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Original Article

An evaluation of an array of viruses and fungi in adult Lebanese patients presenting with various dental infections: A cross-sectional study

Wael Khalil[1], Hani Abd-Ul-Salam[2,3,4], Abd El Hadi Kanj[5], Rémi Safi[6], Mohammad El-Harakeh[6], Mazen Kurban[7], Elias A Rahal[1], Ghassan M Matar[1]

[1] Department of Experimental Pathology, Immunology, and Microbiology, Center for Infectious Diseases Research, WHO Collaborating Center for Reference and Research on Bacterial Pathogens, Faculty of Medicine, American University of Beirut, Beirut, Lebanon
[2] Faculty of Dental Medicine and Oral Health Sciences, McGill University, Montreal, Canada
[3] Faculty of Dentistry, University of Toronto, Toronto, Canada
[4] College of Dentistry, Gulf Medical University, Ajman, United Arab Emirates
[5] Department of Orthodontics, College of Dentistry, Al-Ayen University, Thi-Qar, Iraq
[6] Department of Dermatology, American University of Beirut Medical Center, Beirut, Lebanon
[7] Department of Anatomy, Cell Biology, and Physiological Sciences, American University of Beirut, Beirut, Lebanon

Abstract

Introduction: The role of bacteria in the pathogenesis of periodontitis, pericoronitis, and periapical infections has been well-established. However, the variation in the severity and prognosis of these lesions could suggest a potential role of other microorganisms, such as viruses and fungi. This study aims to evaluate the presence of adenovirus, human papillomavirus-16, Epstein-Barr virus, *Candida,* and non-*Candida* fungi in these infections.

Methodology: A cross-sectional study including 120 healthy adult patients presenting with dental infections requiring dental extractions were conducted to assess the prevalence and the relative quantity of viruses and fungi in saliva, infected, and healthy tissues using quantitative polymerase chain reaction tests. Samples were collected, and a categorical scale was used for the prevalence and a continuous scale for the relative quantification. Statistical analyses were performed using Chi-square for the prevalence and Wilcoxon rank test for the relative quantification.

Results: Except for the Epstein-Barr virus and *Candida*, the presence of viruses and fungi was significantly associated with dental infections. Adenovirus showed an association with pericoronitis, while human papilloma virus-16 exhibited an association with periapical infections. Non-*Candida* fungi, on the other hand, showed a positive association with all infected tissues and saliva as compared to healthy control lesions except for periapical infections.

Conclusions: According to this study, viruses and fungi were found to be prevalent in dental infections. However, their associations with those infections vary depending on the types of viruses or fungi involved and the category of dental infections.

Key words: Periodontitis; pericoronitis; PCR; microbiology; virus; fungi; peri-apical infection.

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Introduction

Microorganisms, a component of the human body normal microflora, cover various surfaces such as skin, respiratory, urogenital, and gastrointestinal tracts, including the oral cavity. The oral microbiota plays an important role in preserving human health by maintaining homeostasis and preventing local disease. However, oral or systemic diseases may emerge in the event of an imbalance in the microbial flora [1].

It is well-established that bacteria are the primary culprit in all dental infections, including aggressive and chronic periodontitis (AP, CP), periapical infections (PI), and pericoronitis (PC). However, other organisms that could play a role in the pathogenesis of these dental infections, such as viruses and fungi, were overlooked. A variety of viruses could be implicated in oral infections including human papillomavirus (HPV), Epstein-Barr virus (EBV), and human cytomegalovirus [2]. Similarly, fungal involvement in oral pathologies has been reported. Several studies recognized the *Candida* species as the most frequently involved organism in oral fungal infections, followed by *Aspergillus* and *Cryptococcus* [3,4]. *C. Albicans* is usually found in the oral cavity among the normal flora in the form of commensal blastospores. Yet, under certain conditions like immunodepression, HIV

infection, chemotherapy, etc., *C. albican* might become pathogenic and these blastospores may transform into the pathogenic filamentous form [5,6]. Through the literature studies exploring viruses and fungi in various dental infections are scarce and their results are controversial [7].

