

Original Article

Oral and Cervical Human Papillomavirus Infection among Female Sex Workers in Japan

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SUMMARY: It has been reported recently that oral human papillomavirus (HPV) infection is associated with oropharyngeal squamous cell carcinomas. The aim of this study was to determine the prevalence of HPV infection and HPV types in the oral cavity and cervix of female sex workers in Japan. Oral and cervical swabs were taken from 196 female sex workers who visited a clinic for regular medical checkups in 2007, and genomic DNA was extracted from those specimens. The HPV L1 gene was amplified by polymerase chain reaction (PCR) using original and modified GP5⁺/6⁺ primers, and genotyping was performed using the Kurabo GeneSquare Microarray or by sequencing cloned PCR products. HPV DNA was detected in the oral cavity of 12 (6.1%) women, with HPV-56 being the most common type (7/12). Likewise, HPV DNA was detected in the cervix of 103 (52.6%) women, with HPV-52 (30/103, 29.1%), followed by HPV-16 (24.3%) and HPV-56 (18.4%), being the most common. Of the 12 women with oral HPV infection, only two were infected with the concordant HPV genotype in the cervix. These findings suggest that oral HPV infection occurs independently of cervical HPV infection in this population, and that oral HPV infection may play a role in HPV transmission in Japan.

INTRODUCTION

Human papillomavirus (HPV) is recognized as a causal and necessary factor for cervical cancer (1–3). More than 40 HPV types have been identified in the mucosal epithelia of the human genital tract. Cervical cancer is caused by HPV types that belong to a few phylogenetically related “high-risk” species (alpha-5, 6, 7, 9, and 11) of the mucosotropic alpha genus (4–6). Eight HPV types (HPV-16, 18, 31, 33, 35, 45, 52, and 58) are observed most frequently, and four (HPV-39, 51, 56, and 59) are observed less frequently, in cervical cancer. HPV-68 is classified as “probably carcinogenic to humans,” with limited clinical evidence in humans but strong mechanistic evidence. The remaining high-risk alpha HPV types are also considered “possibly carcinogenic.” HPV-6 and HPV-11 (alpha-10 species) are “not classifiable” as regards human carcinogenicity.

It has been reported recently that oral HPV infection is associated with squamous cell carcinomas of the head and neck, particularly oropharyngeal squamous cell carcinomas (OSCCs) (7–10). HPV-positive cases are associated with sex-related risk factors that have also been

linked to cervical cancer and an increased likelihood of orogenital activity, whereas tobacco and alcohol consumption are the key risk factors for HPV-negative cases (11,12). Interestingly, HPV-positive OSCC was reported to have a better prognosis than HPV-negative OSCC (13).

The incidence of OSCC in Japan has gradually been increasing over the last three decades (14), although the rates of tobacco smoking and alcohol drinking have been decreasing (15). This increase in OSCC incidence may be due to an increase in HPV-positive cases. However, despite the fact that the prevalence of oral HPV infection was reportedly 0.8% among healthy individuals on the southern island of Japan in 2003 (16), there is little information about the epidemiological status of oral HPV infection in Japan as whole.

Herein we examine the prevalence of HPV infection and HPV types in the oral cavity and cervix of female sex workers in Japan, the concordance of infected HPV types between these two sites, and the association of HPV type with abnormal cervical cytology. The prevalence of *Chlamydia trachomatis* and *Neisseria gonorrhoeae* infections in the oral cavity and cervix was also investigated.

MATERIALS AND METHODS

Subjects and sample collection: The study subjects were 196 female sex workers (mean age \pm SD, 28 \pm 5.5 years; range, 18–45 years) who engaged in oral- and/or genital sex and visited a sexually-transmitted disease

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clinic in Kyoto, Japan, for a regular checkup in December 2007. All women gave written informed consent for participation in this study. The study protocol was approved by the ethics committee of Kanazawa University (Kanazawa, Japan) in 2006.

One oral specimen and two cervical specimens were collected from each participant. The oral sample and one cervical sample were each suspended in 1 mL of cell lysis buffer (TE buffer, 10 mM Tris-Cl [pH 6.5], 1 mM EDTA, 2% SDS) and stored at -80°C until use. The other cervical sample was immediately placed into a preservative vial containing Liqui-PREP™ (LGM International Inc., Veritas Corporation, Tokyo, Japan) along with preservative and fixation fluid, and stored at 4°C .

Cervical cytology: Cervical scrape smears were applied using Liqui-PREP™ and stained with Papanicolaou stain. Cervical cytology was diagnosed according to the Bethesda system (17) and classified as normal (negative for intraepithelial lesion or malignancy), atypical squamous cells of undetermined significance, low-grade squamous intraepithelial lesion, high-grade squamous intraepithelial lesion, squamous cell carcinoma, or adenocarcinoma in situ.

