Human Papilloma Virus, Herpes Simplex Virus and Epstein Barr Virus in Oral Squamous Cell Carcinoma from Eight Different Countries

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Abstract. *Oral squamous cell carcinoma (OSCC) is a major health problem in many parts of the world, and the major causative agents are thought to be the use of alcohol and tobacco. Oncogenic viruses have also been suggested to be involved in OSCC development. This study investigated the prevalence of human papillomaviruses (HPV), herpes simplex virus (HSV) and Epstein-Barr virus (EBV) in 155 OSCC from eight different countries from different ethnic groups, continents and with different socioeconomic backgrounds. 41 A total of OSCCs were diagnosed in the tongue (26%) and 23 in the floor of the mouth (15%); the other 91 OSCCs were diagnosed in other locations (59%). The patients were also investigated regarding the use of alcohol and smoking and smokeless tobacco habits. Tissue samples were obtained from formalin-fixed, paraffin-embedded samples of the OSCC. DNA was extracted and the viral genome was examined by single, nested and semi-nested PCR assays. Sequencing of double-stranded DNA from the PCR product was carried out. Following sequencing of the HPV-, HSV- and EBV-positive PCR products, 100% homology between the sampels was found. Of all the 155 OSCCs examined, 85 (55%) were positive for EBV, 54 (35%) for HPV and 24 (15%) for HSV. The highest prevalence of HPV was seen in Sudan (65%), while HSV (55%) and EBV (80%) were most prevalent in the UK. In 34% (52/155) of all the samples examined, co-infection by two (46/155=30%) or three (6/155=4%) virus specimens was detected. The most frequent double infection was HPV with EBV in 21% (32/155) of all OSCCs. There was a statistically significant higher proportion of samples with HSV (p=0.026) and EBV (p=0.015) in industrialized countries (Sweden, Norway, UK and USA) as compared to developing countries (Sudan, India, Sri Lanka and Yemen). Furthermore, there was a statistically significant higher co-infection of HSV and EBV in samples from industrialized countries (p=0.00031). No firm conclusions could be drawn regarding the relationship between alcohol, tobacco and virus infections. The significance of our findings must be put in relation to other risk factors and these observations warrant further studies to determine the possible role of viral infections and co-infections with HPV, EBV and HSV as risk markers for the development of OSCC.*

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Oral squamous cell carcinoma (OSCC) is a major health problem in many parts of the world, with an incidence rate higher in developing countries, and with males being more affected than females (1). However, the incidence of OSCC is also increasing in many developed countries. An increase can be seen in Southern and Eastern Europe (2), Scandinavia (3), and also in Afro-Americans (1, 4). The primary risk factors for development of OSCC have not been fully elucidated, but tobacco use and alcohol consumption are considered to be the main etiological factors (5, 6). All over the world, a variety of smokeless tobacco products are used, for example *toombak* in Sudan (7, 8), betel in India (9), snuff in Sweden (10) and chewing tobacco in the United States (11). Smokeless tobacco products include different components, but all of them have in common the carcinogenic tobacco-specific nitrosamines (TSNA) (12). Both epidemiological and molecular data suggest that certain types of human papillomaviruses (HPV), as well as some members of the Herpesviridae family, such as Epstein-Barr virus (EBV) and human herpes simplex virus (HSV), have an oncogenic capability (13-18). HSV-2 has been demonstrated

Table I. *Data on the anatomical location and the personal habits of the 155 patients with OSCC in the study.*

Country	Age, years	Anatomical location			Personal habits		
					Tobacco use		
	Mean	T	FM	OL	Smokeless[a]	Smoking	Alcohol
Industrialized countries							
Norway (n=20)	70.50	4	4	12	-	13	13
UK (n=20)	62.33	6	1	13	-	2	5
Sweden (n=17)	69.71	4	5	8	3	4	7
USA (n=20)	66.10	7	6	7	-	17	11
Developing countries							
Sri Lanka (n=20)	59.10	3	2	15	7	14	8
India (n=20)	60.0	6	0	14	13	11	6
Sudan (n=20)	57.15	7	0	13	6	15	-
Yemen (n=18)	61.56	4	5	9	-	16	-
Total		41	23	91	32	92	50

T, Tongue; FM, floor of mouth; OL, other locations: palate (5), buccal (17), lip (4), gingiva (28), mandible (1), alveolus (13), larynx (7), maxillarysinus (1), tonsil (1), labial sulcus (13), jaw (1). [a]Including use of *toombak*, snuff and betel quid.

to display an oncogenic potential *in vitro* and has been proposed as cofactor in the development of cervical cancer (15). HPV has been shown to cause nearly 100% of cervical cancer cases (19), while EBV is the aetiological factor of Burkitt lymphoma (20) and nasopharyngeal carcinoma (21). The role of HPV, EBV and HSV in the development of OSCC has been investigated in earlier studies. The presence of HPV genome in OSCC has been reported with conflicting results (22, 23). However, two recently published review articles on this subject concluded that HPV is very likely to be involved in the development of OSCC (24, 25). EBV has been associated with OSCC in some studies (16, 26, 27), while in others no clear association between EBV and OSCC was found (28). The role of HSV in the development of OSCC has been less investigated. However, animal studies (29-31), as well as clinical studies (32), have shown a possible interaction between the use of smokeless tobacco and HSV-1 in the development of OSCC. Co-infection by two or more virus species has also been suggested as a factor for increased risk of cancer development in general (33), but also of OSCC (26, 32, 34, 35).