This cross-sectional study aims to examine the prevalence and relative quantification (RQ) of adenovirus (ADV), EBV, HPV-16, *Candida,* and non-*Candida* fungi in saliva and infected tissues pertaining to those infections as compared to healthy tissues.

Methodology

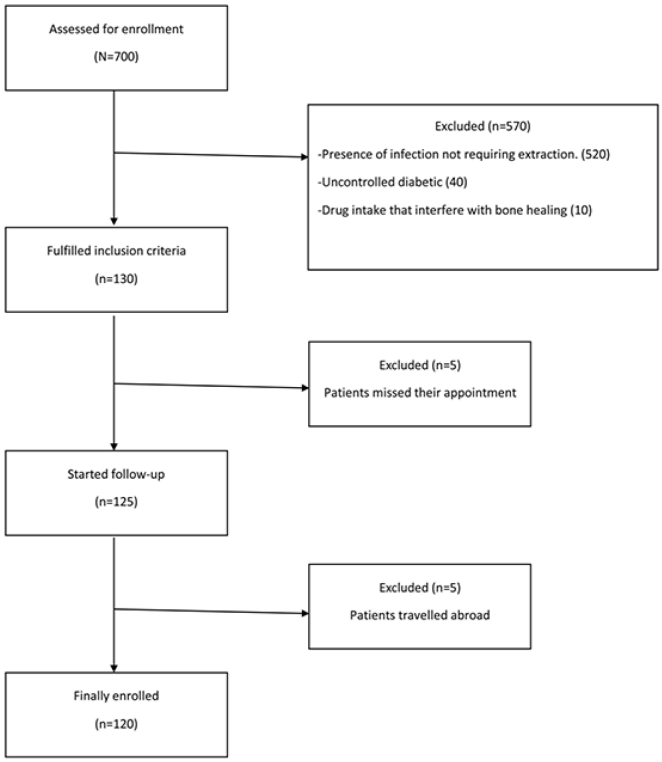
Study Design

This study was designed as a cross-sectional study that included 120 adult healthy patients, presenting to a private clinic in Beirut, Lebanon, and conducted between January 2020 and January 2022. Participants were recruited via a non-probability convenient sampling technique. The tissue or saliva specimens were collected by one clinician and all samples were evaluated by one examiner who was blinded to the disease conditions of the patients.

Ethics Statement

The study was designed and conducted based on the Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) and a flow diagram prepared [8]. The study was performed according to the most recent Declaration of Helsinki guidelines for clinical trials involving human subjects. Ethical approval was obtained from the Institutional Review Board at the American University of Beirut, Beirut, Lebanon (Protocol number: BIO-2019-0511). Patients fulfilling the inclusion criteria were recruited in the study and a consent form was explained verbally in detail including information on the scope and benefits of the study as well as the associated risks. The form was signed by all participants in the study.

Figure 1. Study Design and STROBE Flowchart.



Inclusion Criteria

Adult healthy patients, ASA I, presenting with dental infections that included AP, CP, PI, or PC infections requiring dental extractions.

Exclusion Criteria

Exclusion criteria included children and medically compromised patients (ASA II and III), including non-consenting patients with a history of cognitive dysfunction, pregnant, or lactating women, as well as patients undergoing chemotherapy or radiotherapy.

Sample size determination

The sample size was determined based on the primary outcome, prevalence, with a 95% confidence interval assuming an alpha of 5%, an estimated prevalence of 50%, and a margin of error of 10%.

Study arms

Study Arm I: 40 patients undergoing dental extractions distributed into two groups and differentiated based on the presence or absence of AP, CP, PI, or PC. In the absence of a dental infection, teeth were extracted for orthodontic or prosthodontic reasons.

Study Arm II: 80 patients presenting with AP, CP, PI, or PC and requiring dental extractions.

The difference between study arms I and II is that in arm I, the patients preferred not to have any other specimen collected not related to the dental treatment.

