ORIGINAL ARTICLE

Retrospective case–control study of viral pathogen screening in proliferative verrucous leukoplakia lesions

García-López, R.,*†‡ Moya, A.,*†‡ Bagan, J.V.§ & Pérez-Brocal, V.*†‡

*Área de Genómica y Salud de la Fundación para el Fomento de la Investigación Sanitaria y Biomédica de la Comunidad Valenciana (FISABIO)-Salud Pública, †Institut Cavanilles de Biodiversitat i Biologia Evolutiva, Universitat de València, Valencia, ‡CIBER Epidemiología y Salud Pública (CIBERESP), Madrid, §Oral Medicine, Department of Stomatology, Hospital General Universitari, Universitat de València, Valencia, Spain

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Objective: This study aimed to survey the presence of known oncoviruses in oral biopsies from patients diagnosed with the aetiologically undetermined proliferative verrucous leukoplakia and compare results to those from milder oral leukoplakia (OL) cases, oral squamous cell carcinoma, a common outcome of the lesions of interest, and healthy controls.
Design: Blind, retrospective, case–control study.
Setting: A stomatology unit in an academic Hospital and a Public Health laboratory.
Participants: Forty patients were divided in four groups. Ten patients had been diagnosed with proliferative verrucous leukoplakia, 10 with OL and 10 with OSCC, and 10 were healthy subjects.
Main outcome measures: The presence or absence of oncovirus DNA was assayed with the amplification of viral genetic markers using PCR and subsequent gel electrophoresis confirmation. Amplified fragments were sequenced and identified bioinformatically.
Results: No DNA from the herpesvirus, papillomavirus or polyomavirus species was detected in the samples.
Conclusions: No association between proliferative verrucous leukoplakia and target viruses was detected. A higher throughput viral metagenomic approach may prove valuable for future analyses, as it would not be restricted to *a priori* knowledge of potential targets.

Proliferative verrucous leukoplakia (PVL), first described by Hansen in 1985, is a rare high-risk variant of oral leukoplakia (OL) affecting multiple zones in the oral cavity. It is characterised by the initial formation of white asymptomatic hyperkeratotic plaques that may change into exophytic wart-like forms with time, eventually becoming multifocal.[1] They have been mainly reported on gingiva[2] but can also appear on other oral mucosal tissue.[3] Long-term studies have revealed ≈70% of PVL lesions develop oral carcinoma, especially oral squamous cell carcinoma (OSCC).[1,2,4]

The worryingly high rate of malignant transformation is aggravated by the inefficacy of clinical procedures in the long term and a particularly complicated diagnosis. Treatment is largely ineffective as PVL has a high recurrence rate, rendering scalpel or laser removal insufficient. Local block tissue removal, often including nearby teeth, is sometimes the only viable procedure.[2]

Lesions are preponderant in elderly women, and contrary to common OL, PVL has not been associated to tobacco-related habits.[1,4,5] Its complex diagnostics makes it particularly elusive, and diagnosis is commonly established retrospectively after years of follow-up. Early lesions appear as isolated white hyperkeratosis patches that may or not exhibit verrucous profiles. As lesions progress, they may affect other areas and transform into verrucous lesions. The disease is usually underdiagnosed as PVL is not suspected until lesions become recurrent and atypical. Biopsies are required for patients' follow-up along with regular analyses during their lifetimes as PVL may take months or years to become histologically malignant.[1] Malignant transformation is unpredictable, but prognosis in OSCC can be correlated to aneuploidy in cells from the affected area.[3,6]

The recurrence, multifocal features and oral locations of this pathology have led to several studies focusing on finding correlations between viral infections and PVL development, although a potential viral agent has yet to be identified.[2,7–9]

Correspondence: V. Pérez-Brocal, Área de Genómica y Salud de la Fundación para el Fomento de la Investigación Sanitaria y Biomédica de la Comunidad Valenciana (FISABIO)-Salud Pública, Avenida de Cataluña 21, 46020 Valencia, Spain. Tel.: +34 961925914; Fax: +34 9619259778; e-mail: perez_vicbro@gva.es

This study attempted to survey the presence of oncogenic DNA viruses in the samples using a PCR approach.

Materials and methods

Ethical considerations

This study was approved by the Institutional Review Board of the Universitat of València. All of the participants signed the informed consent documents.