The objective of the present study was to examine the prevalence of HPV, HSV and EBV in OSCC obtained from eight different countries from different ethnic groups, continents and with different socioeconomic backgrounds.

Materials and Methods

Patients and oral tissue specimens. The study population consisted of 155 patients diagnosed with OSCC from eight different countries in Europe, Asia, Africa and North America, respectively (Table I): UK (n=20, age range 43-91, mean 62.33±SD 13.33 years), Sweden (n=17, age range 56-84, mean 69.71±9.14 years), Sudan (n=20, age range 18-94, mean 57.15±16.97 years), Norway (n=20, age range 45-86, mean 70.50±12.42 years), the USA (n=20, age range 44-89, mean 66.10±12.93 years), Yemen (n=18, age range 25-91, mean 61.56±15.00 years), India (n=20, age range 41-70, mean 60.0±7.97 years) and Sri Lanka (n=20, age range 40-70, mean 59.10±8.73 years). The anatomical location of the tumour was recorded and the alcohol habits, smoking and smokeless tobacco habits were requested or obtained from patient records (Table I). Of the 155 OSCCs, 41 were diagnosed in the tongue (26%) and 23 in the floor of the mouth (15%). The other 91 OSCCs were diagnosed in other locations (59%) (Table I). Due to differences in socioeconomy between the eight countries, we also divided the patients into two groups: industrialized countries, namely Sweden, Norway, UK and USA (n=77), and developing countries, namely Sudan, India, Sri Lanka and Yemen (n=78) (Table I).

DNA extraction. Tissue samples were obtained from formalin-fixed, paraffin-embedded samples of the oral carcinomas. From all the samples used in this study (n=155), DNA was extracted using a standard protocol as described in the DNeasy Tissue Kit Handbook (cat. no., 69506; Qiagen GmBH, Hilden Germany). Purified DNA was quantified by spectrophotometry (DU 530 Life Science UV/Visible Spectrophotometer; Beckman Coulter, Fullerton, CA, USA).

Nested and semi-nested polymerase chain reaction (PCR) assay for detection of EBV and HSV (types 1 and 2). DNA extracted from OSCC samples was used to amplify HSV (types 1 and 2) and EBV by two-step (semi-nested and nested) PCR amplifications. Before testing the samples, the specificity of the methods was examined using positive and negative control samples. As positive control, Raji cell DNA for EBV and green monkey kidney (GMK) cell DNA for HSV was used (Virology, Gothenburg university, Sweden). The primer sequences used in the PCR reactions are shown in Table I. EBV was amplified with EBV-1/EBV-2 in the first round and with

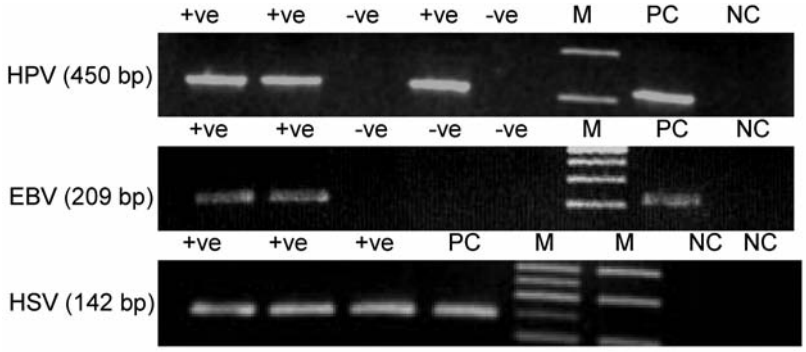

Figure 1. *An example of PCR amplifications for HPV, EBV and HSV viral genomes from (OSCC). +ve: Positive sample; –ve: negative sample; M: PCR marker; PC: positive control (HeLa cell lines for HPV; Raji cell line for EBV; GMK, infected cell with HSV); NC: negative control.*

Table II. Primer sequences used in the semi-nested and nested PCR techniques for detection of EBV-DNA and HSV-DNA and single PCR technique for detection of HPV-DNA.