Preparation of genomic DNA: Genomic DNA was extracted from cervical and oral cells using a DNA extraction kit (SMI test; Genome Science Laboratories, Fukushima, Japan) according to the manufacturer's instructions. The quality of the extracted DNA was evaluated by amplifying the glyceraldehyde-3-phosphate dehydrogenase gene (primers: 5'-ACCACAGTCCATGCCATCAC-3' and 5'-TCCACCACCTGTGCTGTA-3') (18). All extracted DNA samples were confirmed as suitable for HPV, *C. trachomatis*, and *N. gonorrhoeae* testing.

Detection and typing of HPV DNA: Initially, HPV DNA was detected by polymerase chain reaction (PCR) using the original and three pairs of modified GP5⁺/6⁺ primers (19–21), namely GP5 + M1-2 (5'-TTTRTTACTGTTGTWGATACTAC-3'), GP5 + M2-2 (5'-TGTWACTGTTGTWGATACCAC-3'), GP5 + M3-2 (5'-GTWACTGTTGTRGACACCAC-3'), GP6 + M1-2 (5'-AATTGAAAWATAAACTGTAAWTCATATTC-3'), GP6 + M2-2 (5'-GAAACATAAAATGTAAATCAWATTC-3'), and GP6 + M3 (5'-GAAAATYTGCAAATCAWACTC-3'). These modified GP5⁺/6⁺ primers were designed to minimize the mismatches between primer sequences and complement target HPV L1 genes and to amplify a 140-bp fragment of the HPV L1 gene. Amplification was performed as follows: one cycle at 95°C for 10 min, followed by 45 cycles at 95°C for 30 s, 45°C for 30 s, and 74°C for 30 s, with a final extension at 74°C for 10 min. The presence of HPV DNA was confirmed by ethidium bromide staining of the PCR products following agarose gel electrophoresis.

HPV genotyping was performed using a Kurabo GeneSquare Microarray system (Kurabo, Okayama, Japan), which utilizes multiplex PCR targeting different genes from type to type (22). The sensitivity and specificity of the system are reportedly equal to those of the Roche Linear Array HPV Genotyping Assay. The GeneSquare Microarray contains 23 type-specific probes, 13 high-risk HPV types (HPV-16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68), and 10 low- or

unknown-risk HPV types (HPV-6, 11, 30, 34, 40, 42, 53, 54, 61, and 66) (5).

The original and/or modified GP5⁺/6⁺ PCR products of the samples found to be HPV-negative by the GeneSquare were cloned using the TOPO TA Cloning Kit (Invitrogen, Carlsbad, Calif., USA) and sequenced as described previously (23). The similarity between the L1 sequences obtained by PCR and those of various HPV genotypes in the GenBank database was determined by BLAST analysis (<http://www.ncbi.nlm.nih.gov/BLAST/>).

Accession numbers: The sequence described in this article have been deposited in GenBank/EMBL/DDBJ under accession nos. AB601041–AB601063.

Detection of *C. trachomatis* and *N. gonorrhoeae*: *C. trachomatis* and *N. gonorrhoeae* DNA was detected using the loop-mediated isothermal amplification (LAMP) method, which amplifies DNA with high specificity, efficiency, and rapidity under isothermal conditions (24,25).

Statistical analysis: Statistical analysis was performed using SPSS version 15.0 J for Windows. The odds ratio (OR) and 95% confidence interval (CI) were calculated as approximations of relative risks. Univariate analyses were performed to assess the association between abnormal cervical cytology and HPV types. Any variables shown to be significant in univariate analysis were analyzed using a multivariate model. The comparisons of HPV infection rates in the oral cavity with those in the cervix and HPV infection rate with *C. trachomatis* or *N. gonorrhoeae* infection rate were analyzed using Student's *t* test. The level of statistical significance was set at $P < 0.05$.

RESULTS

Prevalence of HPV, *C. trachomatis*, and *N. gonorrhoeae* infection: The prevalence of HPV, *C. trachomatis*, and *N. gonorrhoeae* infection was evaluated in the oral cavity and cervix of 196 Japanese female sex workers in 2007. HPV DNA was detected in the oral cavity of 11 of the 196 women by PCR using the original primers and in one additional woman when the modified GP5⁺/6⁺ primers were used. Finally, 12 (6.1%) women were positive for HPV DNA. *C. trachomatis* and *N. gonorrhoeae* infection was found in 12 (6.1%) and eight (4.1%) women, respectively, by LAMP assays (Fig. 1). There was no significant difference in the prevalence of these three sexually transmitted infections in the oral cavity.

