Prevalence of human papillomaviruses in the mouths of New Zealand women

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ABSTRACT

AIM: Human papillomavirus (HPV) in the oral cavity has been retrospectively associated with an increased risk of developing HPV-positive head and neck squamous cell carcinoma (HNSCC). The aim of this study was to determine the prevalence of oral HPV infection in a local population of New Zealand women aged 18 to 25 years, including determination of HPV genotypes, and to assess potential risk factors for oral HPV infection using participant questionnaire responses.

METHODS: Oral brushings and questionnaire responses were collected from 234 women recruited from sexual health and student health centres. Questions covered age, ethnicity, sexual partners, alcohol consumption and smoking. PGMY primers were used for HPV detection by PCR, and results confirmed by sequencing and the cobas® 4800 HPV system.

RESULTS: The prevalence of HPV infection was 3.2% of 216 women (95% CI: 1.6%–6.5%). Samples from two women (0.9%, 95% CI: 0.3%–3.3%) contained oncogenic HPV, and another five (2.3%, 95% CI: 1.0%–5.3%) were positive for HPV 13. No significant associations were found between putative risk factors and the presence of oral HPV infection.

CONCLUSION: The prevalence of HPV in the oral cavity of New Zealand woman was comparable to results of other studies, but showed an unusual distribution of HPV types. The comparatively high detection rate of HPV 13 suggests that further work into clinical significance of oral HPV 13 infection is warranted.

Head and neck squamous cell carcinomas (HNSCCs) are the sixth most common cancers worldwide, with an estimated incidence of 405,000 cases and 200,000 deaths annually.[1] These cancers comprise tumours of the oral cavity, oropharynx, hypopharynx, and larynx.[2] Head and neck SCCs are a heterogeneous group of cancers showing two distinct pathways to malignancy. The first pathway involves smoking and alcohol as risk factors, and the second is mediated by infection with a high risk human papillomavirus (HPV) type.[3] The incidence of HPV-positive HNSCC in developed countries has increased significantly in the past few decades.[4,5] For example, data from the US have shown a 225% increase in cases of HPV-positive HNSCC between 1984 and 2004.[1]

Although detection of HPV DNA in the oral cavity has been found to be associated with an increased risk of HPV-positive HNSCC,[6,7] the pathogenesis of HPV-positive cancers of the oral cavity is not completely understood.[8] Risk factors for HPV-positive HNSCCs include sexual behaviours, such as an increased number of sexual partners and practising oral sex.[6,9,10] HPV-positive HNSCCs are most common in the oropharynx, and in particular the tonsils.[6,11] HPV-positive HNSCCs appear to show some similarities with cervical cancer, including a pre-requisite for a persistent infection with a high-risk HPV type for a varied period of time before the development of cancer.[1] As is the case with cervical HPV infection, only a very limited number of primary HPV infections persist and develop into malignancy.[5]

The prevalence of HPV in the mouths of healthy individuals varied from 0% to 81% in overseas-based studies,.[12,13] Both low- and high-risk HPV types were detected

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in the oral cavity.[14-16] High-risk HPV types, such as 16 and 18, have been shown to be associated with the development of oral cancer, with over 90% of HPV-positive HNSCC linked to infection with HPV 16.[3,8,11] Infection with low-risk HPV types, such as 6 and 11, is causally associated with respiratory papillomatosis, a condition characterised by the growth of multiple papillomas, usually in the larynx.[17]

There is currently no data on the prevalence on HPV in the oral cavity among New Zealanders. Thus, the aim of this study was to determine the prevalence of oral HPV infection in a local population of New Zealand women aged 18 to 25 years, including determination of HPV genotypes, and to assess potential risk factors for oral HPV infection using participant questionnaire responses.

Methods

Study design and recruitment

A cross-sectional design was used for this study. Eligible participants were women aged 18 to 25 years. Written consent was obtained from all participants prior to their participation. The study was approved by the Massey University Human Ethics Committee Southern A (approval 13/12). Recruitment took place between June and November, 2013, at sexual health and student health centres in the Manawatu region. All participants were asked to complete a questionnaire which encompassed demographic information and putative risk factors for oral HPV infection, including smoking, alcohol consumption, and the number of sexual partners. Information on whether the participants had been vaccinated with HPV vaccine Gardasil® was also collected. Alcohol consumption was measured in standard drinks per week, as defined by the New Zealand Health Protection Agency (330 mL can of beer, a 100 mL glass of wine, or a 30 mL glass of straight spirits). Being a current or previous smoker was defined as smoking more than 20 cigarettes in a lifetime. A sexual partner was defined as a partner of either sex, and the definition of sexual activity included vaginal, anal, and oral sex.

