with skin warts in the Basrah and Missan provinces - Iraq during the period from October 2018 to May 2019. The researchers identified the samples genetically using the FAP PCR system followed by multiplex PCR and confirmed by DNA sequencing.

Results: A 120 (78.95%) samples were positive according to the FAP PCR method, while a 131 (86.2%) samples were positive by using the multiplex PCR system; a 99 (75.6%) samples as single genotypes and 32 (24.4%) samples as multiple genotypes. HPV57, 1, 2, and 27 were the most detected HPV genotypes among cutaneous skin wart patients.

Conclusion: HPV57, 1, 2, and 27 were the most common HPV genotypes associated with cutaneous skin warts in patients of the Basrah and Missan provinces. Multiple and single HPV genotypes were detected in the same sample.

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INTRODUCTION

The cutaneous warts are an infectious skin disease commonly found in a dermatology clinic throughout the world. They are caused by human papillomaviruses (HPVs) of the *Papillomaviridae* family of viruses [1].

Over 100 HPV types have been described based on their DNA sequences, distributed over 5 genera (alpha, beta, gamma, mu, and nu), and the genus *Alphapapillomavirus* includes HPV genotypes that infect both genital and oral mucosa [2,3]. In the cutaneous warts, HPV 2, 7, 27 and 57 of the alpha genus, HPV 4 and 65 of the gamma genus and HPV 1 of the mu genus were most often detected [4,5]. HPVs were classified into skin and mucosal types depending on the anatomical detection sites [6]. HPVs have also been classified according to their oncogenicity into high and low risk genotypes [7].

Direct skin- to- skin contact or sexual contact primarily transmits HPV infections [8]. Four main types of warts are common warts, plantar warts, flat warts and anogenital condylomata [9]. Common warts are often found on feet and hands, but can also be found in other areas [10]. HPV-2, -27, and -57 are usually detected in common warts [3]. Flat warts are developing on the face, dorsal hands, or distal forearms of adolescents. HPV-3, -10, -28, -29, -77, -78, -94, and -117 primarily cause them [2]. Plantar warts are benign epithelial tumors which

usually develop in the plantar area. Infection by human papilloma virus types 1, 2, 4, 60, or 63 generally causes these warts, but types 57, 65, 66, and 156 also cause them [11,12].

MATERIALS AND METHODS

Patients and samples

A total of 152 cutaneous wart samples collected from patients of both sex attending the dermatology clinic at main hospitals in Al-Basrah province (Al-Basrah teaching hospital and Al-Fayhaa hospital) and the main hospitals in Missan province (Al-Sadir teaching hospital and Al-Zahrawi hospital) during the period from October 2018 to May 2019. Each sample was placed in a tube containing 300 µl phosphate buffer saline (PBS) and immediately transferred to the laboratory of the virology branch of the College of Science at Al- Basrah university, which were stored at -20^{0}C.

FAP PCR system

DNA was extracted by using geneaid viral DNA extraction kit (Viral Nucleic Acid Extraction Kit II). The mixture of PCR to the FAP gene was contained 2 µl of primer (FAP59 and FAP64), 25 µl Master Mix, 10 µl of the extracted DNA sample, and the mixture was complete by 11 µl nuclease free water to 50 µl. The researchers used nuclease free water as negative control. They applied PCR by using thermal cycler machine using the following PCR condition; 94°C for 10 min and then 45 cycles of 94°C for 1.5 min, 50°C for 1.5 min, and 72°C for 1.5 min. the final step was 72°C for 10 min [13]. The researchers analyzed the amplification products by gel electrophoresis and visualized it under UV. They regarded a 480 DNA band as positive for the FAP PCR system.

