Case Report: Co-Infection of Streptococcus Dysgalactiae Subspecies Equisimilis and HPV11 in Laryngeal Papilloma

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Abstract

We report microbial and immune cell findings from a 59-year-old male with a history of recurrent HPV11-positive laryngeal papilloma (LP) for 48 years. During the years 2019 and 2020, surgery for LP was performed at frequency of 4.5 per year (9 times in 2 years). In March 2021 a swab sample was obtained from LP during surgery, and Streptococcus dysgalactiae subspecies equisimilis (SDSE, EMM type stG840) was isolated. The identical SDSE strain (stG840) and Hemophilus parahaemolyticus were detected in LP during the next surgery in August 2021. After both occasions (March and August 2021), antibiotic treatment for 10 days was prescribed, and the patient had two surgeries per year (every 6 months). Furthermore, Gram-positive cocci, likely SDSE, were scattered throughout superficial layers of a formalin-fixed-paraffin-embedded LP-sample from January 2021. At surgery in February 2022, Streptococcus agalactiae (GBS) and Staphylococcus aureus were identified in a swab from the LP. Fresh biopsies were further analyzed over two years, where increased myeloid cells, neutrophils, and reduced cytotoxic T cells were observed by flow cytometry. Staining of LP tissue revealed cytotoxic T cells in the periphery and within vascular regions of the LP. An average of 47 (range 22-74) HPV11 DNA copies/cell among nine LP-samples was observed, whereas a paired healthy larynx tissue sample demonstrated 0.0013 HPV11 DNA copies/cell as well as an increased proportion of cytotoxic T cells. In conclusion, the human pathogen SDSE was present in a case with a long history of LP. Antibiotic treatment coincided with fewer surgeries per year. Neutrophils were present in LP tissue, and cytotoxic T cells appeared to have limited infiltration capacity. HPV11 levels were stable during the patient’s clinical symptoms. Further research is needed to investigate a possible role of concomitant bacterial species in LP and if appropriate antibiotic treatment could reduce frequency of surgeries.
Introduction

Laryngeal papillomatosis (LP) occurs in the larynx and sometimes extends to the nasopharynx and lungs, and when it recurs it is known as recurrent respiratory papillomatosis (RRP) [1]. Papilloma can occur in childhood (juvenile-onset RRP) as well as in adults (adult-onset RRP) and may lead to difficulty in breathing, hoarseness, and obstruction of the respiratory tract [2].

Management usually involves surgical interventions. Some adjuvant therapies, such as cidofovir, interferon, proton pump inhibitors, and therapeutic and prophylactic human papillomavirus 9-valent vaccines may, in some patients increase the time between surgeries [3].

LP is associated with low-risk human papillomavirus (HPV), especially types 6 and 11. HPV11 is the more aggressive of the two types and is usually associated with younger patients at the onset and a more detrimental course [4, 5]. There is also evidence that HPV is transmitted vertically from mother to firstborn and through oro-genital contact [6]. Despite the presence of HPV DNA in the larynx of 5% of the healthy population, only a small proportion develops RRP [7].

Interestingly, the exclusion of CD8 T cells may play a role in persistence of HPV in the cervix [8, 9]. Also, the presence of vaginal Peptostreptococcus anaerobius has been linked to cervical cancer progression by inducing macrophage polarization [10]. Some studies have investigated immune cells within the RRP [10-13]. The RRP lesions have been shown to have a suppressive microenvironment for T helper 1-like responses, thereby allowing for recurrent lesions [10]. An infiltration of T regulatory cells (CD4+/CD25+/Foxp3+/CD127+low) have also been observed [11]. Additionally, the severity of RRP has been linked with the presence of CD83+ dendritic cells in the epithelium [12] and an increased amount of neutrophils [13]. Although knowledge of the role of the microbiome among HPV-driven cancers is emerging [14-16], no such studies have been reported with context to laryngeal papilloma. Here we present a case with co-infection of HPV11 and pathogenic bacteria in recurrent LP, whose reduced frequency of surgery coincided with antibiotic intervention.
Figure 1: **Time axis.** (A) Bar graph showing the last 58 surgery dates and time to subsequent surgery (in days) over the years. (B) Depicts points of analysis and antibiotic treatments conducted since March 2020, as described in the case report.
Case Presentation

A 59-year-old male with recurrent LP has been treated since 1974 at the Department of Ear, Nose, and Throat Disease, Head and Neck Surgery, Region of Skåne, Sweden.