Sample collection and processing

After thorough explanation of the procedure by a registered nurse or the researcher, oral brushings were collected by self-sample. The left and right buccal surfaces and the base of the tongue were brushed using a cytobrush (Thermo-Fisher). Brushes were vigorously rinsed in ThinPrep® vials containing 20 mL Preservcyt® (CYTYC Corp). Samples were stored at room temperature until further processing. After mixing, 10 mL of each sample was centrifuged at 13,000xg for 10 minutes. The pellet was resuspended in 200 µL of phosphate buffered saline pH 7.0 (PBS), then transferred to a microtube for DNA extraction using High Pure™ PCR template preparation kit (Roche Diagnostics) as per the manufacturer's instructions. The quality and quantity of extracted DNA was assessed using a NanoDrop™ spectrophotometer (Thermo Scientific).

Screening PCR with PGMY primers

Initially, PCR with PGMY09 and PGMY11 primers[18] was used for detection of HPV DNA in test samples. Each 20 µL reaction contained 2 mM $MgCl_2$, 0.3 mM of each dNTP, 0.2 µM of each primer, one unit of Platinum® *Taq*, and 3 µL of template DNA in 1 x reaction buffer. The PCR was carried out using a SensoQuest® lab cycler (SensoQuest GmbH). An initial denaturation at 95 °C for 2 minutes was followed by 40 cycles of denaturation (95 °C for 5 seconds), annealing (55 °C for 5 seconds), and elongation (72 °C for 10 seconds for 30 cycles, increased to 30 seconds for the last 10 cycles), followed by a final elongation at 72 °C for 7 minutes. Positive (3 µL cobas® positive control, and 3 µL HPV 16 DNA that had been extracted from a cobas® HPV 16 positive cervical specimen) and negative (3 µL cobas® negative control, and 3 µL water) controls were included in each run. PCR products were visualised following electrophoresis through a 1.0% agarose gel containing 0.5 µg/mL ethidium bromide in 0.5% Tris-borate-EDTA (TBE) buffer at 100 volts for 45 minutes. Any sample that produced a 450 base pair band was considered a suspect positive.

Beta-globin PCR

The presence of amplifiable DNA in samples negative for HPV DNA by PGMY PCR was assessed by PCR targeting a human beta-globin gene using the PCO4 and GH20 primers.[19] Each 20 µL reaction contained

1.5 mM $MgCl_2$, 0.2 mM each dNTP, 0.5 µM of each primer, 0.4 units of Platinum® *Taq*, and 3 µL of template DNA in 1 x PCR buffer. PCR was performed as described for the PGMY PCR. Positive (3 µL cobas® positive control) and negative (3 µL water) controls were included in each run. A sample was considered positive if a product of the expected size (268 bp) was visible on a gel. Samples that did not produce the expected band were removed from the analysis.

Roche cobas® 4800 testing

Suspect positives based on PGMY PCR were sent for testing using the Roche cobas® 4800 system, which detects 14 high-risk HPV types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68).[20] The remaining ~10 mL of oral brushings from each suspect positive was centrifuged at 13,000xg for 10 minutes, the pellet was then resuspended in 1.5 mL Preservcyt®, and this preparation was sent for cobas® testing at a commercial laboratory (MedLab Central, Palmerton North) according to standard protocols. Types 16 and 18 were reported individually while the other detectable HPV types were reported as 'other high risk type/s detected'.

Confirmatory testing

All suspect positives that tested negative using cobas® system were re-tested using PGMY PCR. Samples that did not produce the expected band on re-testing were considered negative for HPV DNA. The identity of the 450 base pair bands produced by the remaining samples was confirmed by sequencing. DNA was purified from the gel using a freeze-squeeze procedure. Briefly, the excised band was placed in a 1.5 mL microtube on the top of a filtered pipette tip, which had been cut short to fit into the tube. The tube was snap-frozen in liquid nitrogen, then centrifuged at 13,000xg for 3 minutes. The filtrate containing DNA was used for sequencing, either directly or after cloning into a pCR4-TOPO vector using TOPO TA cloning kit for sequencing with Top10 competent cells (Life Technologies Inc.), according to the manufacturer's instructions.