Virus	Target gene	Product size (bp)	Primer sequence
EBV	*ebna 1*	209	EBV-1 5' ATC GTG GTC AAG GAG GTT CC EBV-2 5' ACT CAA TGG TGT AAG ACG AC EBV-3 5' AAG GAG GGT GGT TTC GAA AC EBV-4 5' AGA CAA TGC ACT CCC TTA GC
HSV	*D*	142	HSV-1 5' TGC TCC TAC AAC AAG TC 3' HSV-2 5' CGG TGC TCC AGG ATA AA 3' HSV-3 5' ATC CGA ACG CAG CCC CGC TG 3' HSV-4 5' TCT CCG TCC AGT CGT TTA TCT TC 3'
HPV	*L1*	450	MY 11: 5' GCM CAG GGW CAT AAY AAT GG MY 9: 5' CGT CCM ARR GGA WAC TGA T M=A+C, R=A+G, W=A+T, Y=C

EBV-3/EBV-4 primers in the second. HSV was amplified with the primers HSV-1/HSV-2 in the first round and with HSV-3/HSV-4 in the second round. The final products included a 209 bp product of the Epstein-Barr nuclear antigen (*ebna 1*) gene from EBV and a 142 bp fragment of the *D* gene of HSV. The PCR mixture contained 0.4 M of the appropriate primer (10 nmol), 1 x PCR buffer (Gene Amp 10× PCR buffer II), 200μM of each dNTP (Gene Amp, dNTP Mix with dTTP), 1.25 units of Taq DNA polymerase (Ampli Taq Gold, 5U/μl), nuclease-free water and 1.5 mM (HSV), 2.5 mM (EBV) of $MgCl_2$ (25 mM $MgCl_2$ solution), all from PE Applied Biosystems, Foster City, CA, USA, in a final volume of 25 μl. Viral DNA, human DNA and reaction controls were included in each run. DNA amplification was performed in an automated thermal cycler (Gene Amp PCR system 2700; PE Applied Biosystems). Reactions were incubated at 95˚C for 10 min, followed by 30 cycles consisting of a denaturing step for 30 s at 94˚C, an annealing step for 30 s at 60˚C (EBV), 50˚C (HSV first round) or 60˚C (HSV second round), and an extension step for 30 s at 72˚C. A final extension step at 72˚C was carried out for 5 min. A total of 2 μl of the first round product was used in the second round of amplifications. The amplified PCR products were found to be 209 bp long for positive EBV and 142 bp long for positive HSV samples (Figure 1).

Single PCR assay for HPV detection. For the detection of HPV, a single PCR assay was used. Before testing the samples, the specificity of the methods was examined using positive and negative control samples. As positive control, HeLa cell DNA was used (Virology, Gothenburg university, Sweden). The primer sequences used in the PCR reactions are shown in Table II. Each PCR mixture was diluted with 2.5 μl 10× PCR buffer, 0.6 μl 10× dNTP mix, 3.5 μl $MgCl_2$ (25 mM), 0.3 μl oligonucleotide primer 1 (100 mM), 0.3 μl oligonucleotide primer 2 (100 mM) and 14.2 μl water all from PE Applied Biosystems, in a final volume of 21.4 μl. Furthermore, 3.5 μl of the sample and 0.15 μl of the *Taq* polymerase (0.75 U) were added to the reaction mixture. Each cycle consisted of 1 min at 95˚C, 1 min at 55˚C and 2 min at 72˚C. A final extension step at 72˚C was carried out for 5 min. The amplified PCR products were 450 bp long for positive HPV samples (Figure 1).

Gel electrophoresis. Aliquots of 15 μl of the PCR product were analysed on 2% agarose gel (DNA Agar; Marine Bio Products Inc, Quincy, MA, USA) containing 0.5 g/mol of ethidium bromide (Merck KGaA, Darmstadt, Germany), and visualized under ultraviolet light. The size of the amplified product was determined by comparison with a bp ladder size marker for HPV, HSV and