The patient has undergone 135 anesthetic surgeries for HPV11-positive LP. On average, the patient returns to the clinic three to four times per year for surgical removal of the lesions, including COVID-19 pandemic in 2020 (Figure 1A). The patient had nonavalent HPV-vaccine (Gardasil) in February, April, and August of 2019.

Bacterial findings

Screening for bacterial species was conducted by collection of swabs (ESwab™, Red Cap, and COPAN Cat No 482CE Copan Diagnostic Inc) from the papilloma and clinically healthy laryngeal mucosa in close vicinity to the papilloma, in March and August 2021 as well as February 2022 (Figure 1B). Samples were incubated and analyzed according to the standard protocol for lower airway bacterial diagnostics at Clinical Microbiology, Lund, Sweden. On both occasions in 2021, Streptococcus dysgalactiae subspecies equisimilis (SDSE) (EMM type stG480, Beta-streptococcus G) was isolated from the LP. No growth of any bacteria was noted from healthy larynx in March, whereas the SDSE was also isolated from healthy larynx as well as from a throat swab from August 2021. In addition, Hemophilus haemolyticus and Hemophilus parahaemolyticus were isolated from the LPs in March and August 2021, respectively. Furthermore, Gram-positive cocci, likely SDSE, were found to be scattered throughout superficial layers of a formalin-fixed-paraffin-embedded (FFPE) LP sample from January 2021 (Figure 2). Thus, the cultivated SDSE strains from the two first occasions were identical whereas at the last visit in February 2022, when the patient had COVID-19, we isolated Streptococcus agalactiae (GBS) and Staphylococcus aureus from the LP. There was only growth of normal bacterial flora at the healthy laryngeal site and throat.

Figure 2: Gram staining on the pathological glottis specimen from January 2021. (A) Original magnification 600x. (B) Original magnification 1000x. The black arrows point towards Gram-positive coccoid bacteria.
Due to the presence of pathogenic bacteria, antibiotics were then administered to the patient. Since the first single antibiotic medication Amoxicillin/Clavulanic acid (Bioclavid) 875 mg/125 mg x 2 for 10 days in March 2021, the time between surgery procedures increased to six months. After the second antibiotic, Clindamycin (Dalacin) 300 mg x 2 for a 10-day dose that was prescribed due to its good penetration into most tissues, in August 2021, the patient reported less discomfort and did not require subsequent debridement for six months. Similarly, again after the last February 2022 visit, the patient was prescribed the same antibiotic: Clindamycin 300 mg x 2 for 10 days. The isolated SDSE- and GBS-strains were all susceptible, by disk-diffusion method, to the prescribed antibiotics. Due to logistic reasons, the microbial swabs were only taken before the surgical procedures and not after completion of antibiotics treatment.

**Immune cell characterization and HPV11-quantification**

From March 2020 to February 2022, the patient’s fresh tissue samples were analyzed by flow cytometry with parallel HPV11-DNA-quantification at five separate time points: March 2020, October 2020, January 2021, March 2021, and February 2022, as described previously [17] (Figure 1B).

In March and October 2020, the patient had routine debridement of lesions, and due to the extensive distribution of the lesions in the larynx, only papilloma from the posterior supraglottic wall and the laryngeal surface of epiglottis were sampled. Different laryngeal sampling sites were included in January and March 2021 to observe any anatomical variations of immune cell distribution and HPV11-copy numbers. In October 2020 and March 2021, the patient complained of “ill-health” and extensive obstructive lesions were observed in the respiratory tract. Clinically healthy laryngeal control tissue was only obtained in March 2021. Fresh biopsies were obtained again in February 2022 but were limited to immune cell distribution analysis only (remaining samples were dismissed due to the patient testing positive for COVID-19).