The colonies were screened for the presence of the 450 bp insert using PCR with M13 primers. Up to 10 colonies were randomly picked into 10 µL Luria Bertani (LB) broth containing 50 µg/mL ampicillin, and incubated at 37 °C for approximately 3 hours. DNA from PCR reactions that produced product of the expected size (616 bp) was prepared using ExoProStar™ (GE Healthcare, Life Sciences) as per the manufacturer's instructions, and sent for sequencing using the Big Dye Terminator 3.1 chemistry at the Massey Genome Service (Massey University). Initially, two colonies were sequenced from each ligation reaction. If neither of these two colonies contained HPV sequences, an additional three colonies were sequenced, for a total of 5 colonies. The sequences were compared with other sequences available in GenBank using Basic Local Alignment Search Tool (BLAST).[21]

Data analysis

Initial exploration of data was by summary statistics, tables and plots. Descriptive analysis was performed for each variable using statistical software R 3.0.2 (R development core team 2011, R foundation for statistical computing, Vienna, Austria). Categories were collapsed for analysis to avoid issues associated with data scarcity for the variables ethnicity, alcohol consumption, and number of sexual partners. Associations between categorical variables and the detection of HPV were explored using univariable logistic regression. Variables were allowed to enter a multivariable model if the Likelihood Ratio Test (LRT) was statistically significant at a p-value ≤0.20.

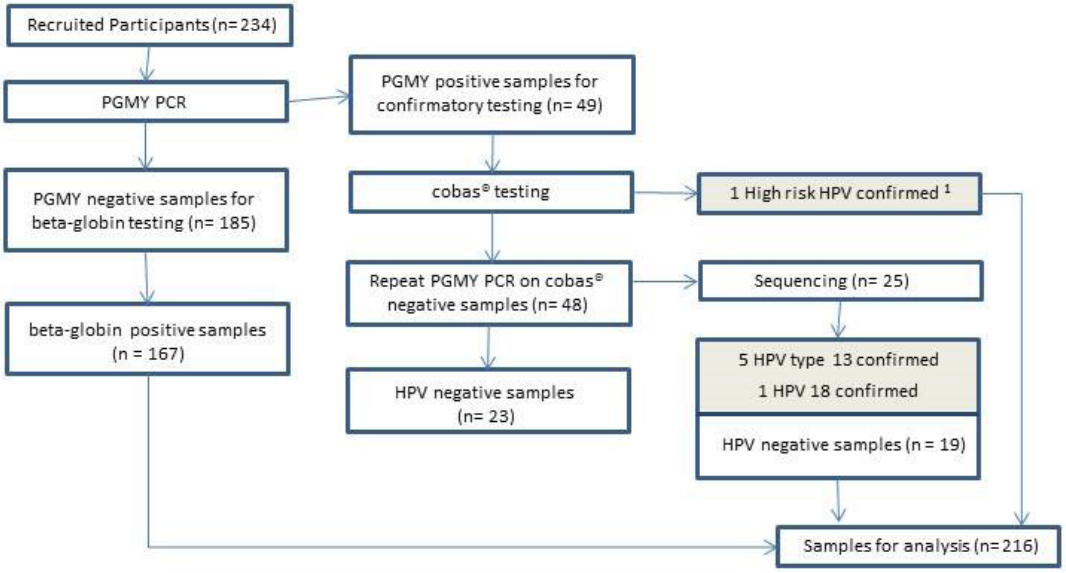
Results

A total of 234 participants were recruited as part of this study. Participants were from sexual health (n=55) and student health (n=179) centres. Each participant provided an oral brushing and at least partially filled in the questionnaire.

A summary of the results obtained at each stage of the testing process is shown in Figure 1. A total of 49 samples were considered suspect positives from the initial PGMY PCR. Of the 185 PGMY negative samples that were subjected to the beta-globing PCR, 18 samples were excluded due to a lack of amplifiable DNA. This resulted in 216 samples for inclusion in the analysis.

After all confirmatory testing was completed, seven of 216 samples were

Figure 1: Flow chart of testing methods and results obtained from each step.