HPV DNA was detected in the cervix of 80 and 95 of the 196 women by PCR using the original and modified GP5⁺/6⁺ primers, respectively. Finally, 103 (52.6%) women were positive for HPV DNA (Fig. 1). *C. trachomatis* and *N. gonorrhoeae* infections were found in 32 (16.3%) and 11 (5.6%) women, respectively (Fig. 1). The prevalence of cervical HPV infection was significantly higher than those of cervical *C. trachomatis* or *N. gonorrhoeae* infection ($P < 0.01$) and the prevalence of HPV infection was significantly higher in the cervix than in the oral cavity ($P < 0.05$).

Profiles of HPV types in the oral cavity and cervix: HPV typing was successfully performed in four of the 12 oral HPV-positive women using a Kurabo

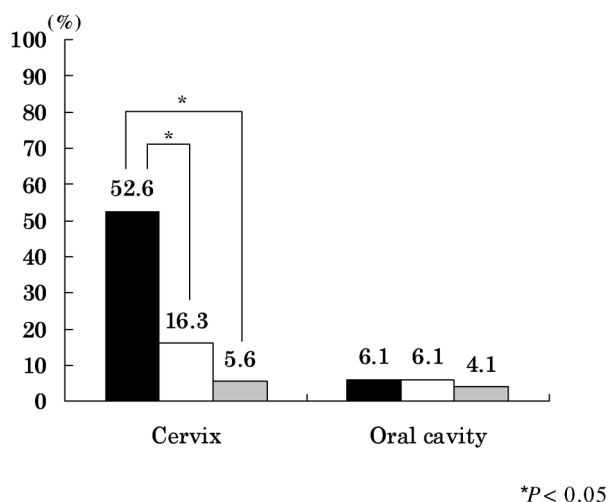


Fig. 1. Prevalence of HPV, *C. trachomatis*, and *N. gonorrhoeae* in oral cavity and cervix among female sex workers in Japan ($n = 196$). ■: HPV; □: *C. trachomatis*; ■: *N. gonorrhoeae*.

GeneSquare Microarray system. For the remaining eight samples, the PCR products were further cloned, sequenced, and genotyped. A total of 10 (83.3%) women were infected with high-risk HPV types in the oral cavity, with HPV-56 (7/12, 58.3%) being the most common (Table 1).

HPV typing was successfully performed in 96 of the 103 cervical HPV-positive women using the GeneSquare system. As above, the PCR products were further cloned, sequenced, and genotyped for the remaining seven samples. A total of 35 (34.0%) women had single-type HPV infections in the cervix, with remaining 68 (66.0%) having multiple-type HPV infections. High-risk HPV types were detected in 84 (81.6%) women, with HPV-52 (29.1%) being the most prevalent, followed by HPV-16 (24.3%), -56 (18.4%), -68 (17.5%), and -58 (15.5%) (Table 1). Seven women whose HPV strains could not be successfully genotyped using the GeneSquare system were found to be infected with HPV-62, -70, -83, and/or -90 by sequencing the cloned PCR products. The detection probes for these HPV types are not included in the GeneSquare system (Table 1).

Six women were infected with HPV in both their oral cavity and cervix (Table 2), although only two women showed concordance of HPV genotypes (HPV-56) between these sites.

Cervical HPV infection and abnormal cervical cytology: Of the 196 women tested, 136 (69.4%) had normal cervical cytology, 35 (17.9%) had atypical squamous cells of undetermined significance, and the remaining 25 (12.8%) had abnormal cytology (low-grade squamous intraepithelial lesion, $n = 22$; high-grade squamous intraepithelial lesion, $n = 2$; adenocarcinoma in situ, $n = 1$). HPV DNA was detected in 57 (41.9%) of the 136 women with normal cytology, 22 (62.9%) of the 35 women with atypical squamous cells of undetermined significance, and 24 (96%) of the 25 women with abnormal cytology. One woman with low-grade squamous intraepithelial lesion tested negative for HPV DNA.