The immune cells distribution was assessed using flow cytometry (Figure 3A). Overall, in the LP, a relative decrease and increase of the cytotoxic T cells (CD8+ T cells) and of neutrophils were observed, respectively. This held stronger on instances when the patient was not feeling well i.e., in October 2020 and March 2021. In October 2020, 39% of neutrophils and 4% of CD8+ T cells of CD45+immune cells were observed. A similar trend was observed in a supraglottic sample from March 2021, 73% and 2% of neutrophils and CD8+ T cells of CD45+immune cells, respectively (Figure 3B and 3C). FFPE-biopsies were also prepared and observed microscopically for neutrophils and cytotoxic T cells (Figure 4A). Haematoxylin and eosin staining on formalin-fixed paraffin-embedded tissue from
Figure 3: Flow cytometric analysis at different time points. (A) Representative gating strategy for flow cytometric analysis. After single-cell gating to exclude doublets, viable immune cells (CD45+ cells) were further gated for T cells (CD3+), B cells (CD19+CD20+) and non-T/B cells (CD3-CD19-CD20-). Myeloid cells were further categorized into DCs (HLA-DR+ CD14-), macrophages (HLA-DR+ CD14+), and HLA-DRlow CD14int cells. Neutrophils were gated as HLA-DRlow CD14int CD15+ cells. (B) Heat map showing relative percentages of immune cells recorded during each visit. (C) Line graph showing relative percentages of cytotoxic (CD8+) T cells, neutrophils and HPV11 counts over the visits. The left axis depicts the percentage of cytotoxic T cells and neutrophils from CD45+ immune cells. The right axis represents HPV 11 E7 DNA copies over mean cell number per PCR run.
January 2021 did show neutrophils infiltrating the epithelium of the papilloma. (Figure 4A and B). Furthermore, multiplex staining of supraglottic LP-biopsy from March 2021 revealed that neutrophils comprised the majority of the invading immune cells within the epithelium as well as in the core of the papilloma (Figure 4C), whereas few CD8+ T cells were detected in the periphery and within the papilloma core (Figure 4D).

**Figure 4**: Histological staining with various morphological markers in formalin-fixed paraffin embedded LP biopsies. (A and B) Hematoxylin and eosin staining from January 2021 (12/01/2021) for papilloma as seen in 10x and 20x magnification, respectively. Arrows pointing at neutrophils. (C and D) Multi-channel staining on sections from March 2021 (16/03/2021) showing supraglottic LP site (scale of 2mm) for immune cells and specifically CD8+ T cells, in four channels: DNA (FITC; Cyan) PanCk (Alexa 532/Cy3; Purple), CD45 (Alexa 594/Texas Red; Yellow), and NPE and CD8 (Alexa 647/Cy5; Red). In (C) red color depicts neutrophils, whereas (D) shows CD8+ T cells in red. The white arrow points towards the neutrophils and CD8+ T cells in (C) and (D), respectively.
HPV11-quantity PCR-assay was performed, with primers HPV11 E7 634F: 5’TGAGGGTGGGACAGGTGGACAA, HPV11 E7 759R: 5’TGATGCTCCGTCTGCACTC, and HPV11 E7 Probe 677-700 FAM- CAACATTACAAATACGACTGTCMBNQF, with parameters as previously described [18]. HPV11 copies per cell were stable during the patient’s clinical exhibition (Figure 3C). Strikingly, the clinically healthy laryngeal control tissue taken at visit in March 2021, demonstrated tiny amount of HPV11 copies per cell (Figure 3C).

Discussion

In this case study, we isolated Streptococcus dysgalactiae subspecies equisimilis (EMM type stG840) in LP collected with six months intervals. Coccioid bacteria were also found to be scattered throughout superficial layers of LP from the patient. Interestingly, we also noted the reduced frequency of surgery that coincided with antibiotic interventions. Furthermore, staining of the LP revealed that neutrophils were frequently present and that few cytotoxic T cells were found. It was also observed that the HPV11 copy number is stable at different anatomical LP-sites and time points.

