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Loop mediated isothermal amplification (LAMP) for the detection and subtyping of human papillomaviruses (HPV) in oropharyngeal squamous cell carcinoma (OPSCC)

D.M. Livingstone[a,*], M. Rohatensky[b], P. Mintchev[c], S.C. Nakoneshny[d], D.J. Demetrick[e], G. van Marle[c], J.C. Dort[a,d]

[a] Section of Otolaryngology-Head and Neck Surgery, Department of Surgery, Cumming School of Medicine, University of Calgary, Calgary, Alberta, Canada
[b] Undergraduate Medical Education, Faculty of Medicine & Dentistry, University of Alberta, Edmonton, Alberta, Canada
[c] Department of Microbiology, Immunology and Infectious Diseases, Cumming School of Medicine, University of Calgary, Calgary, Alberta, Canada
[d] Ohlson Research Initiative, Southern Alberta Cancer Research Institute, Cumming School of Medicine, University of Calgary, Calgary, Alberta, Canada
[e] Department of Pathology and Laboratory Medicine, Cumming School of Medicine, University of Calgary and Calgary Laboratory Services, Calgary, Alberta, Canada

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ABSTRACT

Background: Human papillomavirus (HPV)-related oropharyngeal squamous cell carcinoma (OPSCC) is a growing problem that presents a significant challenge to Otolaryngologist-Head and Neck Surgeons. Knowledge of HPV status yields critical prognostic information, with potential for treatment selection based on tumour HPV status. The current gold standard of diagnosis, PCR, is expensive, demanding and time consuming. Alternatives such as p16 immunohistochemistry are subjective and potentially inaccurate. Loop-mediated isothermal amplification (LAMP) is a rapid, robust and inexpensive molecular diagnostic technique.
Objectives: Our aim was to verify LAMP as a potential bedside diagnostic assay for subtyping of HPV in OPSCC.
Study design: DNA from 72 formalin-fixed paraffin embedded (FFPE) OPSCC patient samples was tested. PCR and LAMP were then performed to specifically identify HPV 16, 18, 31, 33 and 35.
Results and conclusions: For these high-risk subtypes, LAMP had an overall sensitivity of 99.4% and specificity of 93.2% relative to PCR. LAMP is a promising technology that can accurately diagnose high-risk HPV infection.

1. Background

Over the past two decades, the incidence of oropharyngeal squamous cell carcinoma (OPSCC) continues to rise despite a decline in traditional risk factors, including tobacco and alcohol use [1,2]. Epidemiologic studies implicate human papillomavirus (HPV) infection as a strong risk factor and the likely etiologic agent [1–3]. HPV subtypes 16, 18, 31, 33 and 35 have been identified as high-risk for OPSCC, and are associated with more than half of all cases of OPSCC in North America [1,2]. Of these, subtype 16 is the most common, accounting for nearly 90% of all HPV-positive OSPCCs [4]. HPV positive malignancies appear to have a distinct set of clinicopathologic features and are increasingly viewed as separate biologic entities from HPV negative OPSCCs [5]. Multiple clinical trials have shown that HPV positive OPSCC portends a better prognosis than HPV negative cancer of comparable stage, with improved overall and disease specific-survival [6,7]. Several investigators have suggested that current chemoradiotherapy (CRT) regimens for HPV-positive OPSCC may lead to overtreatment, resulting in unnecessary toxicity and reduced quality of life outcomes [8,9]. Randomized controlled clinical trials evaluating the efficacy of de-intensified treatment regimens for HPV-positive patients are on-going [10]. As the body of evidence supporting alternative treatment protocols continues to grow, therapeutic de-escalation in specific HPV-positive patient cohorts may provide improved quality of life without compromising oncologic outcomes [10,11]. HPV status of a lesion for a patient with OPSCC therefore has both prognostic and potential therapeutic implications [12].

* Corresponding author at: HRIC 2A02, 3280 Hospital Dr. NW, Calgary, Canada, T2N 4Z6. Fax: +1 403 270 3145.
E-mail address: devonlivingstone@gmail.com (D.M. Livingstone).

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Identification of HPV infection in oropharyngeal biopsy specimens is currently accomplished by two different molecular diagnostic techniques: polymerase chain reaction (PCR) and immunohistochemistry (IHC) staining for p16 [13]. PCR, the current gold standard for HPV DNA detection, is highly specific and sensitive, but is costly, time consuming and requires extensive laboratory infrastructure [14]. p16 is an endogenous tumour suppressor protein that is upregulated in response to functional inactivation of Retinoblastoma (Rb) by the viral oncoprotein E7 [15]. IHC staining using *in situ* hybridization for p16 is thus used as a surrogate marker for HPV infection. It is inexpensive by comparison, but lacks the sensitivity and specificity of PCR [5,16]. In addition, p16 staining is subjective, requiring a pathologist to make a determination about what constitutes a positive stain [16–18]. Due to these limitations, p16 staining is often regarded as a suboptimal surrogate for the detection of HPV in the oropharynx [13–15]. Overall there is a need for a sensitive, specific, rapid, and cost-effective assay to determine HPV status in OPSCC.