Multiplex PCR

The researchers used Purified DNA as a template for screening of HPV genotypes (1, 2,27, and 57) by multiplex PCR based on the size of the PCR products after electrophoresis. Multiplex PCR was applied in a final volume of 25 µl of reaction mixture that contained 0.75 µl of each primer, 12.5 µl master mix, 4 µl of the DNA sample, and the mixture was completed by 2.5 µl nuclease free water to 25µl [14]. PCR was performed using the following condition: denaturation for 3 min at 94°C followed by 35 cycles of denaturation step for 40 secs at 94°C, annealing step for 40 secs at 58°C, and extension for 40 secs at 72°C. the final step was final extension for 3 min at 72 °C. The amplification products were analyzed by gel electrophoresis and visualized under UV.

Sequencing of PCR product

The PCR product samples of HPVs were sent to Macrogen Inc. (Macrogen Korea: 10F, 254 Beotkkot-ro, Geumcheon-qu, Seoul, 08511, Rep. of Korea) and to china (yang ling tianrun aoke biotechnology company). The sequence processing was performed using Chromas version 2.6.2 (http:// technelysium.com.au).

Statistical analysis

Statistical analysis was conducted using T- and chi-square tests with differences at P<0.05 which is considered to be statistically significant. This calculation was carried out according to the Statistical Package for Social Science using IBM SPSS statistics version 24.

RESULTS

A total of 152 patients with the age range 5-70 years' old were diagnosed with skin warts, of these patients 101 (66.4%) were males and 51 (33.6% were females and the age group 11-20 years were the most infected group 53 (34.9%) than other groups with significance differences 0.03 at level of 0.05, of which 34 (64.15%) were male and 19 (35.85%) were female (Tables, 1 and 2). The present study showed that the most types of skin warts were common warts (125; 82.24%), appearing as rough surface nodules or papules, skin-colored with hyperkeratotic border, with black dots on their surface followed by plantar warts (27 ;17.76%), appeared as a keratinous lesions located on the sole of the feet. Furthermore, 94 (61.8%) patients had one wart, 32 (21.1%) two warts, 16 (10.5%) three warts, 6 (3.9%) four warts, 2 (1.3%) five warts, and 2 (1.3%) six warts. In addition, a total of 92 (60.5%) warts occurred on the hand of HPVs infected individuals, 43 (28.3%) warts on foot, 10 (6.6%) warts on the head and 7 (4.6%) warts on the neck. The current study showed that the size of the warts ranges from 1 to 7 mm, while the number of warts ranged from 1-6 HPV skin warts on the same patient. The majority of the warts (61.8%) were single HPV warts, while the remainder (38.2 %) were multiple HPV warts with maximum 6 HPV warts per patient.

Table 1: **Characterization of the study population.**

Sex	Male	101 (66.4%)	*P* value 0.000
	Female	51 (33.6%)	
Marital status	Married	41 (27%)	*P* value 0.000
	Single	111 (73%)	
Location of warts	Hand	89 (58.6%)	*P* value 0.000
	Foot	46 (30.3%)	
	Face	10 (6.6%)	
	Neck	7 (4.6%)	
Number of warts	1	94 (61.8%)	*P* value 0.000
	2	32 (21.1%)	
	3	16 (10.5%)	
	4	6 (3.9%)	
	5	2 (1.3%)	
	6	2(1.3%)	

Table 2: **Characterization of sex of patients among age groups.**

Age group	No.	%	male	Female
5-10	37	24.3	21	16
11-20	53	34.9	34	19
21-30	36	23.7	28	8
31-40	15	9.9	12	3
41-50	7	4.6	5	2
51-60	2	1.3	0	2
61-70	2	1.3	1	1
Total	152	100.0	101	51

The molecular detection

FAP PCR: The researchers found that the FAP PCR system is capable of identifying HPV DNA in 120(78.95%) samples and the remainder 32(21.05%) samples were negative in FAP PCR.