Sample Collection

Study Arm I: 40 independent samples of AP, CP, PI, or PC infected tissues (group 1) or gingival healthy tissues (group 2) were collected.

Study Arm II: Paired samples of tissues harvested from AP, CP, PI, or PC infected tissues were collected, as well as control healthy tissues from the same patients.

All patients had saliva samples collected and analyzed for the presence of the studied viruses and fungi. Patients were provided a sterile container in which they spit. Infected and healthy tissues were collected from both the socket and/or the periodontal ligament of the extracted teeth. Sample tissues were then rinsed with a sterile saline solution and all samples were stored at -80 °C for future analysis.

Outcome measures

The primary outcome was the prevalence of ADV, EBV, HPV-16, *Candida,* and non-*Candida* fungi. The prevalence of those microorganisms in saliva, healthy and infected samples was calculated and reported as a ratio.

The secondary outcomes included the RQ of these microorganisms in saliva and the infected AP, CP, PI, and PC tissues, as compared to healthy tissues from the same patient.

Sampling Quantification of Viruses and Fungi

DNA extraction and isolation were performed using the QIAamp® DNA mini kit (Qiagen, Gaithersburg, USA), as per manufacturers' instructions, through three main steps: lysis, followed by DNA precipitation, and DNA elution. DNA purity and yield were evaluated using a nanodrop spectrophotometer (Denovix, Wilmington, USA) in addition to q-PCR analysis (Numelab, Beirut, Lebanon).

Quantitative Polymerase Chain Reaction Analyses (q-PCR)

The q-PCR technique was used to determine the presence of these microorganisms in saliva and tissue samples. The q-PCR technique was conducted using a SYBR green mix in a CFX96 system (Bio-Rad, city USA). Products were amplified using primers detailed in Table 1. PCR settings were set according to the following criteria: a pre-cycle at 95 °C for 3 minutes followed by 40 cycles each consisting of 95 °C (denaturation) for 15 seconds, the annealing temperature for 30 seconds, and 72 °C (elongation) for 30 seconds. Fluorescence threshold cycle value (Ct) was recorded for each microorganism. Positive PCR cases were further classified into four categories: strongly positive, positive, low positive, and negative, based on Ct values. The positive control was the DNA of the microorganism, and the negative control was water. If the Ct value was less than 20, the sample was considered strongly positive, whereas, if the Ct value was between 20 and 29, the sample was considered positive. If the Ct value was between 30 and 34, the sample was considered positive and negative samples had a Ct value greater than 35.

Relative quantification (RQ) of q-PCR

This analysis was conducted only on the 80 samples collected from saliva and infected tissues and compared to healthy control tissues. The quantity of microorganisms in saliva and infected tissues relative to healthy control tissues was designated 2^{Δ}, where Δ refers to the Ct value of the control healthy tissue subtracted from the Ct value of infected tissue.

Statistical Methods

IBM SPSS statistical software tool 26.0 (Armonk, New York, USA) was used for demographic and statistical analyses. Normality was assessed using Shapiro-Wilk test in addition to quantile-quantile plots. As the data were not normally distributed, non-parametric one-sample Wilcoxon signed rank test was used to test if significant differences existed in the RQ values for each tissue pair in each virus. The hypothesized median for the Wilcoxon test was set to 1, as this value indicated that there was no difference in the PCR values between the control and corresponding infected tissue. 2^{Δ} was considered a variable instead of the Ct value. A median value less than one indicated that the PCR value of C is less than its counterpart, while a median value greater than 1 indicated that the

Table 1. q-PCR primers and annealing temperatures of genes used to detect the DNA of the pathogens.

