Epstein–Barr Virus in Periodontitis

Subjects: Microbiology

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Periodontitis, an inflammatory condition that affects the structures surrounding the tooth eventually leading to tooth loss, is one of the two biggest threats to oral health.

Epstein–Barr virus | periodontitis | detection methods | EBER-ISH | PCR-based methods | immunohistochemistry | immunophenotyping

1. Introduction

Clinically periodontitis is defined as a chronic multifactorial inflammatory disease characterized by the progressive destruction of the tooth-supporting apparatus. The disease of periodontitis is portrayed by three factors: 1. the loss of periodontal-tissue support manifested through clinical attachment loss and radiographically assessed alveolar bone loss; 2. the presence of periodontal pockets (PP) and 3. gingival bleeding [1]. The periodontal disease initiates as gingivitis (inflammation of the gingiva), which is highly widespread and readily reversible by effective oral hygiene. When left untreated, it may gradually progress to early-to-moderate periodontitis and irreversible advanced periodontitis [2]. Periodontitis, along with dental caries, is considered one of the two biggest global oral health burdens [3]. Beyond oral health, growing evidence in the literature supports the direct and indirect impact of periodontitis on the overall health and development of extraoral pathologies. Periodontitis has been associated with seemingly unrelated systemic diseases such as diabetes, cardiovascular diseases and stroke, adverse pregnancy outcomes, respiratory diseases, dementia, Alzheimer's disease, rheumatoid arthritis and different types of cancers [4][5]. However, it remains to be further scrutinized how specific periodontal pathogens contribute to the development of systemic diseases. However, as a minimum, the detection of periodontal pathogens in the oral cavity may be used as an attractive tool for the diagnosis of non-oral inflammatory systemic diseases.

There is a long history of the search for etiological agents of periodontitis and different hypotheses of etiopathogenesis have been proposed. Periodontitis was thought to be (i) an infection caused by bacteria; (ii) a specific bacterial infection; (iii) a biofilm infection; (iv) a specific plaque; (v) result from dysbiosis; (vi) caused by complex interactions among bacteria–host–environmental factors and (vii) a viral-bacterial infection (reviewed in [5][6]). One specific recent hypothesis of interest is based on the herpesvirus–pathogenic bacteria–host response axis in which herpesviral–bacterial interactions assume a major etiopathogenic role [5][7][8][9]. This infectious disease model for periodontitis development proposes that bacteria initiate the gingival inflammation triggering further influx and propagation of herpesviruses. Next, a herpesvirus active infection in the periodontium hinders the local immune defenses, thereby permitting the overgrowth of periodontopathic bacteria. In a two-way interaction, the

virulence factors of periodontopathic bacteria reactivate latent herpesviruses and augment the infection. Reactivated periodontopathic herpesviruses and bacteria also modulate host immune reactions and provoke tissue destruction as a result of immunopathologic responses leading to the progression of the disease. In particular, among herpesviruses, human cytomegalovirus (HCMV) and Epstein–Barr virus (EBV) have been closely associated with severe types of periodontitis [10]. The main focus of the discussion in this review is EBV.

EBV belongs to the family of human gamma herpesviruses (systematic name human herpesvirus 4—HHV-4) and is one of the most ubiquitous and successfully adapted human pathogens that are found in approximately 95% of the total human population. EBV can infect a wide variety of cells and tissues, mostly B cells, nasopharynx and oropharynx squamous epithelial cells (ECs), thyroid glandular ECs, salivary and stomach glands and, occasionally, T cells, smooth muscle cells and follicular dendritic cells [11]. EBV has been associated with an extended list of diseases, from transient benign infections to aggressive malignancies. EBV is best known as the causative agent of infectious mononucleosis and has been implicated in several oral pathologies, such as oral hairy leukoplakia (OHL), oral lichen planus, Sjogren's syndrome and periodontitis [9]. It is a known carcinogen implicated in the etiology of several malignancies of both lymphoid and epithelial origin [12]. Infection of B cells with EBV has been linked to Burkitt's lymphoma, Hodgkin lymphoma and post-transplant lymphoproliferative disorders; infection of ECs is implicated in nasopharyngeal cancer, gastric cancer and breast cancer. Furthermore, a recent study suggested the association of EBV with seven different autoimmune diseases—multiple sclerosis, rheumatoid arthritis, inflammatory bowel disease, type 1 diabetes, juvenile idiopathic arthritis and celiac disease [13]. To confirm the etiopathogenic role of EBV in a disease, a better understanding of the EBV biology and molecular mechanism of the associated disease is required.