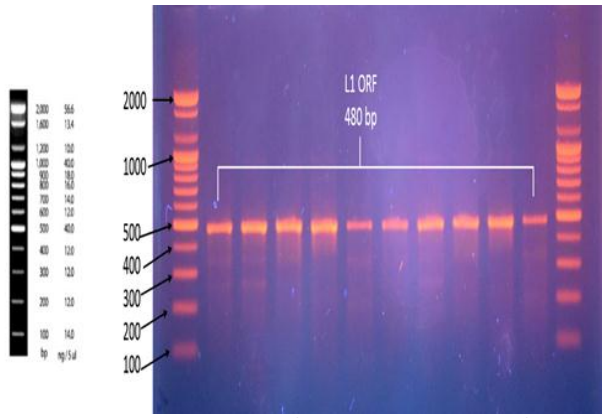

Figure 1. **Gel electrophoresis of FAP PCR products of human papillomaviruses. lane M: DNA marker (100-2000 bp. DNA marker), lanes 1-10: positive results for amplification (480bp).**

The Multiplex PCR system: The researchers used multiplex PCR to detect HPV DNA in the 131 (86.2%) samples and the remainder 21 (13.8%) were negative by using this method. Of the 131 samples single and multiple HPV were detected. Out of the 131 multiplex positive samples 99 (75.6%) samples were single genotypes and 32 (24.4%) samples were multiple genotype with highly significance differences at 0.05 test level.

Out of 99 single HPV genotypes, HPV type 57 was the most common HPV genotype (33.34%) that detected among cutaneous warts patients with significance differences (0.000) between each other, followed by HPV type 1 (32.32 %), HPV type 2 (18.18%), and HPV type 27 (16.16%). Single HPV genotypes were compared with type of warts and found all four HPV genotypes were detected in common skin warts and HPV genotype 27 were not detected in plantar warts. HPV1 were most frequently with plantar warts and HPV57, HPV2 and HPV27 were most frequently with common skin warts (Table 3).

Out of 32 multiple HPV genotypes, HPV 1 and 57 was the most HPV genotypes (31.25%) followed by HPV 2 and 57 (28.125%), HPV 1, 2 and 57 (21.875), and HPV1 and 2 (18.75).

Multiplex PCR were compared with FAP PCR system and found that 112 (73.7%) samples were positive for HPV DNA testing by using multiplex and FAP PCR methods. Eight samples were positive by using FAP PCR method and negative by using multiplex PCR system (Table 4).

Table 3: **HPV genotype according to type of warts.**

		Type of wart				Total	%
		Common wart	%	Plantar wart	%		
HPV genotypes	**HPV1**	15	15.2	17	17.2	32	32.32
	HPV2	14	14.1	4	4	18	18.18
	HPV27	16	16.2	0	0	16	16.16
	HPV57	32	32.3	1	1	33	33.34
Total		77	77.8	22	22.2	99	100

Table 4: **HPV genotyping prevalence.**

		FAP PCR		Total
		Positive	Negative	
Multiplex PCR	Negative	8 (5.3%)	13 (8.6%)	21 (13.8%)
	Positive	112 (73.7%)	19 (12.5%)	131 (86.2%)
Total		120 (78.9%)	32 (21.1%)	152 (100%)
***P* value = 0.000**				

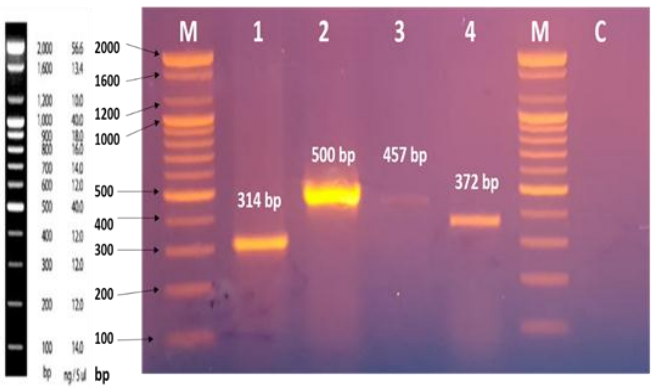

Figure 2. **Gel electrophoresis of the multiplex PCR product of human papillomaviruses. Lane M: DNA ladder (100-2000 bp DNA marker), Lane 1 HPV type 1, lane 2 HPV type 57, lane 3 HPV type 27, lane 4 HPV type 2, lane C negative control.**

Sequencing of PCR products: Thirty-eight PCR product samples were sent to sequencing, including 8 PCR products that were positive in FAP PCR and negative in multiplex PCR and 30 multiplex PCR positive samples.