Participants

Forty patients from the Stomatology Unit in Hospital General Universitari in Valencia were studied. Their average age was 58.20 ± 21.73 years, and 72.50% were female. Samples from four groups of 10 patients each were analysed: (i) patients diagnosed with OL[10] but negative to PVL (age mean = 65.50 ± 9.86, 90% female), (ii) patients matching PVL diagnostic criteria[1] (age mean = 70.30 ± 8.12, 100% female), (iii) OSCC patients with no PVL or OL (age mean = 72.10 ± 14.11, 30% female) and (iv) control group with no related oral precancerous lesions (age mean = 24.90 ± 3.98, 70% female).

Patient diagnosis and sample collection

Diagnoses were determined by histological analysis of incisional biopsies obtained with local anaesthesia. Samples from the OL, PVL and OSCC groups were obtained directly from the lesion area (Fig. 1), whereas control biopsies were obtained from healthy mucosa near lower third molars during teeth removal. Biopsies were separated into two parts for histopathological studies and for storage in RNAlater (Ambion Inc, Austin, TX, USA) at −80°C, respectively.

Frozen biopsies were cut in two using sterile scalpels. One was returned to storage while the rest was immediately transferred to microcentrifuge tubes for extraction. These were rinsed with 300 μL of Hank's balanced salt solution (HBSS) buffer (GIBCO, Gaithersburg, MD, USA) at 4°C to remove RNAlater. Next, 600 μL HBSS was added to each tube.

DNA extraction

The present research was designed as a blind study for the laboratory research group. Tissue was disrupted mechanically using a TissueLysser II (Qiagen, Valencia, CA, USA) (30 Hz, 5–10 min depending on its hardness) with 3-mm tungsten carbide beads (Qiagen), at 4°C. Genomic DNA was extracted following a conventional phenol/chloroform protocol, in which consecutive extraction steps were carried out, adding one volume of chloroform, followed by phenol, a 1 : 1 mixture of phenol-chloroform and a chloroform : isoamyl alcohol 24 : 1. In all cases, the upper aqueous phase containing the DNA was recovered. The DNA was precipitated with 40 μL NaOAc 3 M and 1 mL EtOH at −20°C and incubated in ice for 15 min. After centrifuging (12 100 ***g***, 3 min), the resulting pellet was cleansed with EtOH 70%, dried at room temperature and resuspended in 50 μL TE buffer.

DNA presence was confirmed with PCR amplifications of the 16S rDNA gene of co-extracted bacterial DNA, using universal primers E8F (5′-AGAGTTTGATCMTGGCTCAG-3′) and B530R (5′-CCGCGGCKGCTGGCAC-3′). Reactions were carried out using Biomix PCR kit (Bioline, London,