[1] High risk type from cobas® testing was a non-16/18 and could be any of the following high risk types; 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, or 68.

considered positive for HPV. Thus, the prevalence of oral HPV infection among sampled women was 3.2% (95% CI: 1.6%–6.5%). An oncogenic HPV type was found in two samples (prevalence: 0.9%; 95% CI: 0.3%–3.3%), and HPV 13 was found in five samples (prevalence of 2.3%; 95% CI: 1.0%–5.3%).

A summary of questionnaire responses is shown in Table 1. Questions one to three were answered by all participants. Question four was only applicable to those answering yes for question three (*n*=134). This question had eight missing responses. Questions five to seven had 10, 9, and 11 missing values, respectively.

Of the 216 study participants, 196 (91%, 95% CI: 86%–94%) were eligible for the free Gardasil® immunisation based on their date of birth. Of those eligible, 68 (35%, 95% CI: 28%–42%) were not vaccinated. The majority (*n*=122) of the vaccinated women had completed the course of three injections, while 12 participants received one or two injections. Around two thirds (*n*=82) of the vaccinated women received the vaccine before becoming sexually active. The remaining third (*n*=44) were vaccinated after they had commenced sexual activity. No significant associations (at p<0.2) were found between putative risk factors (smoking, alcohol consumption, and the number of sexual partners) and the presence of oral HPV infection (including all types, an oncogenic type, or type 13) were found.

Discussion

The oral HPV prevalence obtained in this study, although comparable to results of other published studies,[14,16] showed an unusual distribution of HPV types—namely the comparatively high prevalence of HPV 13 and the lack of any HPV 16 among HPV-positive samples. The latter has been reported as the most prevalent HPV type in a number of other oral HPV prevalence studies of healthy individuals.[14,15] Our study found no associations between previously reported risk factors for oral HPV—namely smoking, alcohol consumption, and the number of sexual partners—and the detection of oral HPV. The lack of association may be due to the low oral HPV prevalence seen in this study.

HPV 13 is typically transmitted through oral contact, and the shared use of objects contaminated with saliva.[8,22] HPV 13 is considered an non-oncogenic HPV type, as infection with this type is not associated with malignancy.[23] However, detection of HPV types 13 and 32 in the oral cavity have been linked to development of focal

Table 1: Questionnaire responses from study participants.

Question number	Variable	Level	Overall number (%)	HPV-positive number (%)	HPV negative number (%)
1	Age	Born before 1990	20 (9)	0	20 (100)
		Born during / after 1990	196 (91)	7 (4)	189 (96)
		Missing	0	0	0
2	Ethnicity	NZ European	153 (71)	4 (3)	149 (97)
		Māori	38 (18)	1 (3)	37 (97)
		Other	25 (11)	2 (8)	23 (92)
		Missing	0	0	0
3	Vaccination status	Not vaccinated	82 (38)	3 (4)	79 (96)
		Fully vaccinated	122 (56)	4 (3)	118 (97)
		Partially vaccinated	12 (6)	0	12 (100)
		Missing	0	0	0
4	Vaccine and sexual debut[1]	Before sexually active	82 (61)	1 (1)	81 (99)
		After sexually active	44 (33)	3 (7)	41 (93)
		Missing	8 (6)	0	8 (100)
5	Smoking	Never smoked	157 (73)	6 (4)	151 (96)
		Current smoker	28 (13)	1 (4)	27 (96)
		Previous smoker	21 (10)	0	21 (100)
		Missing	10 (4)	0	10 (100)
6	Alcohol consumption	Doesn't drink	47 (22)	2 (4)	45 (96)
		1-5 drinks per week	128 (59)	5 (4)	123 (96)
		>6 drinks per week	32 (15)	0	32 (100)
		Missing	9 (4)	0	9 (100)
7	Sexual partners	0-5 partners	135 (63)	3 (2)	132 (98)
		6-10 partners	34 (16)	2 (6)	32 (94)
		>11 partners	36 (17)	2 (6)	34 (94)
		Missing	11 (4)	0	11 (100)