In order to understand the mechanisms behind our earlier observation of increased proportions of neutrophils in LP-biopsies [19], we searched for viable bacterial species on separate occasions from distinct laryngeal areas in a patient with a long history of LP. Interestingly, we identified SDSE, which is a beta-haemolytic group G streptococci, which causes a broad range of diseases [20]. The SDSE EMM type stG840 has been associated with the presence of virulence factors [20], which may play a role for the development of LP in our current case. However, Streptococcus dysgalactiae and Hemophilus haemolyticus detected in our patient has been associated with acute pharyngitis [21, 22]. It is plausible that the patient experienced co-incident pharyngitis, which exacerbated the severity of LP symptoms such as difficulty breathing and inflammation. In addition, it is reported that some Streptococcus and Proteobacteria genera in the larynx are commensal [23, 24]. Nonetheless, the existence of the identical pathogenic bacterium over six months (March to August 2021) was noteworthy. The first course of antibiotics given coincided with a prolonged period of six months until the next surgery, but also that the patient qualitatively reported less discomfort after surgery. When the same strain was diagnosed in August 2021, the patient was administered a second antibiotic cure, which extended his surgical visit by another 6 months. However, in February 2022 Streptococcus dysgalactiae was replaced by Streptococcus agalactiae (GBS) and Staphylococcus aureus. Staphylococcus aureus is a human commensal and also a frequent cause of clinically severe infections [25]. However, whether it plays a role in the development of LP remains elusive. To the best of our knowledge, no findings of GBS in the larynx have been reported and its presence remains to be further studied.

Overall, to the best of our knowledge, this is the first report of streptococcus species isolated from LPs. Further studies from LP-patients are required to investigate the significance of streptococcus species, and of other bacterial species, among the LPs. Previously, few studies have discussed T cell variations within LP [10, 11]. By immunohistochemistry (IHC), squamous epithelium in papilloma has been demonstrated to contain a significantly greater number of CD4+ and CD8+ T cells, as compared to normal tissue [26]. Moreover, El Achkar et al. and ourselves independently observed an inverse relation of neutrophils and cytotoxic T cells in the LP compared to that of healthy laryngeal tissue [13, 17]. El Achkar et al. also noted a link between severe disease with increased neutrophils and lowered CD8+ T cells [13]. In our current case report, a similar observation was made at the time point when the patient felt unwell. Using flow cytometry, neutrophils were found 9.75 and 29.2 times more frequently than CD8+ cytotoxic T cells in the laryngeal side of an epiglottis sample from October 2020 and a supraglottic sample from March 2021, respectively.

Among HPV+/- head and neck malignancies, a greater neutrophil to lymphocyte ratio (NLR) is a poor prognostic sign, linked with fewer disease-free periods and higher recurrence, irrespective of other confounding factors (e.g., HPV status, age, sex) [27-29]. An analogous association was observed in both malignant and benign tumours in the larynx [30, 31]. The precise role of neutrophils in cancer and pre-malignant lesions is yet unknown. It may be argued that neutrophils are the primary line of defence against bacterial infections and inflammation [32], and their increase in the inflammatory lesions could be expected.

HPV6 and HPV11 are strongly linked to LP [33]. Despite perceived complaints from our patient of periods of “ill-health”, no indications of a higher HPV11 copy number were observed at these occasions. Our HPV11 quantity findings are similar to those of HPV6 from LP patients, with a range of one log10 per cell in LP and significantly lower in healthy laryngeal tissue [18].

Conclusion

In the current case report, we conducted a two-year thorough follow-up of a chronic HPV11-positive LP patient. Noteworthy, the human pathogen Streptococcus dysgalactiae subspecies equisimilis were isolated six months apart in two consecutive LP-swabs and antibiotic treatments coincided with lower frequency of surgery. In addition, the presence of high load of neutrophils was evident in histological staining of the LP-tissue that contained Streptococcus dysgalactiae subspecies equisimilis. However, it remains to be established whether the bacterial infection was due to an already debilitated immune status within the larynx or an independent co-
infection that transpired. In addition, HPV11 copy numbers were relatively stable at clinically appearing LP at different anatomical laryngeal sites. This case report shows observations that may be useful to pave the way for studies exploring microbial flora and immune cells infiltration of LP.

**Data availability:** Raw data files from the experiment will be available from the corresponding author on direct request.

**Ethics statement:** Informed consent was obtained from the patient, and ethical permission was granted by the Ethical Committee of Lund University (2013/562, amendments 2016/155 and 2020-01420). Ethical permission for cultivation of bacteria was granted by the Ethics Review Authority (Dnr 2021-04941)

**Author contribution:** AS, ML, OF and RR designed the case report. RR recruited the patient and collected samples. OF administered HPV-quantification and bacterial diagnostics. AS performed flow cytometry and multiplex immunofluorescence. FA assisted with evaluation of histological slides. AS wrote the first draft of the manuscript and ML, OF and RR revised manuscript. All authors have read and approved the final version of the manuscript.

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**References**