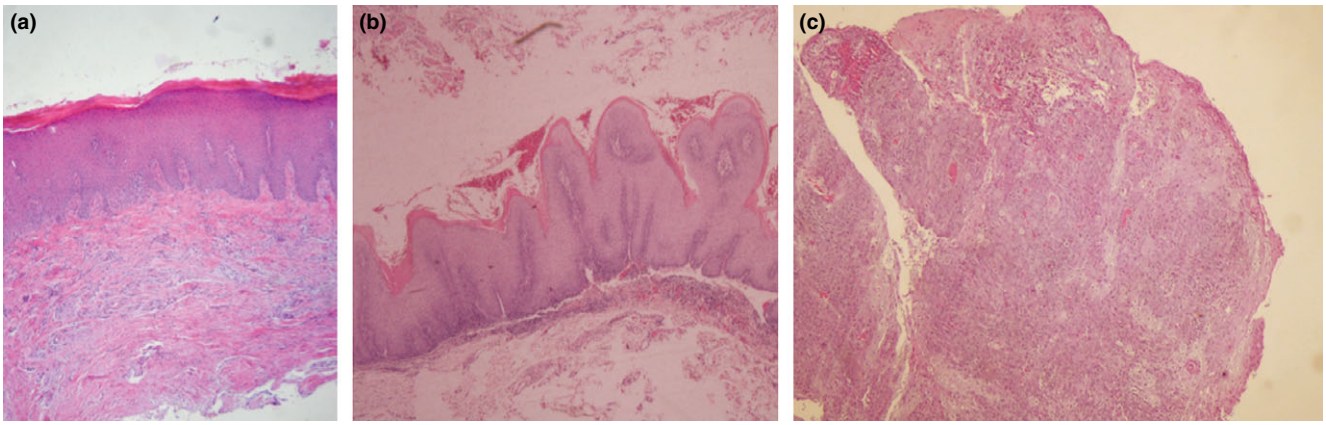

Fig. 1. H&E-stained sections of the lesions in groups oral leukoplakia (OL), proliferative verrucous leukoplakia (PVL) and oral squamous cell carcinoma (OSCC). Haematoxylin and eosin stain of histological sections of affected patients. (a) Patient of group OL displaying hyperorthokeratosis without displastic changes. (b) Patient of group PVL with wavy hyperorthokeratosis, and exophytic warty configuration. (c) Patient of group OSCC showing conventional squamous cell carcinoma.

UK), and conditions were as follows: denaturation at 95°C for 2 min; 30 cycles of denaturation at 95°C for 30 s, annealing at 52°C for 1 min and extension at 72°C for 1.5 min; and final elongation at 72°C for 10 min. Blank controls were included to monitor contamination events.

Resulting amplicons were confirmed by 1.4% agarose gel electrophoresis, stained with GelRed (Biotium, Hayward, CA, USA) and seen under UV light. For samples where amplification failed, the extracted DNA was enriched with Genomiphi (GE Healthcare Life Sciences, Amersham, UK) following the manufacturer's instructions. DNA concentrations were measured with Picogreen (Invitrogen, Carlsbad, CA, USA), and 60 μg/mL DNA was loaded in a new round of 16S rDNA PCR, using identical conditions.

Virus screening

Samples yielding positive bacterial DNA amplicons were considered for screening potential oncoviruses. The presence of papillomaviruses, polyomaviruses (including Merkel cell polyomavirus) and herpesviruses (including Epstein–Barr Virus), was tested using universal primers obtained from the literature. The targeted oncovirus species, primer sequences and PCR conditions are detailed in Table 1.

Two sets of degenerate primers were selected for screening papillomaviruses: FAP59/FAP64[11] and CP4/CP5.[12] DNA from *Eidolon helvum* papillomavirus was included as positive control, expecting a fragment of ≈500–600 bp. Polyomaviruses were surveyed with two sets of degenerate primers designed for nested PCR.[13] The first PCR used primers VP1-1f/VP1-1r, while the internal PCR used VP1-2f/VP1-2r primers (≈250–300 bp amplicon). Additional primers specific for Merkel cell polyomavirus, MCVPS1f/MCVPS1r, were selected.[14] No positive control was available. The screening for herpesviruses was carried out using degenerate primers in nested PCR.[15] For the first PCR, two forward primers and one reverse were used: DFAf/ILKf and KG1r. The internal PCR used primers TGVf/IYGr. The positive control contained DNA from Epstein–Barr virus and Kaposi's sarcoma-associated herpesvirus, expected to produce ≈600 bp fragments.

Samples yielding bands in agarose gels were considered for Sanger sequencing. A representative for each band was selected for the sequencing reactions (generally those yielding the most intense and well-defined bands). Whenever possible, two samples sharing similar size bands were selected. Single bands were cleansed using NucleoFast 96-well plate (Macherey-Nagel, Düren, Germany). Multiple bands were separated and excised from agarose gels and cleansed using a High Pure PCR Product Purification Kit (Roche Diagnostics, Mannheim, Germany).

Sequencing reactions were performed with BigDye Terminator v3.1 sequencing kit (Applied Biosystems, Foster City, CA, USA) following the manufacturer's instructions. Sequencing was carried out in a 3130xl Genetic Analyzer (Applied Biosystems).

Trimming of the nucleotide sequences (reads) was implemented in Staden.[16] Sequences were submitted to the DDBJ Nucleotide Sequence database (AB897841-AB897864). Reads were aligned to their corresponding viral family subset obtained from NCBI's non-redundant database (nr) (11 September 2013), *Papillomaviridae*, *Herpesviridae* or *Polyomaviridae*, using megablast setting (e-value 1e-5) to identify highly similar sequences.[17] Those with no significant match were aligned to the same subset using tBLASTx (e-value 1e-3) in search of distant sequences with similar protein structure. Sequences with no assigned taxonomy were then compared against the whole nr database using BLASTn (e-value 1e-3) to search remote homologous sequences.