EBV is an enveloped DNA virus that has approximately 172 kb double-stranded DNA (dsDNA) genome encoding genes for latent and lytic infection. This virus was the first of the herpesviruses to be completely sequenced [14] to identify over 80 protein-coding open reading frames and around 30 different non-coding RNAs (ncRNAs) [15].

Latent EBV infection allows for long-term viral persistence in the host, owing to tight control of viral gene expression to reduce the antiviral immune recognition. During latent infection, only several different types of RNAs and proteins are expressed. They include ncRNAs (EBV-encoded RNA 1 (EBER1) and EBER2, microRNAs (miRNA), EBV-stable intronic-sequence RNAs (EBV-sisRNA), EBV small nucleolar RNAs (EBV-snoRNA) and RPMS1 messenger RNA), six nuclear proteins (EBV nuclear antigen 1 (EBNA1), EBNA2, EBNA3A, EBNA3B, EBNA3C and EBNA5) and three latent membrane proteins (LMP1 and LMP2A-B) [11][15].

Upon lytic reactivation, EBV genes are sequentially expressed in immediate-early (IE), early (E) and late (L) states. Switch from latent to lytic state is triggered by expression of two IE viral transcription factors, the master regulator ZEBRA (also known as BZLF1, Zta, EB1 or Z) and Rta (BRLF1 or R). ZEBRA and Rta individually or cooperatively activate a subset of E genes many of which encode proteins involved in viral lytic DNA replication, such as the single-stranded DNA-binding protein (BALF2) and five replication enzymes and coenzymes, namely the helicase (BBLF4), primase (BSLF1), primase-associated factor (BBLF2/3), DNA polymerase (BALF5) and DNA polymerase processivity factor (BMRF1) [16]. For more details of E gene products, the reader is referred to the discussion in

Kenney, 2007 [17]. Viral DNA replication is followed by expression of EBV's L genes, which code for viral structural proteins (major capsid protein p160 (BcLF1) and three small capsid proteins, p18, p23 and p40 (BFRF3, BLRF2 and BdRF1)), glycoproteins (gp350/220 (BLLF1), gH (gp85; BXLF2), gp42 (BZLF2), etc.) and tegument proteins and viral interleukin 10 (vIL-10; BCLF1) [16][17][18]. All these latent and lytic determinants (the whole list is out of the scope of this review) are potentially useful for EBV detection and objective diagnosis.

Accurate laboratory tests to detect EBV are needed for purposes of basic and epidemiologic research and clinical management for different diseases. Biochemical, serological, immunological, histological, cytological and molecular detection methods of EBV have been used in the diagnosis and monitoring of patients with EBV-associated diseases [19]. The development of advanced laboratory methods allows timely and accurate diagnosis of clinical manifestations, which, in turn, may contribute to the prognosis and successful treatment. The identification of a suitable methodology that links EBV with different diseases will also advance our understanding of the molecular mechanisms underlying the onset and progression of EBV-associated diseases. (Figure 1)