Loop-mediated isothermal amplification (LAMP) is a single test tube gene amplification method that generates large amounts of target DNA in less than an hour without a thermocycler [19,20]. LAMP utilizes four primers that anneal to six regions of target DNA with high specificity. A positive LAMP reaction can be visualized directly due to magnesium pyrophosphate precipitation, negating the need for costly and time-consuming gel electrophoresis and spectrophotometry. The simplicity of LAMP has made it economical, costing less than 1/100th of a typical PCR reaction [21]. Additionally, *Bst* polymerase, the enzyme that drives the LAMP reaction, is unaffected by the multiple PCR inhibitors commonly found in blood and saliva, obviating the need for extensive sample purification steps [21,22]. LAMP based protocols have been developed to detect other pathogens including malaria and tuberculosis, with sensitivities and specificities equivalent to PCR [22,23]. LAMP has also been used by investigators to detect high-risk HPV subtypes for cervical carcinomas, genital lesions, and anterior tongue carcinomas, but has not yet been utilized for OPSCC [24–26].

2. Objectives

Our aim was to compare LAMP to PCR and validate it as a rapid, low-cost and accurate test for the detection and subtyping of HPV in clinical OPSCC specimens.

3. Study design

3.1. OPSCC specimen deparaffinization and purification

A CONSORT diagram (Fig. 1) illustrates our inclusion and exclusion criteria, yielding a final study cohort of 72 patient samples obtained from the Ohlson Research Initiative (ORI) tumour bank. The ORI tumour bank is a collection of surgically excised patient tumours that have been stored, with appropriate patient consent, for the purposes of medical research. This study was reviewed and approved by the Conjoint Health Research Ethics Board at the University of Calgary.

Slides from cases of p16-positive, oropharyngeal squamous carcinoma were evaluated by a surgical pathologist (DJD) and 1.5 mm cores of the paraffin block from foci of invasive carcinoma were extracted and used for the PCR analysis. New core punches and dermatome blades were used for each sample to prevent cross-contamination of HPV DNA between samples. DNA extraction and purification was accomplished using the QiaAMP™ DNA Mini Kit (Qiagen) per the manufacturer's protocol with the following modifications to improve DNA yield; (1) Block cores were deparaffinized using two 1 mL xylene washes (5 min at 54 °C then 5 min at 22 °C),

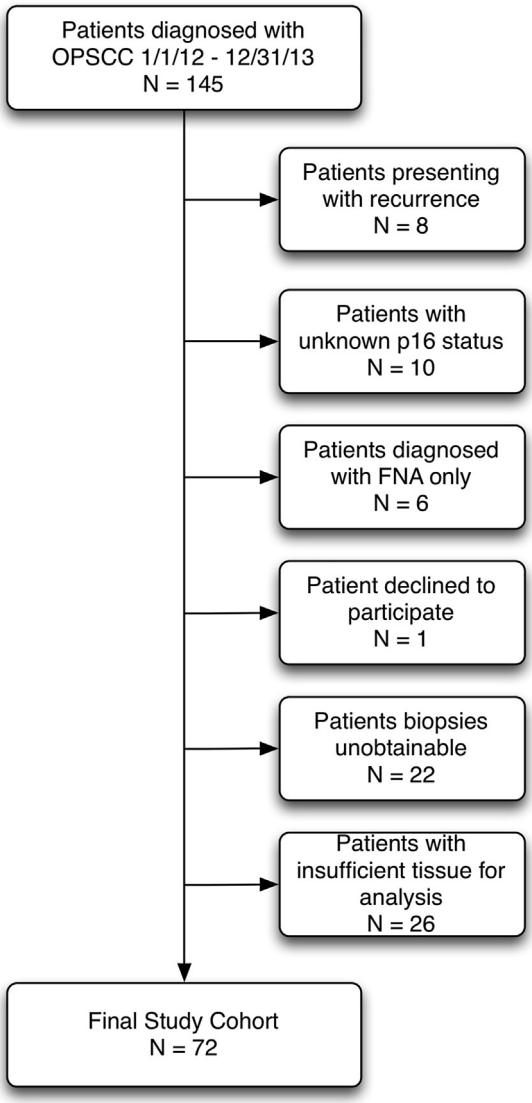

Fig. 1. CONSORT chart of FFPE samples obtained from ORI Tumour Bank.

followed by two 100% ethanol washes, (2) Samples were then suspended in 160 μl of ATL buffer (Qiagen) and 20 μL proteinase K (Qiagen), (3) Samples were then incubated overnight at 55 °C with shaking, centrifuged and treated with 20 μL proteinase K for 30 min. Purification then proceeded according to the manufacturer's protocol. DNA concentration was established using a NanoDrop™ Spectrophotometer (Thermoscientific).

4. PCR of OPSCC specimens

The Molecular Pathology Laboratory (Calgary Laboratory Services (CLS), Calgary, Alberta, Canada) analyzed all paraffin specimens using a standardized PCR protocol [27]. PCR products were then electrophoresed on a 1.5% polyacrylamide gel and compared to known positive control amplicons.

5. Primers for LAMP

Previously established type specific LAMP primers for HPV 16 [28] along with 18, 31, 33, and 35 primers designed by our group were utilized [29]. Each set of primers consisted of two outer primers (F3, B3), two inner primers (FIP, BIP) connected by a TTTT linker, and two loop primers (LF, LB) to improve the amplification