Results

Forty samples of variable sizes and tissue composition were processed under identical conditions. DNA extraction was confirmed for 38 samples, 25 successfully amplified during direct 16S rDNA amplification and the remaining 13 after enrichment with Genomiphi and subsequent 16S rDNA amplification. The remaining two were discarded as no genomic DNA could be confirmed to be present. After their origin was disclosed, they were identified as belonging to the OSCC and control group.

The screening for papillomaviruses with primers FAP59/FAP64 resulted in the amplification of two bands for 32 of them (Fig. 2a). Fragments were ≈300 and 400 bp long, contrary to the expected size of the papillomavirus amplicon (≈600 bp) only seen in the positive control. A representative sample was selected for each band size for sequencing. No significant similarity was found in the alignments with megablast against the *Papillomaviridae* family sequences in the nr database, contrary to the positive control, which matched the *E. helvum* papillomavirus 1 major capsid protein (L1) gene with e-value 0.0, 100% identity. Alignments with tBLASTx yield no significant results for unknown bands either, whereas alignment to the whole nr database using BLASTn resulted in *Enterobacteria* phage phiX174 as the best alignments (e-value 3e-145, 88% identity for the longest fragment, and 9e-25, 85% identity for the shortest one).

The second set of primers for papillomavirus primers, CP4/CP5, produced a single band (≈250 bp) in 33 samples (Fig. 2b). The positive control generated a ≈600 bp band, as expected. A similar band was also detected in one sample. A

Table 1. Virus screening primers and PCR conditions

Virus	Primers	Sequence	Initial denaturation	Cycle	Denaturation	Annealing	Elongation	Final elongation
Papillomavirus	FAP59	5′-TAACWGTIGGICAYCCWTATT-3′	94°C–5 min	35x	94°C–15 s	45°C–1 min	72°C–20 s	72°C–10 min
	FAP64	5′-CCWATATCWVHCATITCICCATC-3′						
	CP4	5′-ATGGTACARTGGGCATWTGA-3′	94°C–5 min	35x	94°C–15 s	45°C–1 min	72°C–20 s	72°C–10 min
	CP5	5′-GAGGYTGCAACCAAAAMTGRCT-3′						
Polyomavirus	VP1-1f	5′-CCAGACCCAACTARRAATGARAA-3′	95°C–12 min	45x	95°C–30 s	46°C–30 s	72°C–2 min	72°C–15 min
	VP1-1r	5′-AACAAGAGAACACAAAT(N/I)TTTC C(N/I)CC-3′						
	VP1-2f	5′-ATGAAAATGGGGTTGGCCC(N/I)C T(N/I)TGYAARG-3′	95°C–12 min	45x	95°C–30 s	50°C–30 s	72°C–2 min	72°C–15 min
	VP1-2r	5′-CCCTCATAAACCCGAACYTCYTC (H/I)ACYTG-3′						
Merkel cell Polyomavirus	MCVPS1f	5′-TCAGCGTCCCAGGCTTCAGA-3′	95°C–5 min	35x	95°C–30 s	55°C–30 s	68°C–90 s	72°C–7 min
	MCVPS1r	5′-TGGTGGTCTCCTCTCTGCTACTG-3′						
Herpesvirus	DFAf	5′-GAYTTYGCNAGYYTNTAYCC-3′	94°C–5 min	45x	94°C–30 s	46°C–1 min	72°C–60 s	72°C–7 min
	ILKf	5′-TCCTGGACAAGCAGCARNYSGCN MTNAA-3′						
	KG1r	5′-GTCTTGCTCACCAGNTCNACNCCYTT-3′						
	TGVf	5′-TGTAACTCGGTGTAYGGNTTYACNG GNGT-3′	94°C–5 min	45x	94°C–30 s	46°C–1 min	72°C–60 s	72°C–7 min
	IYGr	5′-CACAGAGTCCFTRTCNCCRTADAT-3′						