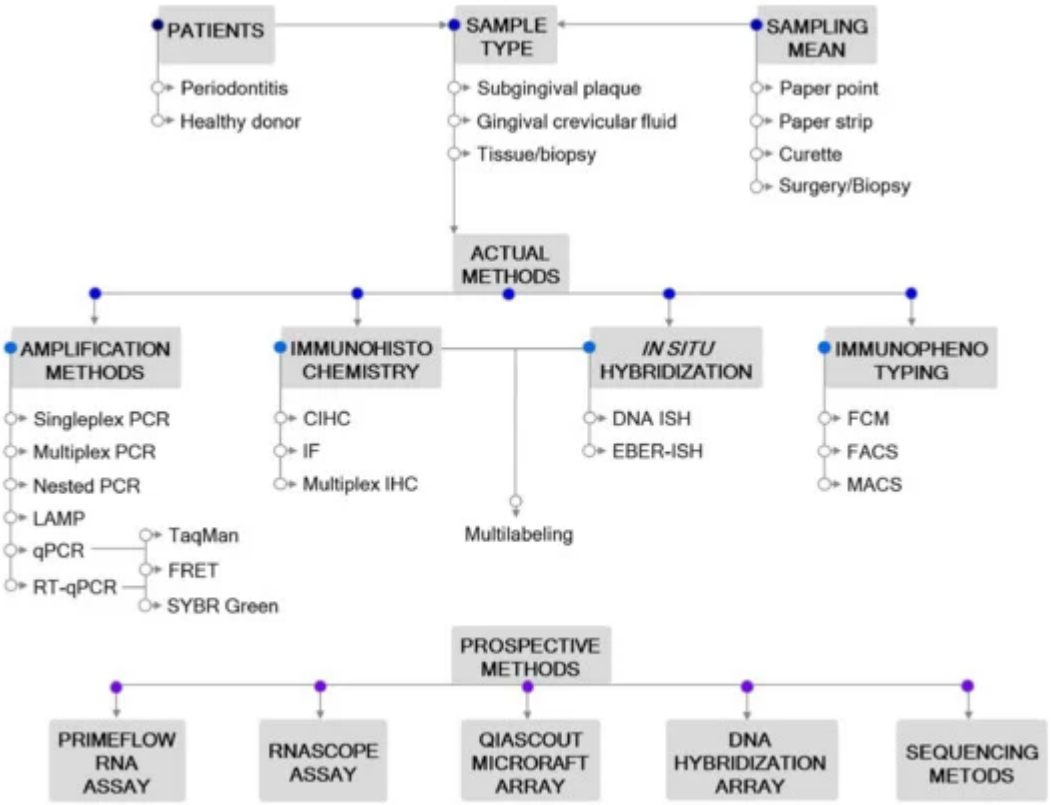

Figure 1. Outline of the methodological approaches for Epstein–Barr virus (EBV) detection in periodontitis. Actual and prospective methods are listed. Abbreviations: CIHC, chromogenic immunohistochemistry; EBER—EBV-encoded RNA; FACS—fluorescence-activated cell sorting; FCM—flow cytometry; FRET—fluorescence resonance energy transfer; IF—immunofluorescent detection; IHC—immunohistochemistry; ISH—*in situ* hybridization; LAMP—loop-mediated isothermal amplification; MACS—magnetic-activated cell sorting; PCR—polymerase chain reaction; qPCR—real-time quantitative PCR; RT-qPCR—reverse transcription qPCR.

2. Sampling

The physical presence of EBV in periodontal lesions suggests that EBV may be implicated in the etiopathogenesis of periodontitis. As such, samples need to be taken from the periodontal environment. The sample types and sample extraction methods may influence the identification and enumeration of microbes. The most eligible articles studying the association of EBV with periodontitis used subgingival plaque (SbgP), gingival crevicular fluid (GCF) and tissue/biopsy samples as sample type/sampling location [20], and curette, paper point, paper strip and surgery/biopsy as sample-extracting methods [21]. There is also a good deal of literature retrieving EBV from peripheral blood and saliva of periodontitis patients. Considering EBV is ubiquitous in the human population, the blood will not be reviewed here as a sample relevant to periodontitis, consequently, the serological tests are not discussed in this review. Though salivary EBV load may be very relevant to periodontitis, in this review, the saliva as a sample and salivary EBV load detection methods are not discussed either, because EBV is ubiquitous in humans, it is transmitted through saliva and EBV DNA is commonly detected also in the saliva from healthy adults [22].