Primers	Sequence	Annealing T (°C)
Adenovirus	F: GCCACGGTGGGGTTTCTAAACTT R: GCCCCAGTGGTCTTACATGCACATC	58
Human papillomavirus	F: CCCAGCTGTAATCATGCATGGAGA R: GTGTGCCCATTAACAGGTCTTCCA	52
Epstein-Barr virus	F: GGAACCTGGTCATCCTTTGC R: ACGTGCATGGACCGGTTAAT	57
Fungi	F: CTCGTAGTTGAACCTTGG R: GCCTGCTTTGAACACTCT	52
Candida	F: CAACGGATCTCTTGGTTCTC R: CGGGTAGTCCTACCTGATTT R: CAACAGCCAGCTGCACACAG	56

control has a higher PCR value. Statistical significance was set at a *p* value less than or equal to 0.05.

Results

700 patients were screened of which 570 patients were excluded and 130 patients included. Among the 130 patients recruited, 120 were finally enrolled in the study. The tissue samples obtained were distributed among 26 CP, 35 PI, 22 PC, and 14 AP (Table 2) in addition to a collected saliva sample from each patient.

Prevalence of Viruses and Fungi

Among the ADV positive PCR cases, the prevalence of ADV was highest in PI tissues (42.86%) as compared to PC (37.82%), CP (30.77%), control (26.67%), and AP (7.14%) tissues. ADV was detected in 22.79% of the saliva samples and no strongly positive ADV samples were detected (Table 3).

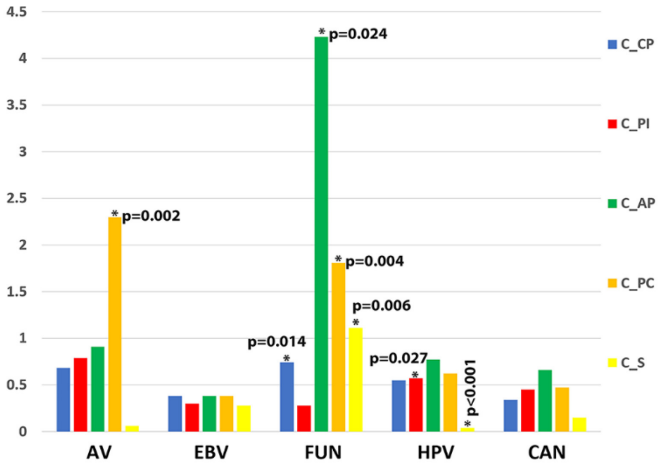
Among the HPV-16 positive PCR cases, the prevalence of HPV-16 was highest in PI (14.28%) tissues as compared to CP (11,54%), AP (7.14%), PC (4.55%), and control (4.00%) tissues. HPV-16 was detected in 1.26% of the saliva samples and no strongly positive cases of HPV-16 were detected in any of the samples (Table 3).

Among the EBV-positive PCR cases, the prevalence of EBV was highest in control healthy tissues (29.33%) as compared to AP (28.57%), PI (25.71%), CP (23.07%), and PC (13.63%) tissues. EBV was detected in 30.38% of the saliva samples and there were not strongly EBV-positive PCR cases in all tissue samples (Table 3).

Among the non-*Candida* fungi positive PCR cases, the prevalence was highest in PI (85,71%) tissues as compared to CP (83,41%), PC (81,81%), control (77.32%), and AP (71,42%) tissues. Positive non-*Candida* fungi were detected in 89.87% of the saliva cases. There were no strongly positive PCR for non-*Candida* fungi in all samples (Table 3).

Among the *Candida-positive* PCR cases, the prevalence of *Candida* was highest in PI (37.14%) as compared to control healthy tissues (34.67%), PC (27.26%), CP (23.07%) and AP (21.42%) tissues as well. *Candida* was detected in 31.65% of the saliva samples. None of the infected or healthy tissues as well as saliva samples were strongly positive for the presence of *Candida* (Table 3).

Figure 2. Comparison between the quantity of the studied microorganisms in infected teeth and saliva samples.



CP: chronic periodontitis, AP: aggressive periodontitis, PI: periapical infection, PC: pericoronitis, C: Healthy Control, AV: Adenovirus, EBV: Epstein-Barr virus, FUN: non-Candida fungi, HPV: Human Papillomavirus, CAN: Candida fungi.