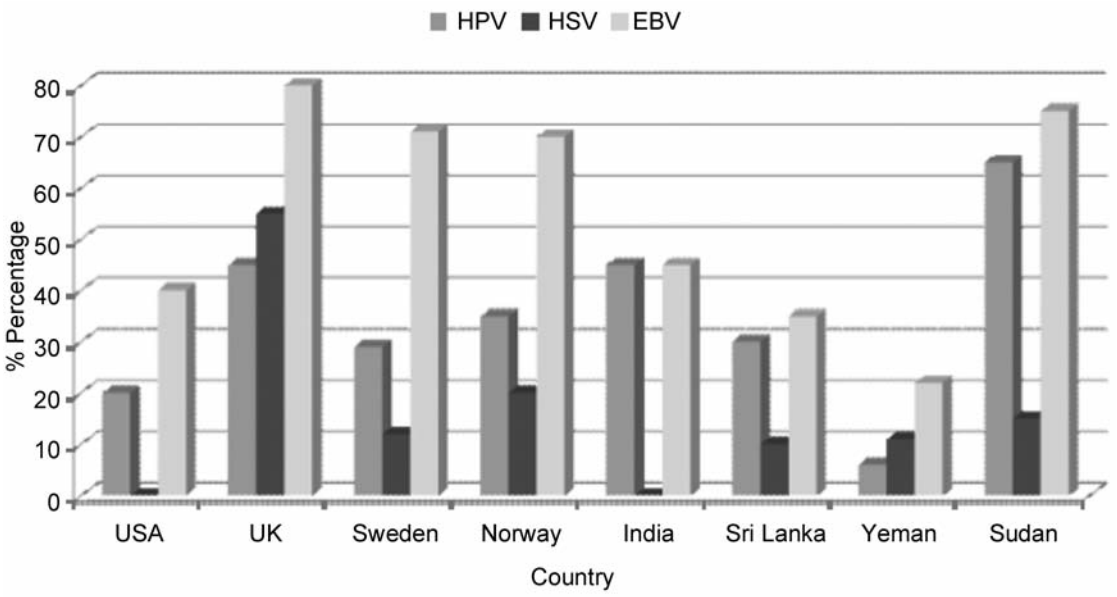

Figure 2. *Prevalence of viral infection in oral squamous cell carcinoma in each study country.*

EBV (Gene Ruler, 100 bp, 50 bp DNA Ladder Plus; Fermentas, St, Leon-Rot, Germany).

Sequencing of the Double stranded (ds) DNA PCR product. PCR products from the OSCC samples were sequenced for HPV, HSV and EBV with fluorescent dye-labelled dideoxynucleotides and cycle sequencing methods utilizing Big Dye Terminator Cycle Sequencing kit (PE Applied Biosystems). Direct DNA sequence analysis was performed using a capillary sequencer (ABI Prism 310); with MY09/MY11 primers for HPV DNA; HSV1/HSV2, HSV3/HSV4 primers for HSV DNA and EBV1/EBV2, EBV3/EBV4 primers for EBV DNA. Sequencing products were purified of unincorporated dyelabelled dideoxynucleotides by processing through Centri-Sep spin columns (PE Applied Biosystems). Sequence analysis was automatically performed on an ABI PRISM™ 310 Genetic Analyzer.

Data analysis. Fischer's exact test was used to evaluate the distribution of viral infections (HPV, HSV and EBV) among the samples studied. For all statistical analyses, SPSS statistical programme version 16.0 for Windows (Statistical Package for the Social Sciences, IBM, Chicago, IL, USA) was used. The values were considered significantly different if $p<0.05$.

Results

Alcohol, smoking and smokeless tobacco habits. In six of the countries, a majority of the patients were smokers, but in the UK and Sweden only two and four patients, respectively, smoked. The majority of the patients with OSCC from India (65%) were smokeless tobacco users; 35% of OSCC patients from Sri Lanka and 30% from Sudan used smokeless tobacco. The product used by patients from India and Sri Lanka was betel (or similar products) and the Sudanese patients used *toombak* (or similar). Only in USA and Norway did over 50% of the OSCC patients use alcohol in high amounts (Table I).

HPV, HSV and EBV infections. Of all the 155 oral carcinomas examined, 85 (55%) were positive for EBV, 54 (35%) for HPV and 24 (15%) for HSV. Regarding the prevalence of HPV, HSV and EBV in OSCC samples from the specific countries, the highest prevalence of HPV was seen in Sudan (65%), while HSV (55%) and EBV (80%) were most prevalent in the UK (Figure 2). In 34% (52/155) of all the samples examined, we detected co-infection by two (46/155=30%) or three (6/155=4%) virus specimens. The most frequent double-infection was HPV with EBV in 21% (32/155) of all OSCCs. HPV with EBV co-infection was the only co-infection detected in all eight countries examined, with Sudan (40%) and Norway (30%) having the highest prevalence (Table III).

In six samples (4%), all three virus types were detected, with the majority of these being detected in the UK. In 4 of 20 samples (20%) from the UK, all three viruses were detected (Table III).

Virus infections in industrialized vs. developing countries. Virus prevalence in OSCC from the industrialized countries, namely from Sweden, Norway, UK and USA (n=77), was compared that from the developing countries, namely from Sudan, India, Sri Lanka and Yemen (n=78). There was a statistically significantly higher proportion of samples with HSV ($p=0.026$) and EBV ($p=0.015$) from the industrialized countries as compared to the developing countries (Figure

Table III. *Frequency of the viral infections found in OSCCs examined from each country.*

	Group I				Group II				
	USA	UK	Sweden	Norway	India	Sri Lanka	Yemen	Sudan	Total
Virus	N=20 n (%)	N=20 n (%)	N=17 n (%)	N=20 n (%)	N=20 n (%)	N=20 n (%)	N=18 n (%)	N=20 n (%)	N=155 n (%)
HPV	4 (20)	9 (45)	5 (29)	7 (35)	9 (45)	6 (30)	1 (6)	13(65)	54 (35)
HSV	0 (0)	11 (55)	2 (12)	4 (20)	0 (0)	2 (10)	2 (11)	5 (15)	26 (17)
EBV	8 (40)	16 (80)	12 (71)	14 (70)	9 (45)	7 (35)	4 (22)	15 (75)	85 (55)
HPV+HSV+EBV	0 (0)	4 (20)	0 (0)	1 (5)	0 (0)	0 (0)	0 (0)	1 (5)	6 (4)
HPV+HSV	0 (0)	0 (0)	0 (0)	1 (5)	0 (0)	0 (0)	0 (0)	2 (10)	3 (2)
HPV+EBV	3 (15)	3 (15)	4 (24)	6 (30)	4 (20)	3 (15)	1 (6)	8 (40)	32 (65)
HSV+EBV	0(0)	6 (30)	2 (12)	3 (15)	0 (0)	0 (0)	0 (0)	0 (0)	11 (7)