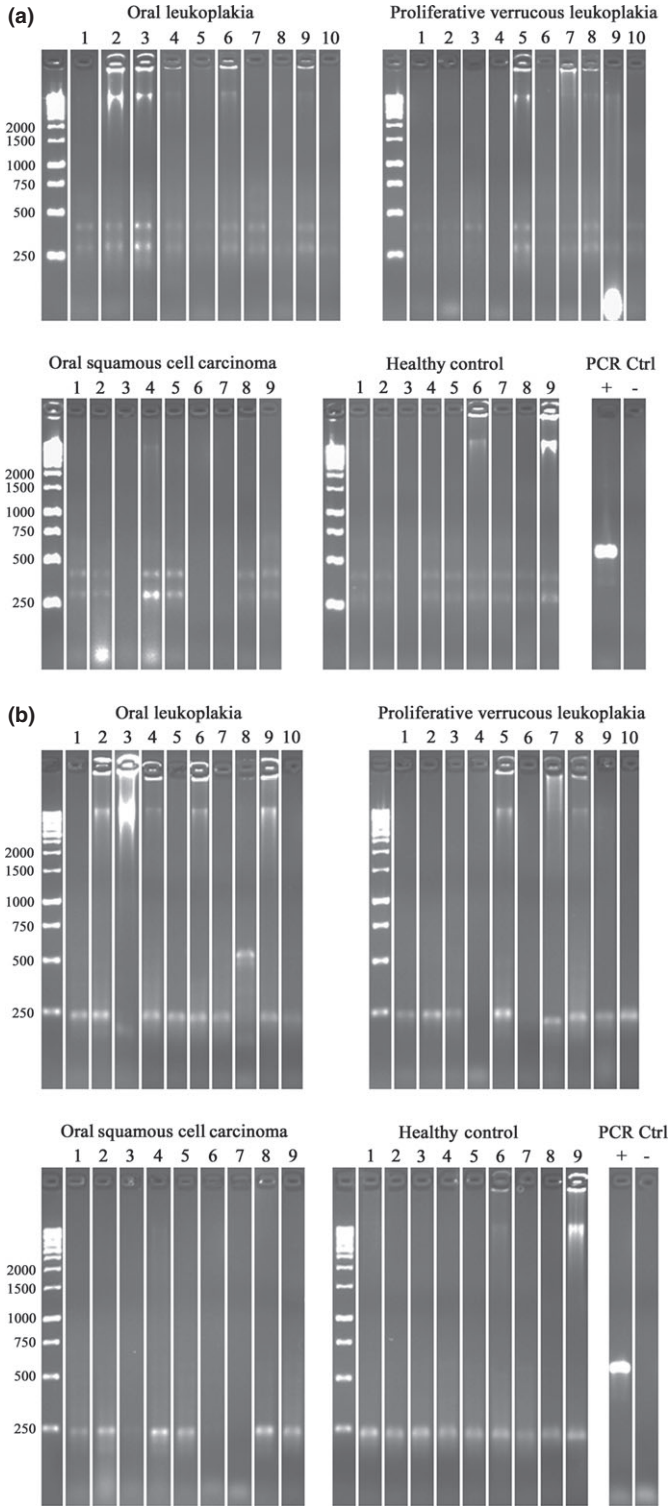

Fig. 2. Papillomavirus screening. Samples containing DNA extracted from biopsy samples belonging to OL, PVL, OSCC and control groups were stained with GelRed, loaded in 1.4% agarose gel for electrophoresis and photographed under a UV light. Exposure was increased to capture a sharper image of the bands. DNA from *Eidolon helvum* papillomavirus was used as a positive control. (a) Amplicon yield of PCR with primers FAP59 and FAP64 for papillomavirus amplification. (b) Amplicon yield of PCR with primers CP4 and CP5 for papillomavirus amplification. OL, oral leukoplakia; OSCC, oral squamous cell carcinoma; PVL, proliferative verrucous leukoplakia.

representative sample was selected for each band. The alignment with megablast against *Papillomaviridae* sequences confirmed the successful amplification of the control, matching *E. helvum* papillomavirus E1 gene (e-value 0.0, 100% identity) but yielded no results for the remaining reads neither with megablast nor with tBLASTx. When BLASTn was used to detect remote homologues in the complete nr database, the sequence obtained from the band that closely resembled that of the positive control was partially aligned to *Leuconostoc mesenteroides* (spanning <20% of the read length with e-value 1e-3, 85% identity). The band detected in most other samples aligned partially (86% query coverage, e-value 5e-28, 80% identity) against *Homo sapiens* steroid receptor RNA activator 1.