Among all studied samples, the only significant difference was noted in HPV-16 in PI tissues (p = 0.032).

qPCR Relative Quantification Analysis of microorganisms

The RQ of ADV in PC tissues (2.3) was significant (p = 0.004) as compared to healthy controls, whereas RQ was not significant for CP (1.18, p = 0.103), AP (0.88, p = 0.507), 1.07 for PI (1.07, p = 0.109). The RQ of saliva samples was also not significant (0.47, p = 0.579).

PI was the only infection showing a significant association with HPV (p = 0.027) as compared to healthy controls, whereas, RQ was 1.07 for CP (p = 0.137), 0.73 for AP (p = 0.807) and 0.58 for PC (p = 0.952). The salivary RQ (0.09) was significant (p < 0.001) with a magnitude 10 times less than that of the healthy control.

The RQ of EBV was not significant in any of the samples including PI (0.91, p = 0.647), CP (0.56, p = 0.353), AP (0.36, p = 0.507), PC (0.36, p = 0.135)

Table 2. Distribution of infected tissues samples per age and gender.

Dental infection	Total	Males	Females	Age range
Pericoronitis	22	11 (9)	13 (11)	17-35
Chronic periodontitis	26	19 (16)	12 (10)	50-67
Aggressive periodontitis	14	8 (6)	6 (5)	16-28
Periapical infection	35	18 (14)	17 (11)	18-70

Values between brackets refers to paired samples.

tissues as well as saliva (0.48, $p = 0.852$) as compared to healthy controls.

The RQ of non-*Candida* fungi was significant in CP (1.96, $p = 0.001$), AP (5.87, $p = 0.048$) and PC (2.04, $p = 0.007$) when compared to healthy controls, as well as in saliva (1.25, $p = 0.006$). In contrast, the RQ of PI tissues was not significant (0.43, $p = 0.778$).

The RQ of *Candida* in all infected tissues was not significant. This includes CP (0.46, $p = 0.6$), PI (0.51, $p = 0.545$), AP (0.62, $p = 0.507$), and PC (0.45, $p = 0.765$) tissues as compared to healthy controls. Similarly, saliva RQ (0.88) was not significant ($p = 0.062$).

Discussion

The aim of this study was to detect the prevalence of viruses and fungi, and the subsequent RQ of viral and fungal loads in saliva and tissues of three common dental infections: PC, AP, CP, and PA infections as compared to healthy tissues from each participant. The quantification was performed using a PCR technique for categorical classification of the levels of positivity of viruses and fungi in tissues as well as relatively quantifying them using q-PCR. To our knowledge, there were no previous studies that relatively quantified the viral or fungal load in those dental infections.

Although the results of this study suggested that there was no significant difference in the prevalence of ADV in all samples, it is worth noting that the high prevalence seen in PI was in the low positive category, which clinically bordered a negative result. In contrast, there was a significant association in ADV relative quantitative viral loads between PC and healthy tissues. This could be plausibly attributed to the fact that ADV has a high affinity to epithelial cells [9]. ADV will eventually adhere and attach to the epithelial cells of the peri-coronal sac which will allow its entry and replication in these cells [10]. The discrepancy noted between the RQ and prevalence is related to the fact that the former was a continuous variable, whereas the latter was categorical.

Since this study is the first study to examine the presence of ADV in dental infections, further exploration is needed to examine whether the ADV was present in high concentration in peri-coronal sacs prior

Table 3. The prevalence of strongly positive, positive, and negative cases of different pathogens among the control healthy tissues, infection lesions, and saliva samples.