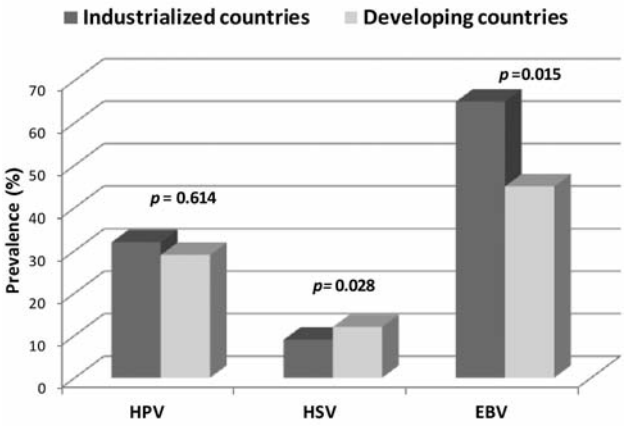

Figure 3. *Prevalence of viral infections in industrialized and developing countries of the study.*

3). Furthermore, there was a statistically significant higher co-infection of HSV and EBV in samples from the industrialized countries (p=0.00031) (Table IV).

Sequencing of ds DNA from the PCR product. For ds DNA sequencing, five samples for each group of viruses (HPV DNA-positive, HSV DNA-positive, and EBV DNA- positive) (Figures 4-6) were randomly selected from the OSCCs (n=15) and were subjected to DNA sequencing. We used the Basic Local Alignment Search Tool (BLAST) to find and compare the similarity between the sequencing results from HPV, HSV and EBV from the OSCC PCR products using nucleotide sequence databases. Following sequencing of the positive HPV positive, positive HSV and positive EBV PCR products, a 100% homology between sample was found. An example for each viral DNA sequence homology found is presented in Figures 4-6.

Table IV. *Comparison of viral infection between industrialized and developing countries*.

	Yes	No	p-Value*
HPV			
Industrialized countries	25	52	0.6139
Developing countries	29	49	
HSV			
Industrialized countries	17	60	0.02761
Developing countries	7	71	
EBV			
Industrialized countries	50	27	0.01547
Developing countries	35	43	
HPV+HSV+EBV			
Industrialized countries	5	72	0.1165
Developing countries	1	77	
HPV+HSV			
Industrialized countries	1	76	1
Developing countries	2	76	
HPV+ EBV			
Industrialized countries	16	61	1
Developing countries	16	62	
HSV+EBV			
Industrialized countries	11	66	0.00031
Developing countries	0	78	

*Fishers' exact test.