A total of 14 different HPV types were detected in the women with abnormal cervical cytology, with HPV-16

Table 1. HPV types detected in the oral cavity and in the cervix among female sex workers in Japan

HPV type	Cervix ($n = 103$)	Oral cavity ($n = 12$)
	no. (%)	no. (%)
High-risk HPV	84 (81.6)	10 (83.3)
16	25 (24.3)	0
18	15 (14.6)	2 (16.7)
31	15 (14.6)	1 ²⁾ (8.3)
33	7 (6.8)	0
35	4 (3.9)	0
39	11 (10.7)	0
45	6 (5.8)	0
51	15 (14.6)	0
52	30 (29.1)	0
56	19 (18.4)	7 ³⁾ (58.3)
58	16 (15.5)	0
59	7 (6.8)	0
68	18 (17.5)	0
Low-risk HPV	10 (9.7)	1 (8.3)
6	8 (7.8)	1 (8.3)
11	3 (2.9)	0
Other	55 (53.4)	1 (8.3)
30	10 (9.7)	0
34	9 (8.7)	0
40	7 (6.8)	1 ²⁾ (8.3)
42	11 (10.7)	0
53	15 (14.6)	0
54	10 (9.7)	0
61	5 (4.9)	0
66	10 (9.7)	0
62 ¹⁾	2 (1.9)	0
70 ¹⁾	1 (1.0)	0
83 ¹⁾	1 (1.0)	0
90 ¹⁾	4 (3.9)	0

¹⁾: GeneSquare is unable to detect these HPV types, which were detected by cloning and sequencing.

²⁾: Detected by cloning and sequencing.

³⁾: Six samples were examined by cloning and sequencing.

Table 2. HPV types detected in the oral cavity among female sex workers in Japan

Sample ID	HPV type	
	Oral cavity	Cervix
KU 12	6	negative
KU 45	18	negative
KU 61	56	negative
KU 77	18	90*
KU 78	56*	negative
KU 80	56*	33, 52, 58
KU 81	56*	56
KU 82	56*	56
KU 84	40*	negative
KU 90	56*	66
KU 93	56*	negative
KU159	31*	16

*Detected by cloning and sequencing.

and -51 being the most prevalent (28.0%), followed by HPV-68 (24.0%), -18 (20.0%), -52 (20.0%), and -56 (20.0%). Multivariate analysis using a logistic regres-

sion model revealed that HPV-51 was significantly associated with abnormal cytology (OR, 9.7; 95% CI, 2.6–37).

One of the two women with high-grade squamous intraepithelial lesion was infected with HPV-16, -39, -56, and -58, whereas the other was infected with HPV-16 and -18. The woman with adenocarcinoma in situ was infected with HPV-42 and -52.

DISCUSSION

The prevalence of HPV infection among Japanese female sex workers in this study was 6.1 and 52.6% in the oral and genital mucosal areas, respectively. This is one of the first studies to compare oral and cervical HPV infection among such subjects.

Kurose et al. (16) reported that the prevalence of HPV infection in the oral cavity of a population of women who visited a dental clinic in the southern part of Japan was 0.8%. The prevalence in our subjects (6.1%) was about eight times higher, which is probably mainly due to the difference in the study population. Our study subjects were female sex workers who provided oral-and/or genital sex, a behavior which has been reported to be associated with oral HPV infection (26–28). The difference between our and Kurose’s results may also be partly due to the difference in the primer set for HPV PCR. Thus, Kurose et al. conducted HPV PCR using the MY09/11 primer set (16), whereas we used the modified GP5⁺/6⁺ primer set as well as the original GP5⁺/6⁺ primer set (21) in this study. MY09/11 and the original GP5⁺/6⁺ primer sets have been used in many epidemiological studies. However, some HPV types, such as HPV-52, may not be amplified by these primer sets as effectively as HPV-16 and -18 because of sequence mismatches between the target gene and the primers (29). We therefore used the modified GP5⁺/6⁺ primer set together with the original set to broaden the spectrum of detectable HPV types in this study (20,21).

The reported prevalence of concomitant HPV infection in oral and cervical mucosal areas among women in

various countries is summarized in Table 3 (30–33). The prevalence of oral HPV infection was much higher among outpatients (23.6%) than among the general population of women (1.4, 2.4, and 9.0%). These differences in prevalence are mainly due to the difference in study populations, as discussed above. However, considering the differences in published oral HPV prevalence among women who are not sex workers, the sampling procedures may also have affected the outcomes of these studies. It has been reported that oral rinse sample collection and storage methods improve DNA yield and quality (34–36). In this study, however, we collected oral and cervical samples by scraping, since superficial cervical scraping is the standard technique for HPV DNA detection in the cervix (37). The prevalence of oral HPV infection in our study group may therefore have been underestimated.

The majority of women in this study had discordant oral and cervical HPV type infections. This is consistent with the findings of previous studies (30–33), in which no women had the same HPV genotype at the two sites (Table 3). In addition, HPV-56 was the most prevalent genotype in the oral cavity in this study, although it was only the third most prevalent type in the cervix. These findings suggest that oral HPV infection can occur independently of cervical HPV infection. Therefore, although the prevalence of oral HPV infection (6.1%) was not as high as that of cervical HPV infection (52.6%), oral-genital contact may play some role in HPV transmission in Japan.