FAP-positive/multiplex negative samples: Out of eight FAP-positive PCR products, seven isolates were successfully sequenced. The sequence was identified as

genotype 7, 65, and 84 of human papillomavirus (Table 4).

The result showed that HPV genotype 84 that belong to the genus *Gamma papillomavirus* where detected in 2 isolate of 8 FAP PCR positive/multiplex PCR negative. The 2 HPV84 isolate were detected in Basrah province in patients presented with common skin warts. HPV65 from the genus *Alpha papillomavirus* were detected in 3 isolate in patients presented with common skin warts in Missan province. HPV genotype 7 where detected in 2 isolate in patents presented with common skin warts in Basrah and Missan provinces.

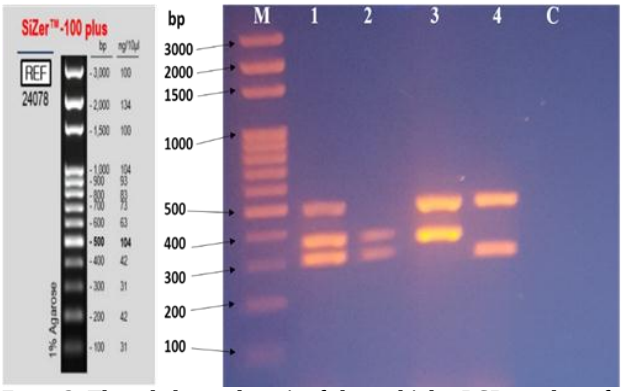

Figure 3. **The gel electrophoresis of the multiplex PCR product of HPVs. Lane M: DNA ladder (100-3000 bp DNA marker), Lane 1: HPV type 1,2 and 57, lane 2: HPV type 1and 2, lane 3: HPV type 2 and 57, lane 4: HPV type 1 and 57, lane C negative control.**

Table 4: **Nucleotides Sequencing Data for Isolates.**

No	Isolate No.	HPV type	Compatible with	E value	Identity %	Query cover %	Strand
1	20-F59	HPV7	X74463.1	0.0	99.30%	98%	Plus/Plus
2	22–F59	HPV7	X74463.1	0.0	99.76%	97%	Plus/Plus
3	7-FAP64	HPV65	X70829.1	2e-128	92.19%	79%	Plus/plus
	7-FAP59	HPV65	X70829.1	1e-75	82.20%	84%	Plus/minus
4	49-F596	HPV65	X70829.1	0.0	97.70%	98%	Plus/plus
5	56–F59	HPV65	X70829.1	0.0	93.93%	97%	Plus/Plus
6	15-F59	HPV84	MH777227.1	0.0	99.30%	100%	Plus/Plus
7	18-F59	HPV84	MH777227.1	0.0	99.54%	97%	Plus/Plus
8	85-F59		Failure				

DISCUSSION

The study revealed that the majority of infection was found in age group patients 11-20 years. This elevated prevalence in the age group 11-20 years may be due to elevated communication among school students. This result was an agreement with [15] that reported cutaneous HPV infection are prevalent in 11-30 years age group. and disagreement with [16] that reported age group 1-10 years the most prevalent age group.

The prevalence of HPV based on sex revealed that the males were 66.4%, while the proportion of female infection was 33.6%. The prevalence of infection among males is higher than that of females due to regional factors and repeated work-related communication among males in society. This distinction in outcomes may be due to the absence of education and the absence of attention to health, particularly when such diseases are not only essential because elitist understanding is limited in health matters. Several studies reported that there is no evidence of sex difference in cutaneous skin warts prevalence [15, 17, 18]. This result was a disagreement with the Dutch study which recorded that the prevalence of HPV infection females 58.9% while the proportion of males was (41.1%) [19].