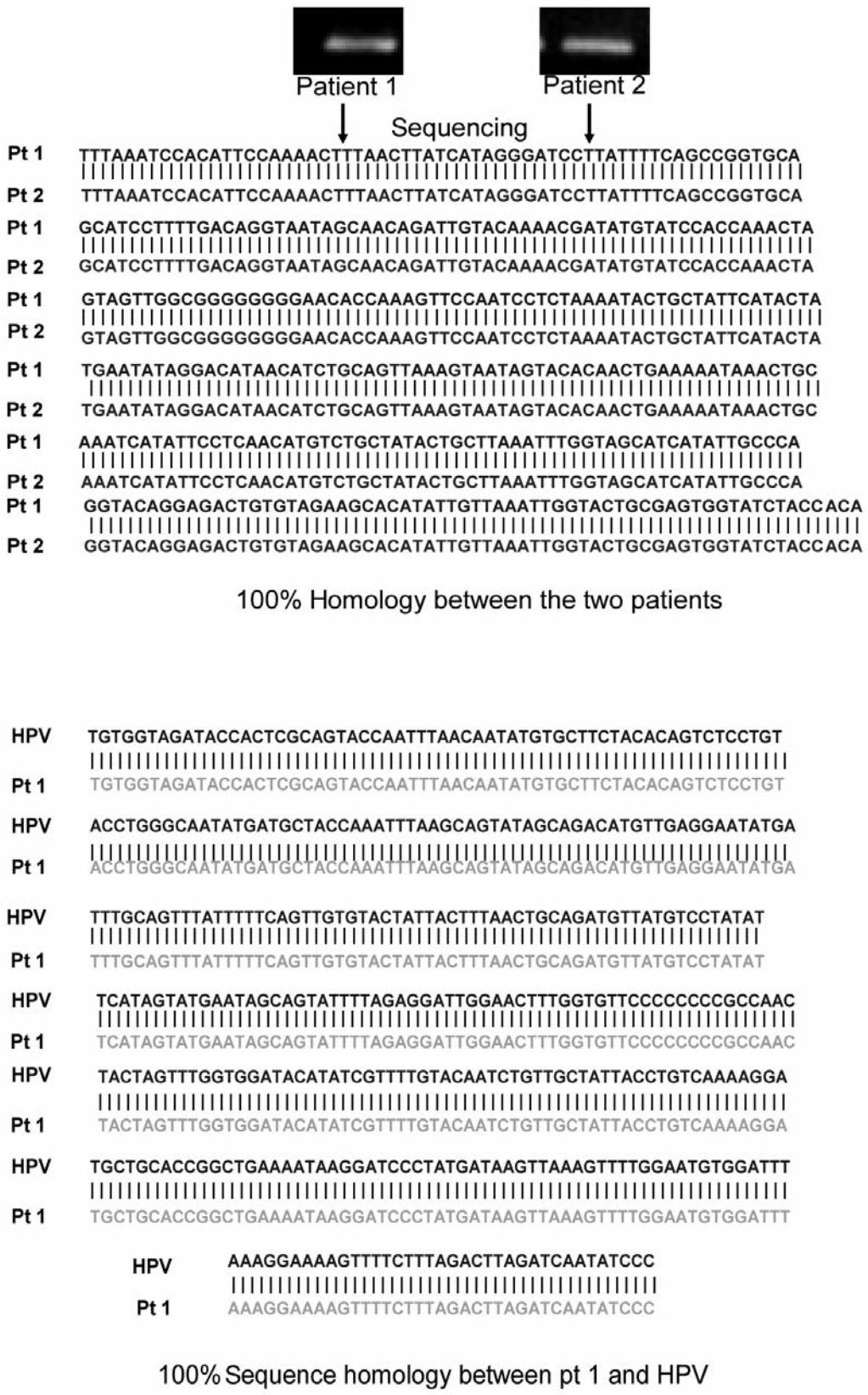

Figure 4. *Results of HPV-DNA-positive genome sequencing in samples from two patients, demonstrating 100% homology.*

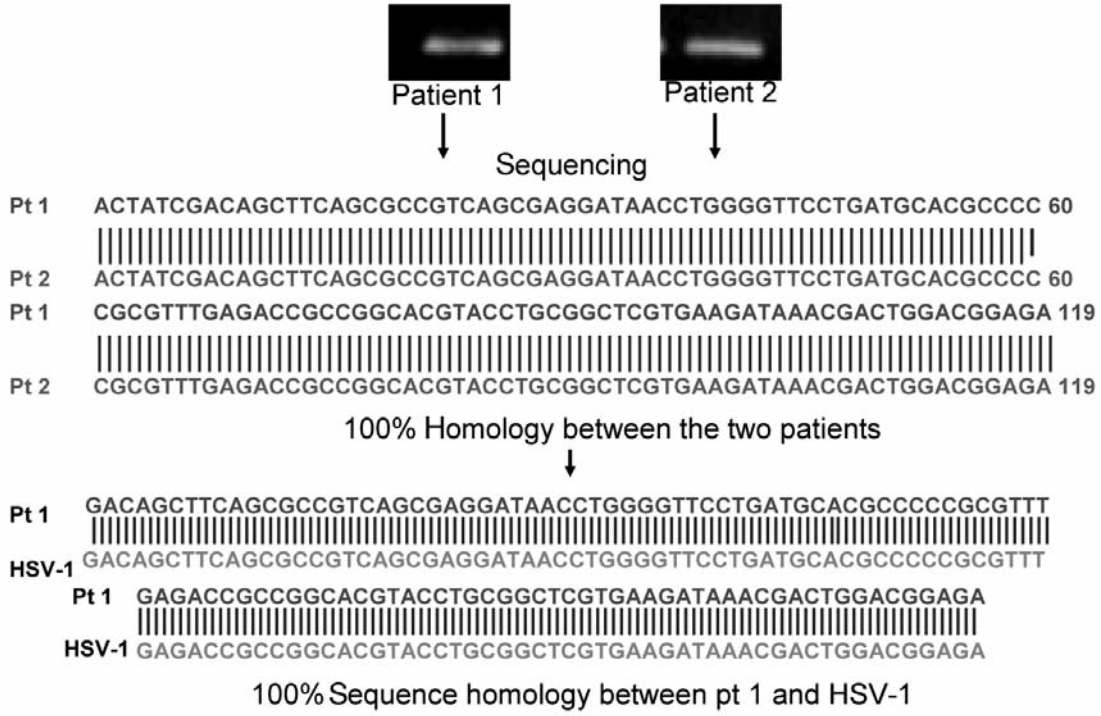

Figure 5. *Results of amplified HSV-DNA-positive genome sequencing in samples from two patients, demonstrating 100% homology.*

Discussion

In this work, we investigated 155 OSCC from eight different countries. The countries are located on four continents, and have different socioeconomic background and religious traditions. We investigated the presence of viral (HPV, HSV-1+2 and EBV) infections, and alcohol and tobacco habits.

