Detection of Human Papilloma Virus in the Tonsils of Children Undergoing Tonsillectomy

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Human papilloma virus (HPV) is related to respiratory mucosal diseases, such as recurrent respiratory papillomatosis, as well as to upper-respiratory-tract malignancies. There are few reports concerning the prevalence of HPV in the upper respiratory tract of non-affected individuals. We examined the prevalence of HPV in the tonsils of children of the general population scheduled for tonsillectomy. Samples were taken from the tonsils of 100 children undergoing tonsillectomy and were then tested for HPV with the polymerase chain reaction (PCR) technique, utilizing the generic primers MY09 and MY 11. The study excluded children known to have HPV and HIV-related diseases. Parents and legal guardians completed a standardized socio-demographic questionnaire. The questionnaire revealed that 84% of the mothers had at least one risk factor for genital HPV. None of the tonsil samples were positive for HPV. Apparently HPV does not commonly colonize the tonsils of children undergoing routine tonsillectomy.

Key Words: HPV, tonsils, children.

Human papilloma virus is currently classified as a DNA virus with more than 90 distinct types. These types are classified according to their relationship with benign and malignant lesions into low and high-risk HPV respectively [1]. HPV is also related to certain skin lesions and other lesions of the respiratory, genitourinary and gastrointestinal tracts. The skin lesions include common warts (verruca vulgaris), plantar warts (myrmecias), flat warts (verruca plana) and verruciform epidermal dysplasia (epidemodysplasia verruciformis). In the respiratory tract, HPV is related to papillomatous lesions that occur from the nasal to the bronchial mucosa. In the larynx, this virus is responsible for recurrent respiratory papillomatosis (RRP) in adults and children. HPV also has been linked to several head and neck carcinomas [2-10]. Gastrointestinal tract manifestations include papillomas, focal epithelial hyperplasia and leucoplakia. In the genitourinary tract, HPV has been detected in cervical, vaginal and penile mucosa and in perianal skin. It is linked to the development of condyloma acuminatum, certain pre-cancerous squamous intra-epithelial lesions, and also to intra-epithelial squamous neoplasia of the cervix, the second-most-common malignant tumor in women [11].

Currently, the diagnostic methods to detect HPV are based either on morphological effects of the virus on tissues or on documenting the presence of HPV DNA. One of the most commonly utilized techniques to detect HPV DNA is PCR, which allows the amplification and identification of small quantities of specific sequences of DNA.

Although several medical modalities have been utilized to treat HPV lesions, the most common treatment for HPV lesions is still surgical removal. Even though HPV is well known to be related to several diseases with significant morbidity and mortality, few researchers have attempted to determine the frequency that HPV colonizes other groups. In order to understand the epidemiology and the prevalence of HPV in children, we we used PCR to look for HPV in the tonsils of children scheduled for tonsillectomy.

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Material and Methods

This study was carried out at the University Hospital of the Federal University of São Paulo. Surgeons performed routine tonsillectomies on 100 children between ages 2 and 13 years and removed a small portion of the tonsils with a surgical scalpel. The physician then the specimen in a cryo-tube containing 5 mL of a media transport solution (tris-HCl 10mM, pH 7.5, EDTA 5 mM, Na Cl 150 mM and sodium azide 0.1%), and arranged for the specimen to be taken immediately to the Molecular Biology Laboratory for storage at minus 70º C.

Before participation in the study, the legal guardians of each child read, discussed and signed an informed consent. They also reviewed specific information about risks for the transmission of HPV and completed a standardized socio-demographic questionnaire designed to evaluate the presence or absence of risk factors. The study excluded any child known to be HIV positive, or having any HPV-related disease and any child born to an HIV carrier.

Laboratory procedures. DNA extraction was accomplished by using a commercial kit for extraction of nucleic acid from tissue "NucleoSpin Tissue Kits", CLONTECH, by following the manufacturer's procedures.

Polymerase Chain Reaction. 5mL of the extracted DNA was added to 45mL of buffer solution [1X], MgCl2 [1.25nM], dNTP (dATP, dCTP, dGTP, dTTP) [200mM/base], 4 pmol/ mL of each

probe and 0.75 U of restriction enzyme taq DNA polymerase, Life Technologiesâ. The probes used were the universal probes MY9 (5'GCTCC AA GG AAC TGATC) and MY11 (5'GC CAGGG CAT AAAATGG), which amplify the L1 gene of HPV [12]. Oligonucleotides were manufactured by Life Technologiesâ. The DNA samples were submitted to 35 cycles of amplification in a thermocycler model MJ Research PTC 200. For each trial, a negative control (water) and a positive control (pool of positive samples) were used. After amplification, the products of the reaction were analyzed with 1.5% agarose ethidium bromide gel eletrophoresis. Detection of the amplified product was done by comparison of the molecular weight relative to the pattern of the 100pb ladder provided by the manufacturer, considering the estimated molecular weight of 450 base pairs of amplified HPV.

Results

Social demographic data. The ages of the 100 children included in this study ranged from 2 to 13 years. Forty-nine girls and 51 boys participated in the study. The educational level of the mothers was low; 62% attended but did not complete elementary school, 12% completed elementary school; 8% attended high school for some period of time; 14% completed high school; 2% attended a university for some period, but none finished their university degree. Nearly 40% of the women were smokers.

The mothers began to be sexually active between 13 and 30 years of age, (mean 17.74, confidence interval 17.71 +/- 1.28); 54.4% of them initiated this activity before age 18. The number of sexual partners ranged between 1 and 11, (mean 2.4, confidence interval 2.4 +/- 0.41). Only a minority of the mothers (38%) used contraceptive techniques. A very small number of the mothers (5%) reported having sexually-transmitted diseases, either condyloma or syphilis. Nine per cent of the mothers delivered their first child before they were 18 years old. Fifty-eight of the study children had a vaginal delivery and 40% were born by Caesarian section.

