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In Situ Hybridization for Molecular Detection of Human Papilloma Viral 6 / 11 DNA in Adenoctomized Tissues from A group of Iraqi Pediatric Patients

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Abstract:

Among more than 200 different human papilloma viral genotypes, the association of low oncogenic risk-HPV genotypes have been recognized with a variety of oral, oropharyngeal, nasopharyngeal benign tumors as well as non-neoplastic polyposis and papillomas and adenoid hypertrophy. This prospective case-control study aims to determine the rate of DNA detection of HPV genotype 6/11 in nasopharyngeal adeno-tonsillar tissues from a group of patients subjected to adenoctomy for adenoid hypertrophy . A total number of 60 nasopharyngeal adeno-tonsillar tissue specimens from pediatric patients with adenoid hypertrophy were enrolled; 40 nasopharyngeal adeno-tonsillar tissues from patients with adenoid hypertrophy, and 20 normal nasal tissue specimens were obtained from pediatric patients following trimming operations of their inferior nasal turbinates' with unremarkable pathological changes (as an apparently healthy control group). The molecular detection methods for HPV detection were performed by using DNA probes via a recent version of chromogenic in situ hybridization specified for low- risk HPV genotypes. Among total adenoid hypertrophied tissue specimens group, 8 out of 40 were found to contain positive results for DNA of HPV 6 / 11 genotype, constituting 20% of the total screened nasopharyngeal adenotonsillar tissues. No positive–CISH reactions were detected in the control nasal tissues. The statistical analysis of results in this research showed significant difference when compared to the control apparently healthy tissues. The significant rate of low- oncogenic HPV genotypes detection in those adenoid hypertrophied tissues could play, in part, a role in their pathogenesis and / or constituting a herald focus for the spread of such important virally transmitted infection.

Keywords: Adenoid hypertrophy, Chromogenic in situ hybridization, HPV 6/11, Nasopharyngeal adenotonsillar tissues.

Introduction:

The adenoids are a group of lymphoid tissue, along with the palatine tonsils, lingual tonsils, and tubal tonsils make up what is known as Waldeyer's ring. Adenoiditis is a condition of childhood, where adenoid hypertrophy (an increased in the size of adenoids with or without an acute or chronic infection) is responsible for the most common health issues that are associated with the adenoids [1].

Infectious as well as non-infectious etiologies were allocated in the etiology of adenoid hypertrophy. Among their viral infectious causes, an association of adenoid hypertrophy with adenovirus, coronavirus, Coxsackie virus, cytomegalovirus, Epstein-Barr virus, herpes simplex virus, human Boca virus, para influenza virus, Rhinovirus, Parvovirus B19, and novel KI and KU polyomaviruses have been reported [2-5]

Over 200 types of human papillomavirus (HPV) are recognized to infect the skin as well as epithelia, at least 13'high- oncogenic risk' types are cancer- causing while the rest are found to have

low- oncogenic risk and causing non-malignant diseases, among them, HPV 6 and 11 are the most common types [6]

The rates of human papilloma viral -related cancers in the head and neck region increased in last years, where the majorities arise in oropharynx, and the tonsils are mostly affected tissues [7-9]. The rate of human papillomavirus infection in adenoid hypertrophy still currently is poorly defined [10].

The aim of the present research is to assess the possible associative role of human papilloma viral genotypes 6/11 infections among adenoidal tissues obtained from patients underwent adenoidectomy.

Material and Methods:

Patients and control

This prospective case- control study has enrolled sixty nasopharyngeal adeno-tonsillar tissue specimens from pediatric patients with adenoid hypertrophy; 40 nasopharyngeal adeno-tonsillar tissues from pediatric patients with adenoid hypertrophy, and 20 normal nasal tissue specimens were obtained from pediatric patients following trimming operations of their inferior nasal turbinates' used as a healthy control tissue group.

Methods

Chromogenic In-Situ Hybridization (CISH) for detection of HPV 6\11 was done by digoxigenin-labeled oligonucleotide probe, that targeting HPV 6\11-DNA, on 4μm paraffin tissue sections and as stated by the manufacturing company (Zyto Vision, Germany).