In previous global studies, the single most common localisation of intraoral SCC was the tongue (36). This was confirmed in our study where 26% of the OSCCs were diagnosed in the tongue. In six of the countries, the majority of the OSCC patients were smokers, which was expected since smoking has been shown to be a major risk factor in OSCC development (6). Smokeless tobacco has been suggested as a risk factor in OSCC and this finding could be related to the use of *toombak* and betel. The use of smokeless tobacco products was highest in Sri Lanka, India and Sudan. Betel quid use on the Sub-indian continent and *toombak* use in Sudan are major risk factors in OSCC development, which has been emphasized in previous reports (8, 37). The levels of the potent carcinogenic component of TSNAs, in particular *N*-nitrosonor-nicotine and 4-(methyl nitrosamino)-1-(3-pyridyl)-1-butanone, are higher than that reported for North American and European snuff (38). In our study, alcohol consumption was not as high as expected, although previous studies have stated alcohol to be one of the major risk factors in OSCC development (6). However, alcohol consumption varies largely between countries due to ethnic, religious and cultural differences.

In this study, the overall HPV prevalence in OSCC was 35%. The single highest HPV prevalence was seen in Sudan (65%), followed by India (45%) and the UK (45%). There was a statistically significant difference between HPV prevalence in Sudan (65%) and Yemen (6%). No difference in HPV prevalence was seen between industrialized and developing countries. Our study is the first to perform comparison studies between eight different countries. There are numerous earlier studies about HPV and OSCC, and the HPV prevalence ranges between 0-100%. In our study, ethnicity does not seem to be a major risk factor in HPV prevalence in OSCC, which is in line with previous studies where HPV was found at higher frequency in normal oral mucosa in Sudan and India compared to OSCC (34, 35). Furthermore, Sand *et al*. showed that consumption of Swedish snuff and alcohol, as well as smoking, does not seem to facilitate HPV infection in oral tumours or healthy oral mucosa (17). The variable HPV prevalence in OSCC in different studies might be explained by different sampling techniques, divergent PCR methods and also the quality of the sample (*e.g*. whether frozen or fixed) (25).

The highest overall virus prevalence was EBV, which was seen in 55% of the OSCCs. There was a huge span in EBV prevalence in OSCC, ranging from 22% in Yemen to 80% in