The screening of polyomavirus with primers VP1-2f/VP1-2r resulted in the amplification of fragments with different lengths (Fig. 3a). Several samples yielded a band below the 500 bp marker. A fragment in the OSCC group matched the expected length for the polyomavirus amplicon (250–270 bp). Representative samples for each band were purified and sequenced. Alignments with megablast and tBLASTx against the *Polyomaviridae* family sequences yielded no significant results. BLASTn against the whole nr database aligned partially against human sequences. The ≈250 bp fragment aligned to *H. sapiens* chromosome 5 clone CTB-99P17 (e-value 9e-55, 99% identity). The ≈800 bp fragment matched an undetermined sequence in chromosome 19 (e-value 0.0, 95% identity), whereas the ≈700 bp fragments matched *H. sapiens* protein kinase C, epsilon gene (e-value 0.0, >87% identity). The ≈450 bp band aligned to a sequence on chromosome 12 (e-value 7e-145, 95% identity). The ≈300 bp fragment aligned to *H. sapiens* chromosome 5 clone CTB-55A14 (e-value 6e-13, 85% identity).

PCRs for Merkel cell polyomavirus screening with primers MCVPS1f/MCVPS1r generated a fragment of ≈320 bp in most samples, as well as other bands larger than 700 bp (Fig. 3b). As the expected fragment would be ≈70 bp long, bands beyond 800 bp were not considered. Representative samples displaying either 320 or 700 bp bands were selected and sequenced. Alignments with megablast and tBLASTx against the *Polyomaviridae* sequences yielded no significant results. When aligned with BLASTn against the nr database, the shorter sequence aligned with *H. sapiens* PAC clone RP4-539M6 (e-value 1e-104, 98% identity), whereas the larger was aligned to a sequence in *H. sapiens* chromosome 1 (e-value 0.0, identity 99%).

The screening for herpesvirus with primers TGVf/IYGr resulted in the amplification of a single light band in most samples (Fig. 4). The amplicon for herpesvirus, as confirmed by the band in the positive control, was ≈250 bp long. Representative samples were purified and sequenced. The alignment of the resulting reads with megablast against the *Herpesviridae* sequences confirmed the amplification of the control, matching Human herpesvirus 8 isolate KSHV (e-value 2e-69, 97% identity) but yielded no results for the samples. Neither alignments with tBLASTx and the same database subset nor BLASTn against the nr yielded significant results.

Discussion

Comparison with other studies

The multifocal and recurrent nature of PVL has inspired different studies aimed at identifying individual viruses as potential aetiological agents.[2,7–9] These focus on known oncogenic viruses such as human papillomavirus type 16 and 18, but have failed to correlate their presence with the emergence of PVL or OSCC. In this study, no papillomaviruses were detected in the samples, in accordance with previous reports.[7,8]

Strength and limitations of the study

This work presents the screening of a wider viral spectrum based on reported oncogenic species. Oral biopsies from lesion sites in patients diagnosed with OSCC were included to contrast them as the potential outcome of PVL. Additionally, OL biopsies were used to compare PVL to a different condition bearing similar symptoms but that may be caused by unrelated agents.[4,5]

The project was designed as a blind study to avoid experimental biases in the laboratory procedures and bioinformatics analyses.

As laser capture microdissection was not used, it cannot be discarded that some of the extracted DNA may be from tumour infiltrating inflammatory cells or from stroma and not necessarily, from tumour tissue.

The selection of degenerate DNA primers responded to the need of detecting DNA from different species within the viral families of interest. The main limitation of this study was that positive controls were not available for polyomavirus species screening. However, DNA fragments that were amplified were selected and sequenced, allowing for the dismissal of false positives. Furthermore, primers were tested bioinformatically, simulating amplifications in-silico with Primer Prospector[18] using the custom polyomavirus database.