The inspection of the subgingival plaque occupying the PP is considered the gold standard in studying periodontitis-associated microbial communities [23]. As might be expected, site-specific, intra- and interindividual variations of SbgP profiles may occur. In general, several paired SbgP samples are collected from shallow (healthy) and deep (diseased) sites from the same patient. The single-site analysis is preferential, but for practical and economic reasons, pooled SbgP samplings have often been performed [23]. Paper points are widely used for the collection of SbgP. Generally, the area of the collection is isolated with cotton rolls and air-dried to avoid contamination with saliva, then the supragingival plaque and calculus are carefully removed with a scaler to ensure the collection of only the subgingival material [24]. The color-coded paper points of specific sizes are inserted into the base of the PP, left in place for a certain duration and eluted. Basic parameters such as the origin of the paper points (manufacturer/supplier), the ISO size, probing (sampling) time and elution time may influence the optimum conditions for the microbiological sampling of PPs [25]. Samples can be collected as single (one paper point into the PP of each tooth), pooled (several paper points into the PPs of several teeth) or parallel (several paper points at one tooth) samples [26].

Curettes are also commonly used for sampling of subgingival specimens. After isolating the area with cotton rolls, a sterile curette tip is gently introduced through the pocket orifice into the bottom of the pocket and then removed with slight pressure against the tooth in a single vertical stroke to obtain the subgingival material [27][28].

In periodontitis, gingival crevicular fluid is an inflammatory exudate comprised of host-related substances, and from supra- and subgingival located microbes, thus, the analysis of GCF has become more and more important in the diagnosis of periodontitis [29]. Paper strips are used for GCF sample collection. The paper strip is inserted into the gingival crevice (intracrevicular method) or overlaid on the gingival crevice region (extracrevicular method). The intracrevicular method is subdivided to (i) superficial, when the strip is inserted just at the entrance of the crevice or PP and (ii) deep, when the strip is inserted to the base of the pocket or until minimum resistance is felt [30].

Tissue/biopsy specimens are obtained by periodontal surgery, which contain gingival tissue located adjacent to the PP. Careful dissection of the surgical piece can enrich the biopsy specimen with periodontal tissue attached to the tooth while removing the more distant gingival conjunctive areas. Dependent on the EBV detection method, the tissue specimens can further undergo cell dissociation, homogenization or fixation for isolation of macromolecules or histological analyses.

3. Polymerase Chain Reaction (PCR)-Based Detection Methods

PCR-based detection and quantification of EBV nucleic acids in body fluids and tissues have been used in the diagnosis and monitoring of EBV-associated diseases [19]. Extensive literature exists describing the application of PCR-based methods to identify and quantify EBV in periodontitis, which has targeted different genomic regions of EBV and applied different types of PCR methods. While most studies refer to the detection of the viral genomic DNA suitable to estimate the amounts of viruses in a specimen, a few other studies have focused on the detection of viral transcripts that can be more related to stages of viral replication in infected tissues. Specific characteristics and outcomes of recent studies (2010–2020) are summarized in Table 1. The large majority of these studies ascertain a strong association of EBV with periodontitis and its severity, indicating that EBV may serve as an etiopathogenic factor in periodontal diseases.

Table 1. Characteristics of the studies using nucleic acid amplification-based detection methods.

Study	Periodontitis Type	Sample Size	Sample Type	Sampling Type	Amplification Type	Target	EBV Occurrence		Main Findings
[38]	ApP	40 ApP	PApT	Curette	Nested PCR (DNA) RT (RNA) + Nested PCR (cDNA)	BamHI W (DNA) EBNA2 (RNA)	29 ApP DNA	20 ApP mRNA	EBV infection is a frequent event in ApP.
		40 HC	PT	NA			1 HC DNA	1 HC mRNA	
[37]	CP	40 CP: 40 SS + 40 DS	SbgP	Curette	Nested PCR (DNA) AGE	EBNA2	4 SS + 29 DS		Significant association of EBV1 and CP. Association between EBV1 and periodontopathic bacteria.
		40 HC					1 HC		
[31]	AgP, CP	20 AgP	SbgP	Curette	PCR (DNA) AGE	EBNA2	9 AgP		Significantly higher prevalence of EBV1 in AgP and CP subjects compared to HCs.
		20 CP					5 CP		
		20 HC					0 HC		