[1] Question only applicable to those answering yes for initial vaccination question (*n*=134)

epithelial hyperplasia (FEH) or Heck's disease.[24] Although FEH lesions are benign, they can persist for many years leading to cosmetic problems, which to date have limited treatment options.[24] FEH is characterised by numerous painless papules on the lips, tongue, and buccal mucosa.[24] Heck's disease has been described predominantly in people of the Eskimo, American Indian, and Latin American ethnicity, and is strongly associated with poverty and poor living conditions.[25] FEH and Heck's disease are usually seen in children or adolescents.[25] It is unclear what proportion of oral HPV 13 infections progress to FEH or how prevalent HPV 13 is in healthy adults. We have identified five cases of HPV 13 infection among 216 healthy females. By comparison, the prevalence of oral HPV 13 infection among 689 Dutch men with high-risk sexual behaviours was 0.1 % (95% CI: 0%–0.8%).[26] Thus, it seems that the prevalence of HPV 13 infection in our study (2.3%) was comparatively high. There are several possible reasons for the differences between our data and the Dutch data, based largely on differences in the study populations. The majority of our study subjects were university students, who were therefore likely to be living in student flats or hostels, both of which could be considered inferior living situations. Although the living conditions for the Dutch participants were not specified, the comparatively high prevalence of HPV 13 among subjects of the current study may fit with the reported association between FEH and poor living conditions.[25] Secondly, our sampling population consisted exclusively of females, in contrast to the Dutch study, where the population consisted exclusively of males. Although there are no data available for HPV 13 prevalence among woman from other countries, FEH was reported to be more common in females than in males.[24] Lastly, our study population may have included females genetically predisposed to HPV 13 infection. A genetic predisposition among selected non-European populations for FEH has been described by others.[24,25] Published data on FEH in Māori and Pacific people are limited to a case report of Heck's disease in a Polynesian child in 1966.[27] In our study, three of the five HPV 13 cases were of non-European decent (one Guatemalan, one Pacific Islander and one New Zealand Māori), even though non-Europeans constituted only 29% of the study participants. The two remaining HPV subtypes detected in the study were from New Zealand Europeans. However, given the low numbers of HPV 13 positive samples, the apparent predisposition to HPV 13 infection among non-Europeans should be interpreted with caution. In addition, as we did not perform an oral examination, it is unclear if any participant had visible oral lesions suggestive of FEH.

The detection of oral HPV in this study employed a multi test approach. Of the 49 suspect positives in the initial PGMY PCR, only five turned out to contain HPV sequences. This is likely a reflection of the degeneracy of HPV primers and low annealing temperature employed in the assay. While both are necessary for detection of multiple HPV types, they also facilitate non-specific amplification in samples negative for target HPV sequences. Our results underscore the importance of confirmatory testing when assessing PCR data, particularly if they have been generated using degenerate primers.

The prevalence of oral HPV is also of interest with regard to the prevention of HPV-positive HNSCC through vaccination.[28,29] The quadrivalent cervical cancer vaccine (Gardasil®) was introduced in New Zealand in 2008 and was initially offered free to any female born after 1990. The vaccine protects against HPV types 16, 18, 6 and 11[28] and is now routinely offered to 12 year old girls as part of the national immunisation schedule. However, coverage rates are variable and the vaccine's introduction was controversial, largely due to the sexually transmitted nature of HPV.[30] Results of a Costa Rica-based case control study showed that vaccination with Gardasil® resulted in a 93% (95% CI: 63%–100%) reduction in oral HPV infection between the test and control groups.[29] We were not able to assess the effect of the Gardasil® vaccine on oral HPV infection among New Zealand woman due to the low prevalence of oral HPV infection with only one sample positive for HPV vaccine type (HPV 18) detected in the current study. The HPV 18 positive sample was obtained from a

woman who had received all three doses of the Gardasil® vaccine, but after the onset of sexual activity. Although this is only a single case, the finding supports the importance of vaccination prior to exposure to HPV.

The vaccine coverage in the current study population was 56% (95% CI: 50%–63%) which is consistent with between 48% and 52% coverage for the same age group reported nationally.[31] Thus, one of the challenges for successful immunisation with Gardasil® may be achieving the desired coverage of at least 70% as used in models of vaccine effectiveness and cost efficiency.[32]

The results from this study have provided initial data on oral HPV infection among young women in New Zealand. The comparatively high detection rate of HPV 13 suggests that further work to determine the clinical implications of this infection is warranted, especially in the Māori and Pacific Islander groups who tend to be over represented with respect to poverty-associated diseases.

Competing interests: Nil

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