Bioinformatic exploration depends heavily on the database composition, and associated taxonomic labels are references to sequences that closely resemble the query reads, not necessarily the same fragments found in the samples. Therefore, different instances of the algo-

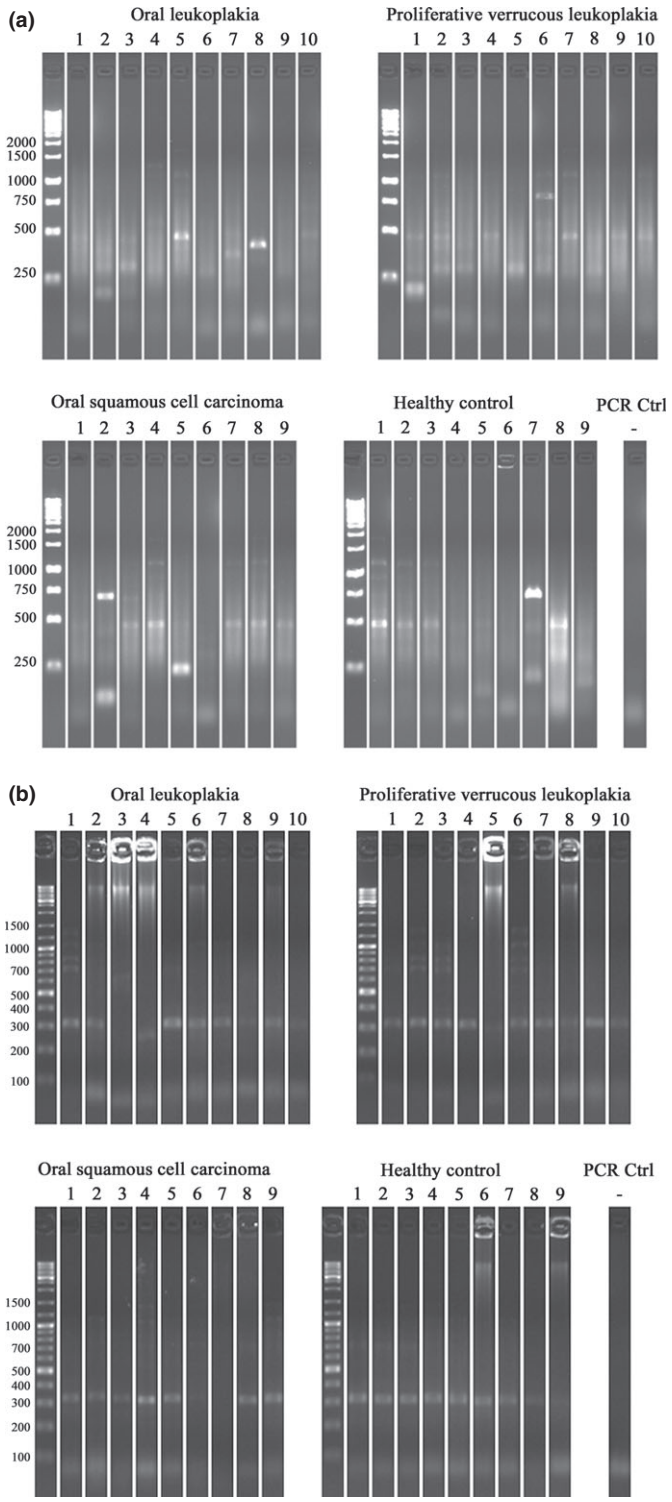

Fig. 3. Polyomavirus screening. Samples containing DNA extracted from biopsy samples belonging to OL, PVL, OSCC and control groups were stained with GelRed, loaded in 1.4% agarose gel for electrophoresis and photographed under a UV light. (a) Amplicon yield of the second round of the nested PCR with primers VP1-2f and VP1-2r for universal polyomavirus amplification. Exposure was increased to capture a sharper image of the bands. (b) Amplicon yield of PCR with primers MCVPS1f and MCVPS1r for Merkel cell polyomavirus amplification. OL, oral leukoplakia; OSCC, oral squamous cell carcinoma; PVL, proliferative verrucous leukoplakia.