The CISH main steps started by incubating the slides for 1 hr at 70°C, then sequential rehydrating the slides at room temperature including their immersion twice in absolute xylene for (15) minute, absolute ethanol (5 minutes), 95% ethanol (5 minutes), 70% ethanol (5 minutes), distilled water (5 minutes), and lastly drying for 5 minutes at 37°C. Then, applying pepsin solution and incubating at 37°C for 45 minute and then immersing in distilled water and air- drying. Then adding probe and denaturizing slides for 5 minutes at 75°C and hybridized for 18 hrs at 37°C and then washed in 1x wash buffer TBS for 5 minutes at 55°C. Then, applying alkaline phosphatase-streptavidin and incubating the slides at 37°C for 30 minutes then, washing in buffer TBS and washing for 5 minutes twice times in distilled water and rinsed in detergent buffer for 5 minutes followed by applying BCIP/NBT and incubating for 30 minutes at 37°C. Color development elicited a dark- blue colored precipitate at positive sites in the examined cells. Then the slides counter- stained by Nuclear Fast Red (NFR) and the sections dehydrated sequentially by ethyl alcohol mounted with Disteren plasticizer Xylene.

To calculate the statistical significance (p value), SPSS-21 package has been performed and p value less than 0.05 denotes a significant relationship.

Results:

I.Pediatric patients with adenoid hypertrophy according to age:

The age of pediatric patients with adenoid hypertrophy ranged from 4 to 9 with mean of (5.77 ± 3.73 years). The mean age of control pediatric individuals (A.H. Control) was (6.35 ± 5.66 years) and the age ranged from 5- 12 years (Table 1) .

Table 1. Distribution of pediatric patients with adenoid hypertrophy according to age

Group	N	Mean Age	Std. Deviation	Std. Error	Age Range		ANOVA test
					Mini.	Maxi.	
Adenoid Hypertrophy	40	5.77	3.73	1.11	4	9	P=0.4 Not Sign. (P≥0.01)
A.H. Control	20	6.35	5.66	2.14	5	12	
Total	60						

II. Gender distribution of the pediatric patients with adenoid hypertrophy:

Pediatric Males with adenoid hypertrophy was higher (60%: 24) than their female counter parts (40%: 16). Also, in control group, the pediatric males were higher (60%: 12) than pediatric females (40%: 8). The statistical analysis showed significant difference (P<0.01) among the studied groups (Table 2).

Table 2. The distribution of pediatric patients with adenoid hypertrophy according to their gender

Gender		Studied Groups		Pearson Chi-Square (P-value)
		Apparently-Healthy Control	Adenoid Hypertrophy	
Male	N	12	24	P=0.007
	%	60%	60%	
Female	N	8	16	Sign.
	%	40%	40%	(P<0.01)
Total	N	20	40	

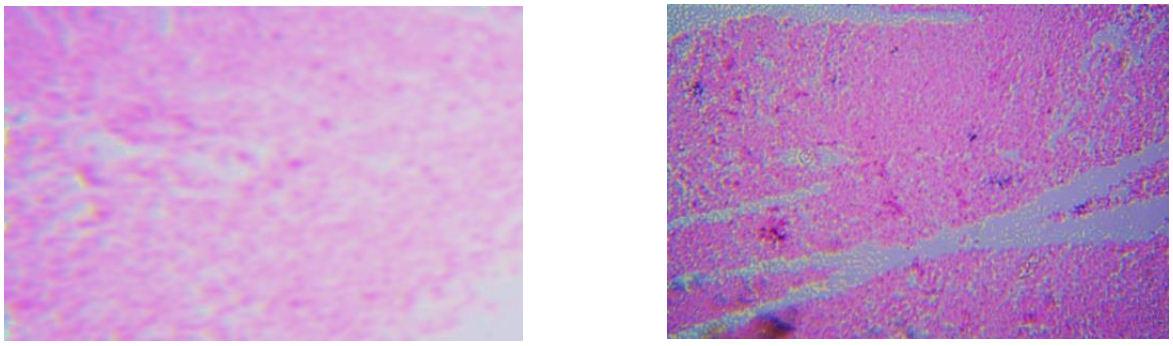
III. Human Papilloma Virus types 6\11 -CISH expression in hypertrophied adenoid tissues

I-Positive HPV 6\11 DNA- CISH signal scoring

The adenoid hypertrophy group revealed 20% positive signals representing 8 out of the 40 tissues while in control tissues group, none showed positive CISH test. Moderate signal scoring noticed in 10% whereas (7.5%) and (2.5%) have low and high signals scoring, respectively as detailed in (Table 3 and Fig. 1). Statistical comparison of HPV 6\11-DNA rates in control and adenoid hypertrophy groups very highly significant difference ($P \leq 0.002$).