HPV molecular assay. All 100 samples of tonsils were negative for HPV.

Discussion

The presence of HPV in the oral cavity and upper respiratory tract mucosa is of great importance, since several studies have demonstrated an association of HPV with a great variety of benign and malignant lesions. Some of the most frequent benign lesions of the oral mucosa related to HPV are oral papilloma, focal epithelial hyperplasia and leukoplakias [13-15]. Frequency of HPV in head and neck cancer patients varies between 8 and 50%; it is believed that its detection may be dependent on several factors including the molecular technique utilized (Southern blot hybridization, PCR, in situ hybridization), the treatment of the sample material (fresh, frozen, paraffin embedded), ethnic and geographical differences, and the anatomic site of the lesion [2-10,13,18].

Because 60% to 64% of individuals with carcinoma of the tonsil have detectable HPV, some researchers have investigated the prevalence of HPV in the oral cavity mucosa [16,17]. An overview of the medical literature results of HPV detection in the oropharynx is shown in Table 1 [1,18-34].

One hundred children previously scheduled for tonsillectomy participated in this research. The size of the cohort was based on the number calculated to achieve statistical significance. Since the aim of the study was to determine the frequency of HPV in healthy children, we excluded all patients with HPV or HIV-related diseases, because HPV prevalence is known to be higher in these groups than in the general population [21,35]. The socio-demographic questionnaire included elements related to the most relevant risk factors for maternal genital HPV infection, including smoking and sexual history [35].

Most of the mothers had stopped their education before completing elementary school, which may mean a lack of knowledge about sexual diseases and preventive methods. The low median family income (approximately 300 US dollars a month) suggests the possibility of poor hygiene, nutrition and limited access to heath care.

According to the literature [35], smoking is also a risk factor for HPV infection in women. Nearly 40% of the women were smokers. It is known that starting sexual activity and giving birth at an early age, having numerous sexual partners, and using oral contraceptives correlate with a higher risk for HPV infection [35]. In our study, maternal sexual activity started between 11 and 30 years of age (median 18 years); 9% delivered their first child before age 18; and the number of sexual partners ranged between 1and 11. Thirty-eight of the mothers were regularly taking contraceptive pills.

Although sexually transmitted diseases were reported by only 5.4% of the mothers, this figure may underestimate the true prevalence because of inadequate medical information and access. It is not clear if delivery by caesarian section acts protects against HPV transmission [1,21,35]. In our study, about 40% of the children were born via caesarian section. The final analysis of the risk factors showed that 84% of the mothers presented at least one risk factor for HPV infection.

The existing reports on the detection of HPV in swabs of oral mucosa and in the nasopharynx of children and newborns using PCR and DNA hybridization techniques show highly variable results, ranging from 0 to 73% [20,21,25-28,30,3-39]. In adults, the results are also variable, ranging from 5.2% to 81% [1,23,29,31-33,34,40]. This suggests that the oral mucosal swab may not yield a reliable sample for detecting HPV, regardless of the technique employed.

On the other hand, biopsies of normal oral mucosa in adults have revealed less variability, with PCR techniques detecting HPV in 3.8% [31]to 23,1% [32], and hybridization techniques

Table 1. Literature findings of human papillomavirus detection in oral mucosa

Reference	Age	Site	Samples	Technique	Frequency
[18]	Adults	Mouth	Biopsy	Hybridization	41.6%
[19]	Adults	Mouth	Biopsy	Hybridization	41%
[20]	Children	Mouth	Swab	PCR	24% & 19%*
[21]	Newborns	Naso-oropharynx	Suction	Hibridization	2.8%
[22]	Adults	Mouth	Biopsy	PCR	3.1%
[23]	Adults	Mouth	Swab	PCR	43%
[24]	Adults	Mouth	Biopsy	PCR	23.1%
			Hybridization	15.6%	
[25]	Children	Mouth	Swab	PCR	31.6%
[26]	Children	Mouth	Swab	PCR	35.4%
[27]	Children	Mouth	Swab	Hybridization	41.6%
[28]	Newborn	Mouth	Swab	PCR	0%
	Children				0%
[29]	Adults	Mouth	Swab	PCR	81.1%
[30]	Children	Mouth	Swab	PCR	51.7%
[1]	Adults	Mouth	Swab	PCR	8.7%**
			Hibridization	0%***	
				5.2%****	
[31]	Adults	Mouth	Swab	PCR	0.6%
[32]	Adults	Mouth	Swab	PCR	2.4%
[33]	Adults	Mouth	Swabs	PCR	51.1%
[34]	Adults	Mouth	Biopsy	PCR	26%

* HPV 6b and 16 respectively. ** Children under 7 years. *** Children between 7 to 12 years. **** Teenagers between 13 to 20 years.

detecting the virus in 15.6% [32], 41.6% [33] and 41% [34]. Based on these results, we elected to use tissue biopsies for our study.

The choice of PCR in-house generic primers MY09 and MY11 was based on the high sensitivity of this method, which allows amplification of small quantities of viral DNA, and also on its specificity compared to other techniques [41-43].

The negative results in all our samples may reflect the fact that when HPV is present in oral mucosa, it might not be due only to colonization, but could be mostly related to disease problems. Possibly, the tonsils are not a frequent site of colonization; concomitant oral swabs might have been useful. Further studies of other areas of the respiratory tract in healthy children and a long-term follow-up are warranted to clarify whether or not the presence of HPV in tissue is a risk factor for the development of HPV-related diseases. We also need to know whether hypertrophic or chronically infected tonsils are refractive to HPV infection.

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