Despite the fact that the GeneSquare system is supposed to be able to detect HPV-31, -40, and -56, eight out of 12 oral samples (six HPV-56 samples, one HPV-31, and one HPV-40) had to be genotyped by sequencing the cloned PCR products (Table 2). This could be due to differences in sensitivity between genotyping methods and due to the low amount of oral sample DNA used for the assay (about 150 ng DNA/assay for cervical samples versus 30–35 ng DNA/assay for oral samples). Type-specific primers and probes designed on the basis of the L2 gene for HPV-31/-40 de-

Table 3. Summary of previous studies: prevalence of oral and cervical HPV infection and concordance of HPV type in both sites

Authors	Subject	Cervical HPV		Oral HPV			Type concordance	
		Positive (%) (no./overall)	Sampling	Detection (Primer)	Positive (%) (no./overall)	Positive (%) (cervical HPV -positive/ no. tested)		Positive (%) (cervical HPV -negative/ no. tested)
Smith et al. [2004] (30)	Pregnant women	28.9 (167/577)	Oral rinse	PCR (MY09/11 or GP5 ⁺)	2.4 (14/577)	42.9 (6/14)	57.1 (8/14)	0/6
Fakhry et al. ¹⁾ [2006] (31)	Women	44.9 (35/78)	Oral rinse	PCR (PGMY09/11) ⁴⁾	9.0 (7/78)	71.4 (5/7)	28.6 (2/7)	0/5
Marais et al. ¹⁾ [2008] (32)	Women ²⁾	86.5 (64/74)	Brushing	PCR (PGMY09/11) ⁴⁾	23.6 (17/72)	n.r	n.r	0/17
Termine et al. [2009] (33)	Women	54.3 (76/140)	Brushing	nested PCR (MY09/11 and GP5 ⁺ /6 ⁺)	1.4 (2/140)	100 (2/2)	0 (0/2)	0/2
Present study	CSWs ³⁾	52.6 (103/196)	Scraping	PCR (original and modified GP5 ⁺ /6 ⁺)	6.1 (12/196)	50.0 (6/12)	50.0 (6/12)	2/6

¹⁾: Control group.

²⁾: The outpatient clinic of the Department of Gynecology.

³⁾: Commercial sex workers.

⁴⁾: Linear array HPV genotyping assay.

n.r., data not reported.

tection, and on the E1 gene for HPV-56 detection, were used in the GeneSquare system, whereas the original and modified GP5⁺/6⁺ primers, followed by cloning and sequencing were used for HPV L1 PCR. In addition, HPV genotyping was attempted only once using GeneSquare, whereas it was attempted several times by sequencing the cloned PCR products with increasing numbers of analyzed clones.

High-risk HPV types were detected in 82.5% of female commercial sex workers with cervical HPV infection in the current study, with HPV-52 being the most prevalent HPV type. This is consistent with previous studies in Japan (38,39). Although HPV-16 is known to be the most prevalent type worldwide, it has been reported that HPV-52 and -58 are also highly prevalent in South Taiwan (40), the Philippines (21), and Vietnam (unpublished data), as well as in Japan. These results suggest that HPV-52 and -58 are common in Asian countries in general, which should be taken into consideration when applying the current HPV vaccines targeting HPV-16 and -18 in this area.

The prevalence of cervical HPV infection (52.6%) was significantly higher in the current study than those of *C. trachomatis* and *N. gonorrhoeae* infection (16.3 and 5.6%, respectively; $P < 0.01$). This finding is consistent with a previous study (41), which reported that the cervical prevalence of HPV, *C. trachomatis*, and *N. gonorrhoeae* among Japanese female sex workers was 55, 13, and 4.1%, respectively. However, there was little difference in prevalence between HPV, *C. trachomatis*, and *N. gonorrhoeae* in the oral cavity (6.1, 6.1, and 4.1%, respectively). Aside from physical elimination by washing out, other factors, such as lysozymes, lactoferrin, IgA, and cytokines, may affect the clearance of HPV in the oral cavity, (42–44).

In conclusion, we found that the prevalence of HPV infection among Japanese female sex workers was 6.1% in the oral mucosa and 52.6% in the genital mucosa. However, the majority of women had discordant HPV infection types in the oral cavity and in the cervix. These findings suggest that oral HPV infection occurs independently of cervical HPV infection in this study population, and that oral HPV infection may play some role in HPV transmission in Japan.

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Conflict of interest None to declare.

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