Table 3. Distribution of signal scores of HPV 6\11-DNA-CISH reactions

HPV 6\11 DNA-CISH reactions scores		Studied groups		P-Value
		A.H. Control	Adenoid Hypertrophy	
Negative	N	20	32	
	%	100%	80%	
Positive	N	0	8	
	%	0.00%	20%	
Low	N	0	3	
	%	0.00%	7.5%	P=0.002 Highly Significant (P<0.01)
Moderate	N	0	4	
	%	0.00%	10%	
High	N	0	1	
	%	0.00%	2.5%	
Total	N	20	40	
	%	100%	100%	



A B

Figure 1. Qualitative results of CISH for HPV 6/11-DNA detection in nasopharyngeal hypertrophied adenoid tissues stained with BCIP/NBT, counter stained by NFR.

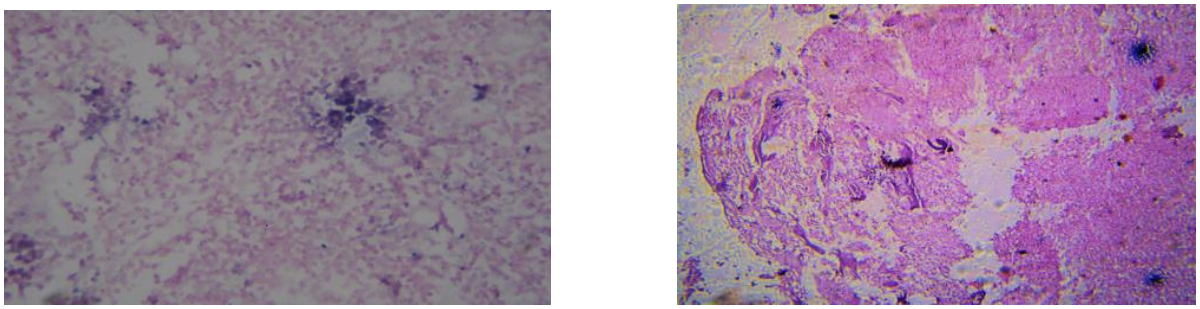
A. Hypertrophied adenoid tissue shows no HPV 6/11-DNA -CISH reaction (20X).B. Hypertrophied adenoid tissue with positive HPV 6/11-DNA -CISH reaction (40X).

II. Signal intensity of HPV 6\11- CISH testing:

Regarding signal intensities of HPV 6\11-CISH signal detection in adenoid hypertrophy tissues group, the weak signal intensity was noticed in (10 %) whereas (5%) have in both moderate and strong intensity. Statistically, significant differences were recorded between studied groups (Table 4 and Fig. 2).

Table 4. Signal intensities of HPV 6\11-DNA-CISH reactions

HPV 6\11 DNA-CISH reactions intensity		Studied groups		
		A.H. Control	Adenoid Hypertrophy	P-Value
Negative	N	20	32	
	%	100%	80%	
Positive	N	0	8	
	%	0.00%	20%	
Weak	N	0	4	
	%	0.00%	10%	
Moderate	N	0	2	P=0.008
	%	0.00%	5%	Sign. (P<0.01)
Strong	N	0	2	
	%	0.00%	5%	
Total	N	20	40	
	%	100%	100%	



A B

Figure 2. Signal intensity and scoring assessment of CISH results of HPV 6/11-DNA in nasopharyngeal hypertrophied adenoid tissues; BCIP/NBT stained and counter stained with NFR; A. Hypertrophied adenoid tissue with positive HPV 6/11-DNA -CISH reaction of score moderate and intensity score moderate (20X).