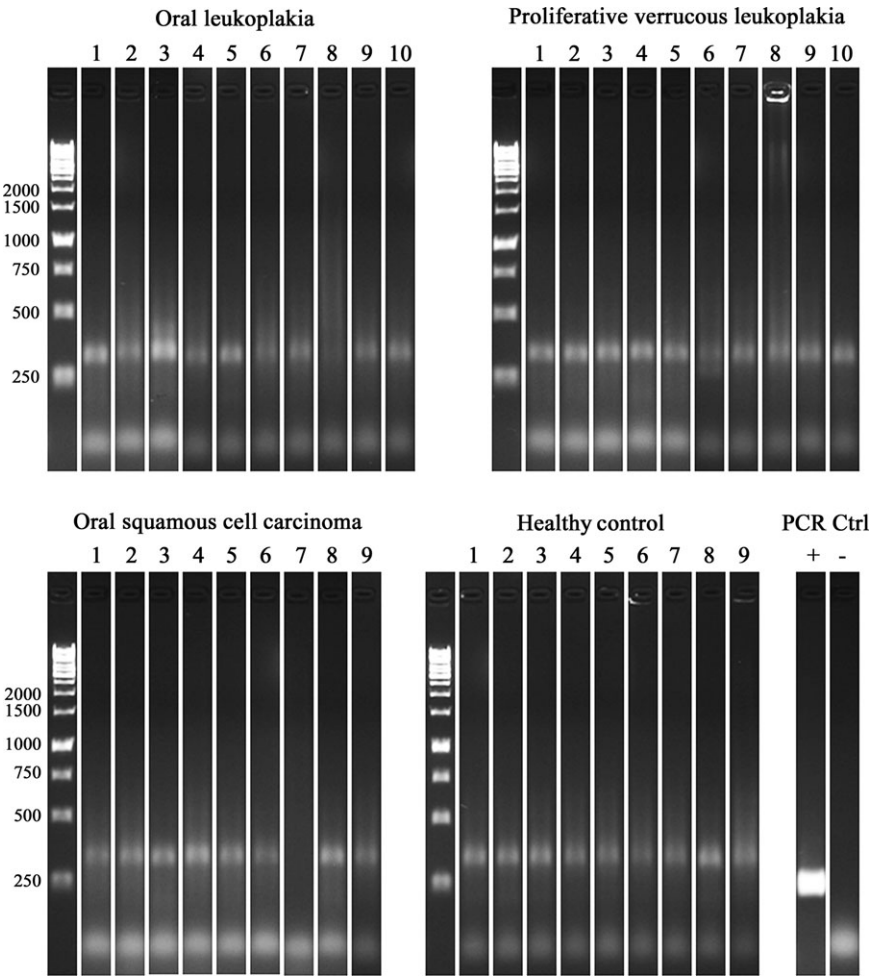

Fig. 4. Herpesvirus screening. Amplicon yield of the second round of the nested PCR with primers TGVf and IYGr for herpesvirus amplification. Samples containing DNA extracted from biopsy samples belonging to OL, PVL, OSCC, and control groups were stained with GelRed, loaded in 1.4% agarose gel for electrophoresis and photographed under a UV light. DNA from Epstein–Barr virus and Kaposi's sarcoma-associated herpesvirus was used as positive control. OL, oral leukoplakia; OSCC, oral squamous cell carcinoma; PVL, proliferative verrucous leukoplakia.

rithm were used, along with different databases. Megablast was the first approach because only highly similar sequences were desired initially. A positive hit would have been normally detected by this method alone. The family subset was obtained from the NCBI database to limit the search to species closely related to the oncogenic targets. Next, tBLASTx was used to search for relatively distant species within the same family that may have similar amino acid sequences. This tool is more flexible because it considers the ambiguity of the genetic code as it compares translated amino acid sequences instead of nucleotide reads both for query and for target. Finally, BLASTn was used to identify sequences not belonging to targeted viruses or to closely related species (as this possibility had been previously discarded). This implementation was intended to determine the taxonomy of PCR by-products.

Synopsis of key findings

No papillomaviruses were detected in the sample, but some by-products present in most samples were apparently human rather than viral and were evenly distributed among groups. Similarly, for polyomaviruses only human DNA matched the sequenced PCR products. This was not totally unexpected as total DNA extractions were carried out, resulting in a mixture of genomic viral, bacterial and human DNA. Reads from the herpesvirus screening produced no significant alignments, but the amplified fragments size did not match that of the positive control, thus suggesting a spurious origin.

Conclusion

Although potential oncoviruses were undetected in this study, the hypothesis of a viral aetiological agent for PVL cannot be ruled out. Future studies using high-throughput analyses with next-generation sequencing platforms might provide an insight to non-cultivable organisms and their role in this pathology.

Keypoints

- The presence of oncoviruses was assayed in samples displaying OL, PVL, and OSCC, following the interest in the recurrence and multifocality of PVL.
- No oncoviruses were detected in any of the samples.
- A metagenomic approach could prove valuable for further exploration of the etiological agent of PVL.

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Conflict of interest

None to declare.

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