FAP PCR system

The molecular detection of HPVs with the FAP PCR system was able to detect HPV DNA in 120 (78.95%) samples, the remainder 32(21.05%) were negative. A clear band of approximately 480 bp were detected. This result is an agreement with the Swedish study which recorded that FAP PCR system is able to identify HPV DNA in 87% of 75 analyzed samples [13]. Another study reported that HPV DNA was detected in 55.6% of analyzed samples using FAP PCR system [20].

Multiplex PCR system

Molecular detection of HPV1, 2, 27, and 57 genotypes with multiplex PCR using four specific sets' primers were able to identify multiple and single HPV genotypes in cutaneous skin samples. The sensitivity of the multiplex PCR system was 86.2 % of analyzed samples. Studies [14] recorded high sensitivity of Multiplex PCR to detect HPV DNA in the clinical samples reported multiplex PCR system was able to detect HPV DNA in 97.6 % of analyzed clinical samples. Study [21] recorded that the multiplex PCR system was able to detect HPV DNA in 95% of analyzed clinical samples.

Among the 131 multiplex positive samples, 99 (75.6%) samples were single HPV genotypes. The most common HPV genotype detected was HPV57 followed by HPV1, 2, and 27. Study [14] recorded that HPV57 was the most detected HPV genotype (35%) among cutaneous skin wart patients followed by HPV1 (32.6%), HPV27 (18.9%), and HPV2 (13.5%). A Japanese study reported that the most common HPV genotype was HPV1 (44.1%) and the less common HPV genotype was HPV 57 (0.47%) that associated with cutaneous skin warts [17]. A British study recorded that HPV 2,27,57 were the most detected HPV genotypes in cutaneous skin warts while HPV1 were the less common HPV genotype [5].

Out of the 131 multiplex positive samples 32 (24.4 %) were multiple HPV genotypes. The study revealed the presence of mixed HPV infection in the same sample including infection with two and three HPV genotypes.

The presence of mixed HPV infection in a single sample may suggest that there is no natural competition on the skin of different HPV genotypes. So far, it has not been known whether these different HPV genotypes exist in the same cell or different cells, whether warts are caused by only one HPV genotypes, while other HPV genotypes are passengers, and whether these passengers are biologically active. However, the majority of the HPV infection were single HPV genotypes 75.6 of analyzed cutaneous warts samples pointing out that

mostly one HPV genotype is accountable for skin lesions. The presence of mixed HPV infection may be due to the presence of passengers HPV on the surface of the warts or this mixed HPV infection is normal phenomenon in this viruses.

Study [16] recorded the presence of multiple HPV genotypes in 16 % of HPV swab samples. Another study reported the presence of HPV mixed infection in 51% of HPV positive samples [22]. Several studies reported the existence of mixed HPV infection in the same sample [14, 21, 23].

The study revealed that HPV1, 2, 27, and 57 were detected in common skin warts and HPV 27 were not detected in plantar warts. HPV1 were most frequently associated with plantar skin warts and HPV2,27, and 57 were most frequently associated with common skin warts. Several studies recorded HPV1 most frequently associated with plantar warts and HPV2, 27, and 57 were most frequently detected in common skin warts [24,25].

5.4 Sequencing

Sequencing of FAP positive sample of L1 ORF result in identification of HPV genotype 7 and HPV 65 from the genus *Alpha papillomavirus* and HPV 84 from the genus *Gamma papillomavirus*. L1 ORF gene encoding for L1 protein has been used for taxonomy and identification of human papillomaviruses and animal papillomaviruses because the L1 ORF is the most conserved region in all papillomaviruses [26]. Several studies reported that HPV genotype7 and HPV genotype 65 takes a part in development of cutaneous skin warts [19]. The result of identification of HPV genotype 84 from the genus *Gamma papillomavirus* was agreement with French Report that reported the identification of HPV genotype 84 in skin lesion [27].

CONCLUSION

The Human papillomavirus infection were mostly infecting males than females and the age group of 11-20 years was more infected than other age groups. HPV 57, 1,2,27 genotypes were the most prevalent HPV genotypes that caused skin warts. Mixed HPV infection were detected in the same sample using multiplex PCR.

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