B. Hypertrophied adenoid shows positive HPV 6/11-DNA -CISH reaction of score high and intensity score strong (40X).

IV. The physical state of HPV-6/11-DNA CISH signal detection

Regarding the physical state of HPV-6/11-DNA as episomal and integrated forms detection in adenoid hypertrophy tissues group, the episomal was noticed in (66.7 %) whereas (33.3%) have an integrated phase (Table 5).

Table 5. Integrated and episomal forms of HPV-6/11

HPV 6\11	Positive (No.)	%
Episomal	4	66.7
Integrated	2	33.3

V. Spearman's Statistical Evaluation of the Studied Parameters In Adenotonsillar Tissues.

A highly significant and strong positive correlation between HPV 6\11 and age of patients with adenoid hypertrophy was found (r = 0.397, P = 0.004), while none- significant relation between adenoid hypertrophy with gender was found (Table 6).

Table 6. Spearman's statistical evaluation of the studied parameters in adenoid hypertrophy group.

Spearman's-rho statistical evaluation		Age	Gender	HPV 6\11
Age groups (Years)	r		0.050	
	P		0.898	
Gender	r			0.186
	P			0.342
HPV6\11	r	0.379		
	P	0.004*		

* Highly- significant Correlation (P<0.01).

Discussion:

Only few studies have assessed normal tonsillar tissues for the presence of HPV DNA and only 200 samples or biopsies from tonsillitis were analyzed by the end of 2002, , where a total of 17 samples (8.5%) contained HPV DNA, and out of them, five samples contained HPV DNA type 6/11 [11] .

To the best of our knowledge, this research represents the first in Iraq that used in situ

hybridization analysis of the rate of HPV 6/11 infection in adenotonsillar tissues of pediatric patients. However, Ali *et al.* [12] have documented HPV DNA detection in 3.2% of those appeared as healthy oral tissues on histopathological examinations. In addition, Ali *et al.* [13] in 2017 have also studied and detected (10%) positive CISH signals for low-risk Human Papilloma Virus DNA in a group of apparently healthy nasal control tissues in Iraq.

The adenoid hypertrophy group showed 20% positive results (8 out of 40 tissues). Control tissues revealed no positive-CISH test (Table 3).

Wojtera *et al.* [10] revealed in a systematic review that the overall rate of HPV in tonsillar tissues of pediatric patients ranged from 0 to 21%. However, and of value is to note that, in a previous study [14] (studied 1670 patients) as well as other four studies (enrolled 1941 patients) were unable to detect this virus in their samples. On the other hand, studies [14-15] that enrolled another largest patient samples have revealed a prevalence of only 1% positive results, pointing for potential sample or selection bias. The rate of HPV in the studied tonsillar tissues from pediatric groups, however, are still poorly defined [10].

The broad-spectrum primers for HPV used for conventional PCR in most of previous studies consequently yields high false positive rates, [16] while RT-qPCR is the gold standard to demonstrate the expression of HPV transcripts [17].In addition, and to explain the differences observed, it could be related to the lack of controls as well as because conventional PCR is still unreliable.

The PV-6 and HPV 11 are two low risk mucosal types which are rare in malignant tumors, yet, have on occasions being reported in carcinomas of vulva as well as cervix, as well as papillomas of larynx and most giant condylomas [18-19]

Brandsma and Abramson [20] and followed by Bercovitch [21] have the first chance to detect HPV-6/11 DNA each in one sample of SCCs of tonsil by Southern blot hybridization while Schwarz [22] and Badaracco [23] have detected HPV-6/11 DNA in 3 and 1 sample of tonsillar SCCs using PCR, respectively.

Both integrated and episomal forms of HPV-6/11 -CISH signal detection in adenoid hypertrophy tissues were noticed in (70 %) and (30%) ,respectively .