Pathogen		Prevalence (%)				
		Strongly Positive	Low positive	Positive	Total Positive	Negative
ADV	C	0.00%	14.67%	12%	26.67%	73.3%
	CP	0.00%	11.54%	19.23%	30.77%	69.23%
	PI	0.00%	42.86%	0.00%	42.86%	57.14%
	AP	0.00%	0.00%	7.14%	7.14%	92.86%
	PC	0.00%	0.00%	37.82%	37.82%	62.18%
	S	0.00%	10.13%	12.66%	22.79%	77.22%
EBV	C	0.00%	28.00%	1.33%	29.33%	70.67%
	CP	0.00%	23.07%	0.00%	23.07%	76.92%
	PI	0.00%	22.86%	2.86%	25.71%	74.28%
	AP	0.00%	28.57%	0.00%	28.57%	71.43%
	PC	0.00%	13.63%	0.00%	13.63%	86.36%
	S	0.00%	21.52%	8.86%	30.38%	69.62%
HPV-16	C	0.00%	4.00%	0.00%	4.00%	96.00%
	CP	0.00%	3.85%	7.69%	11.54%	88.46%
	PI	0.00%	8.57%	5.71%	14.28%	85.71%
	AP	0.00%	0.00%	7.14%	7.14%	92.86%
	PC	0.00%	4.55%	0.00%	4.55%	95.45%
	S	0.00%	1.26%	0.00%	1.26%	98.73%
Fungi	C	0.00%	38.66%	38.66%	77.32%	22.67%
	CP	0.00%	29.57%	53.84%	83.41%	15.38%
	PI	0.00%	60.00%	25.71%	85.71%	14.28%
	AP	0.00%	28.57%	42.85%	71.42%	28.57%
	PC	0.00%	22.72%	59.09%	81.81%	18.18%
	S	0.00%	48.10%	41.77%	89.87%	10.12%
Candida	C	0.00%	34.67%	0.00%	34.67%	65.33%
	CP	0.00%	15.38%	7.69%	23.07%	76.9%
	PI	0.00%	31.43%	5.71%	37.14%	62.85%
	AP	0.00%	14.28%	7.14%	21.42%	78.57%
	PC	0.00%	22.72%	4.54%	27.26%	72.72%
	S	0.00%	10.12%	21.54%	31.65%	68.35%

S: Saliva; CP: chronic periodontitis; AP: aggressive periodontitis; PI: periapical infection; PC: pericoronitis; C: Healthy Control.

to the onset of PC or whether the virus is a contributing factor to the pathogenesis of the disease.

Although the presence of HPV-16 has been explored in AP, CP, and PI, the literature is scarce particularly in PI studies [11,12]. To our knowledge, this is the first study to explore the association between this virus and PC and the results showed no significant association. Similarly, the findings of this study do not support an association between HPV-16 and AP or CP although previous studies remain controversial [13-15].

Among all viruses and fungi examined in this study, only HPV-16 showed significant differences in both prevalence and RQ, particularly in PI. It is worth mentioning that the quantity of HPV-16 in saliva was significantly lower than all other healthy and infected tissue samples. This could be attributed to the fact that HPV-16 is an intracellular micro-organism in contrast to fungi. That could explain its lower prevalence in saliva compared to tissues. The association between HPV-16 and PI reported in this study were in concordance with published studies [11,16].

Although the EBV, part of the human herpes virus's family, has been studied extensively in relation to dental infections, particularly in AP, CP, and PI, the results remain controversial [17-19]. The results of this study suggest that there is no significant association between EBV and all dental infections in both prevalence and RQ as compared to healthy controls. These findings are in concordance with some published studies [17,20,21] and do not support the findings of others [22-24]. This controversy could be attributed to many factors such as the ethnicity and sampling methods such as oral rinses, gingival biopsies, gingival crevicular fluid collection and sub-gingival plaques [25,26]. The presence of EBV in all samples is reflective of the virus's property of surviving in the host's body for a lifetime after the initial infection and its ability to be reactivated. A major difference between EBV and other viruses is that the former could be present in similar viral load in both tissues and saliva which could explain the way of transmission of this virus as in kissing disease [27].