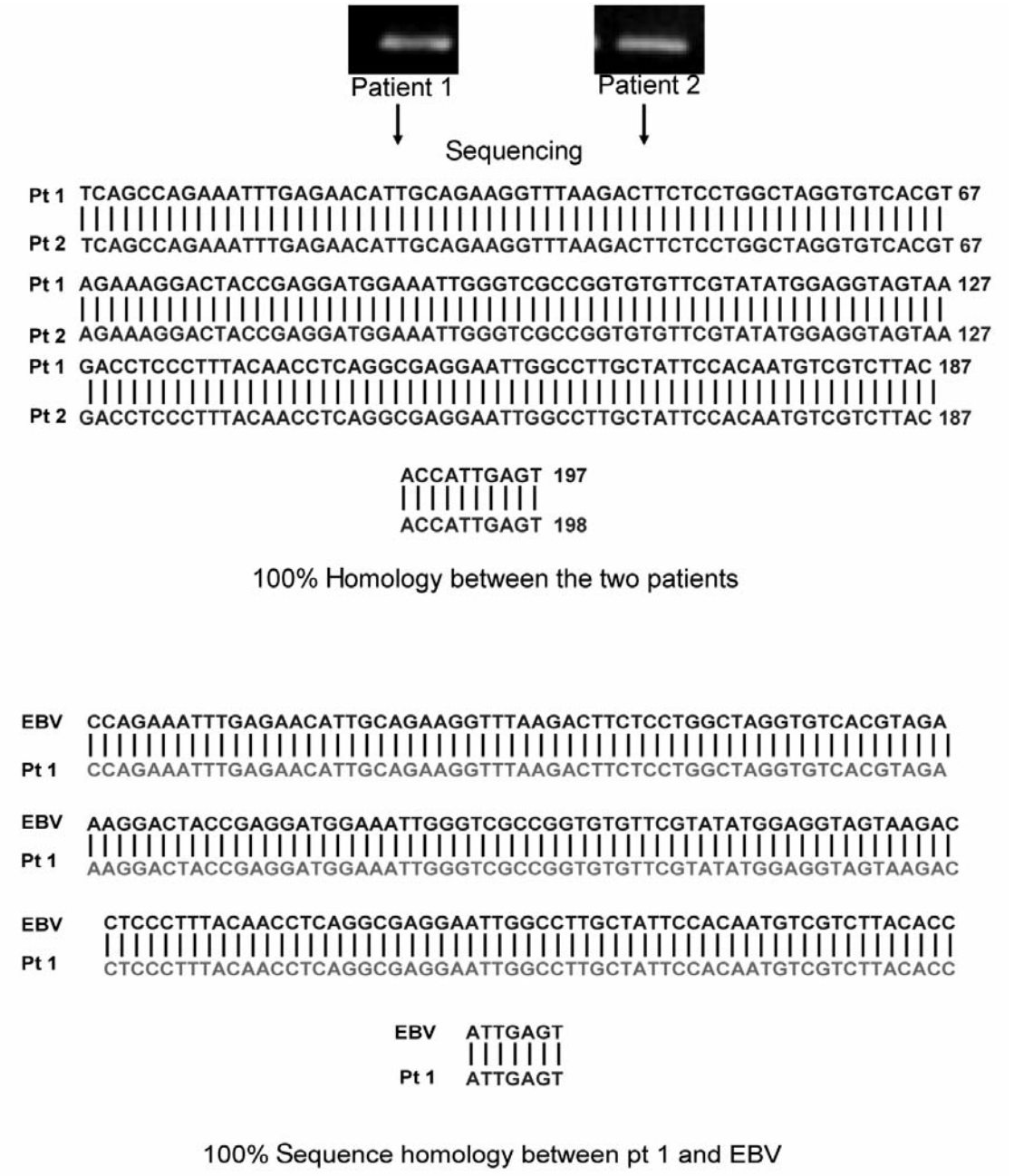

Figure 6. *Results of amplified EBV-DNA-positive genome sequencing in samples from two patients, demonstrating 100% homology.*

the UK, with a statistically significant higher EBV prevalence in the industrialized countries compared to the developing countries. This is comparable to earlier studies performed in Sweden, Sudan and India regarding EBV prevalence in OSCC (16, 34, 35). In a study on immigrants in Sweden, Mousavi *et al*. concluded that "early life infection with EBV in the immigrants countries of origin, and probably a minor contribution by smoking, may be the main exposures influencing nasopharyngeal carcinoma risks among immigrants to Sweden" (39). That study was performed on nasopharygeal cancer, and also reflects the environment as a risk factor for EBV infection. Thus, it seems that the environment has a higher influence on EBV infection than ethnicity alone. Our study focused on OSCC and not on nasopharyngeal carcinoma. Even though ethnicity in the OSCC patients in the industrialized countries was not recorded, it does not seem that EBV is as major a risk factor in OSCC as it is in nasopharyngeal cancer.

Previously, Larsson *et al*. stated that occurrence of HSV-1 infection in association with snuff exposure may be related to development of OSCC (31, 40) and this has also been suggested in clinical studies (41). In an animal study, Larsson *et al*. showed that HSV-1 can act as a carcinogen in the presence of tobacco or any other chemical carcinogen (31). However, in two previous studies from India and Sudan, low HSV prevalence was seen in OSCC (34, 35). In our study, the overall prevalence of HSV-positive OSCC was 15%.

When comparing the different countries, there was a huge span from 0% in USA and India to 55% in the UK. In our work, there was a statistically significantly higher HSV prevalence in the industrialized countries compared to the developing countries. However, this was mainly due to the high prevalence in the UK (55%). We have no information regarding the ethnic composition or socioeconomic situation for the UK samples, hence no safe conclusions can be drawn from this information. In a study from Peru, the low-income population had higher genital HSV prevalence than did the general population (42), and in an epidemiologic, multiethnic study from London, genital HSV-2 was associated with black Caribbean and black African ethnicity, while genital HSV-1 was associated with white ethnicity (43). Hence, based on the limited information in our study, no conclusions can be drawn regarding HSV, ethnicity, socioeconomics and OSCC development.

It has been shown that up to 90% of the general population have antibodies against HSV (44), and Miller *et al*. concluded that shedding of HSV-1 is present at many intraoral sites, for brief periods, at copy numbers sufficient to be transmitted, even in seronegative individuals (45). If HSV plays an active role in OSCC development, or if it is only a passive by-stander in the local immune-deficient tumour area, remains unclear. Co-infection by HPV and EBV was seen in all eight countries in our work, with an overall prevalence of 21%. This finding is very interesting and Al Moustafa *et al*. concluded that high-risk HPV and EBV co-infections play an important role in the initiation of neoplastic transformation of human oral epithelial cells (26). Furthermore, Yang *et al*. concluded that in HPV-induced cervical cancer, other viruses, such as HSV-2, might not be involved in the oncogenic processes directly but might enhance the possibility of oncogenesis (33). Thus, co-infection by two or more viruses may be an important risk factor in OSCC development.

Conclusion

In this work, we examined viral infections by nested and semi-nested PCR techniques, and we have controlled for the results obtained by these methods, where the amplified PCR products of HPV, HSV-1 and EBV were subjected to DNA sequencing of each of the amplified PCR fragment. We found a higher proportion with HSV and EBV in the industrialized countries as compared to the developing countries and also a higher co-infection of HSV and EBV in the industrialized countries.

These observations warrant further studies to determine the possible role of viral infections and co-infections with HPV, EBV and HSV-1 as risk markers for the development of OSCC.

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