Few studies only have analyzed systematically the physical state of HPV in tonsillar carcinoma cases (18). Wojtera *et al.* [10] stated that such HPV finding might be considered as possible confounder, as tonsillar hypertrophy as well as chronic tonsillitis found to be the reasons for their tonsillectomies, where such conditions have no relation to HPV infection. However, the episomal as well as integrated forms of HPV-6 have reported previously in SCC of tonsil [24-25]

To conclude, it is crucial to evaluate HPV prevalence in the pediatric population so as to delineate from where and when such infection of tonsils has been firstly acquired and for how long it has been latent there. It is also important to note that whether these HPV 6/11 infections are either a source for a later non-malignant and benign states or just a transient and / or a latent HPV 6/11 infection are yet to be determined.

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Authors' declaration:

- Conflicts of Interest: None.
- We hereby confirm that all the Figures and Tables in the manuscript are mine ours. Besides, the Figures and images, which are not mine ours, have been given the permission for re-publication attached with the manuscript.
- The author has signed an animal welfare statement.
- Ethical Clearance: The project was approved by the local ethical committee in University of Babylon.

Authors' contributions statement:

- **Dr. Saad hassan**: contributed to collect he samples
- **Dr. Khalil Ismail A. Mohammed**: contributed to make the retrieval and processing the samples
- **Dr. Wifaq M.Ali**: contributed to make sectioning to the samples by microtome device
- **Dr. Suha A. AL-Fakhar & Dr. Shakir Hammad**: make the procedure of immunohistochemistry
- **Biologist Jinan M.Mousa** make the staining of the samples
- **Finally all the team contributed to write this research according to their specialization**

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الكشف الجزيئي بطريقة التهجين الموضعي للحمض النووي للفيروس الورمي الحليمي البشري نوع 11/6 في الأنسجة الغدية البلعومية الأنفية لمجموعة من الأطفال المرضى العراقيين

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الخلاصة:

هنالك أكثر من 200 من الأنماط الجينية لفايروس الورمي الحليمي البشري والتي تم التعرف عليها من خلال الترابط مابين الانماط الجينية ذات الاختطار الواطئ للفايروس الحليمي البشري مع مجموعة متنوعة من الأورام الفموية والبلعوم والبلعوم الأنفي وكذلك الأورام الحليمية غير الخبيثة وتضخم الغدة. هدفت هذه الدراسة من نوع الاستقصائية- السيطرة إلى تحديد الحمض النووي للفيروس الورمي الحليمي البشري 11/6 في انسجة الغدد البلعومية الأنفية واللوزتين لمجموعة من المرضى الذين يخضعون لاستئصال اللوزتين. تم الحصول على 60عينة من أنسجة اللوزتين الأنفية البلعومية من الأطفال الذين يعانون من تضخم اللوزتين الغدي، تضمنت 40 من أنسجة اللوزتين الأنفية البلعومية للمرضى الذين يعانون من تضخم غدي ، و 20 عينة نسجية من الأطفال بعد عمليات التشذيب للانسجة الأنفية السفلية بدون تغييرات مرضية ملحوظة (كمجموعة سيطرة). تم اجراء الكشف الجزيئي عن الحامض النووي للفيروس الورمي الحليمي البشري نمط 11\6 باستخدام تقنية التهجين الموضعي ذات الحساسية العالية في عينات الأنسجة الضخمة الغدية ز تم العثور على 8 من أصل 40 تحتوي على نتائج إيجابية للحمض النووي للفيروس الحليمي البشري 11\6 حيث شكلت 20 ٪ من إجمالي الأنسجة الغدية البلعومية الأنفية.بينما لاتوجد أي اشارة موجبة للحامض النووي للفايروس الحليمي البشري نمط 11\6 في مجموعة السيطرة. أظهر التحليل الإحصائي للنتائج في هذا البحث اختلافًا كبيرًا مقارنةً مابين مجموعتي المرضى والسيطرة عند مستوى احتمالية اقل او يساوي 5%. يمكن أن يلعب فيروس الورم الحليمي البشري ذات الاختطار الواطئ في تلك الأنسجة الغدية المتضخمة دورًا في التسبب في الأمراض و / أو يشكل عامل لانتشار العدوى الفيروسية للانسجة الاخرى

الكلمات المفتاحية: التضخم الغدي، الانسجة الغدية البلعومية الانفية، الفيروس الورمي الحلمي البشري نوع 11,6 ، التهجين الموضعي