The findings related to fungi lack alignment between prevalence and RQ, in which the prevalence of fungi was similar in all samples, whereas the RQ for fungi was significantly higher in PC, AP and CP, and saliva compared to healthy controls. Since fungi can naturally multiply in body fluids [28] as opposed to viruses which can only multiply in living cells [29], this can explain why fungi were found in relatively high amounts in the saliva. This could be explained by the fact that saliva is in communication with the periodontal pocket in case of AP and CP and the operculum in PC, unlike PI where the infection is in bone and not communicating directly with the oral cavity. This is further supported by the outcome that the majority of positive samples were in the low positive category. This in turn confirms that the categorical variables do not entirely corroborate the results of the RQ.

To our knowledge, this is the first study to report on the significant association between fungi and PC compared to previously published reports who studied the microbiological aspects of pericoronitis [30-33]. Fungi were also found to be associated with AP and CP in concordance with the published literature [7,34]. On the other hand, the majority of previous studies documented a positive association between fungi and PI [7,35,36], findings that are not supported by this data.

Furthermore, the prevalence and RQ analyses of *Candida* in this study were not significantly different between all samples. This could be explained by the fact that *Candida* is considered part of the normal oral flora. The results of this study are in alignment with some published studies [37,38] and in disconcordance with other studies [39,40]. The difference in outcomes could be related to the contamination of the specimens by normal oral flora, such as specimens collected from root canals in PI [35,36], and subgingival plaque or tongue swab in AP and CP [39].

Several factors could contribute to the variability in prevalence and the controversies between studies, such as demographic factors, systemic and oral health status, clinical tissue sampling and laboratory techniques.

Demographic and systemic oral health status factors included in this study such as age, gender, medical health status, smoking and oral hygiene were controlled by collecting infected and healthy tissue samples from the same patient [18,41]. Clinical tissue sampling including the location and size of the collected specimens, the harvesting technique, the appropriateness of the collected specimen for the intended examination could contribute to the variability in results.

In this study, we directly collected the specimens from infected tissues associated with extracted teeth and the control was healthy gingival tissues, whereas in other studies, aspiration of pus from peri-apical infections was collected via a needle, a paper point or file inserted in the root canal. In case of AP or CP, published studies collected subgingival plaque, gingival crevicular fluid or gingival biopsies [42-44]. Other investigators used tongue swabs or oral rinses to examine the microbial presence in dental infections. These techniques were indirect and therefore one could

argue that they were not specific enough for infected tissues as compared to the direct tissue sampling technique used. In addition, in many existing research models, authors either did not specify the HPV type examined, or did not have control healthy tissue samples for assessment; while other studies established their comparisons based on saliva samples rather than tissues [41,44-46].

Laboratory techniques used to assess microbial presence could either be qualitative such as microbial cultures and qualitative PCR, or quantitative such as immunofluorescence essays and qPCR. Qualitative tests are limited by utilizing categorical variables and are therefore limited to prevalence, but not the quantification of microorganisms. The advantage of qPCR compared to immunoassays is that the former could allow the normalization of extracted DNA and consequently could eliminate the variability due to the size of the collected specimen.

Based on these findings, the possible association of some viruses and fungi to some dental infections might have an impact on treatment strategy, especially in severe and resistant cases.

There are limitations to the current study including the lack of serotyping of ADV, EBV, fungi, and *Candida*. Moreover, the possible bacterio-viral or bacterio-fungal co-infection status and its association with dental infections was not examined. Furthermore, the correlation between all co-variates such as smoking, age, medical status, the severity of symptoms, etc., in each disease and the corresponding viral or fungal load are controlled using relative quantification analysis but their effect should be assessed on future prospects.

Conclusions

According to this study, viruses and fungi were found to be prevalent in dental infections. However, their associations with those infections vary depending on the types of viruses or fungi involved and the category of dental infections. The findings of this study could modify current treatment protocols of such diseases or lesions.

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Corresponding authors

Elias A. Rahal and Ghassan M. Matar
Department of Experimental Pathology, Immunology and Microbiology
American University of Beirut
P.O. Box 11-0236
Riad El-Solh, Beirut, 1107 2020
Lebanon
Email: er00@aub.edu.lb; gmatar@aub.edu.lb

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