Study	Periodontitis Type	Sample Size	Sample Type	Sampling Type	Amplification Type	Target	EBV Occurrence	Main Findings
[33]	AgP, CP	10 patients: 25 AgPS + 25 CPS 25 HS	SbgP	Curette	Multiplex PCR (DNA) AGE	LMP2	8 AgPS + 8 CPS 2 HS	Significant association of EBV with CP and AgP.
[39]	CP	85 CP: 85 SS + 85 DS	SbgP	Paper point	Nested PCR (DNA) AGE	EBNA2	41 SS + 56 DS	More frequent detection of EBV DNA in patients with DS than in those with SS or HCs. EBV DNA may serve as a pathogenic factor leading to CP.
		20 HC: 40 HS					18 HS	
[56]	AgP	65 AgP	SbgP	Paper point	FRET qPCR (DNA)	BRLF1	7 AgP	No association between EBV and AgP.
		65 HC					9 HC	
[34]	AgP	15 AgP	SbgP, IDPT	Curette	Multiplex PCR (DNA) AGE	LMP2	10 AgP SbgP 11 AgP IDPT	Significant prevalence of EBV in AgP compared to HCs.
		15 HC					1 HC SbgP 0 HC IDPT	
[27]	CP	6 CP: 6 SS + 6 DS	SbgP	Curette	SYBR Green RT-qPCR (RNA)	EBNA1, EBNA2, LMP1, LMP2, BZLF1	6 SS + 6 DS: EBNA1 > EBNA2 ≥ LMP1 ≥ LMP2 ≥ BZLF1	EBV-specific latent (LMP1, LMP2, EBNA1 and EBNA2) and lytic (BZLF1) transcripts detected in all PP but not PalEC samples of CP patients.
		3 CP: 3 PalS	PalECs				3 PalS: EBNA1 = EBNA2 = LMP1 = LMP2 = BZLF1 = 0	
		10 HC: 10 HS	GS			EBNA1	DS > SS > HS	EBNA1 transcripts detected 36- and 5-fold higher in DS and SS, respectively, compared to HS.
[40]	ApP	100 ApP	PApT	Curette	Nested PCR (DNA) PAGE	EBNA2	76 ApP	Significant occurrence of EBV1 genotype in periapical lesions than in healthy pulps.
		25 HC	PT	Endodontic file			6 HC	

Study	Periodontitis Type	Sample Size	Sample Type	Sampling Type	Amplification Type	Target	EBV Occurrence	Main Findings
[32]	CP	100 CP / 100 HC	SbgP	Curette	PCR (DNA) AGE	LMP2	21 CP / 6 HC	Significantly higher levels of EBV in CP as compared to the healthy periodontium.
[28]	AgP	15 AgP / 15 HC	SbgP	Curette	Hotstart PCR (DNA) AGE	NA	6 AgP / 1 HC	EBV occurrence comparable among AgP and HC groups.
[57]	CP	60 CP	Tissue	Surgery	TaqMan qPCR (DNA)	NA	DS > SS	Observed EBV in tissue samples from deep and shallow PPs. Quantification of EBV is high in periodontal tissue samples of severe CP.
[58]	CP	25 CP: 25 SS + 25 DS / 13 HC: 26 HS	SbgP	Paper point	SYBR Green qPCR (DNA)	BNRF1	10 SS + 20 DS / 13 HS	Significantly high EBV DNA in DS than in SS of CP patients and HS of HCs. Association between EBV DNA, *P. gingivalis* and CP.
[35]	CP	40 CP / 20 HC	GCF	Paper strip	Multiplex PCR (DNA) AGE	LMP2	25 CP / 2 HC	Significantly higher prevalence of EBV in GCF of CP patients than in HCs. Strong association between EBV and CP.
[36]	CP (MiP, MdP, SvP)	100 MiP + 100 MdP + 100 SvP / 300 HC	SbgP	Curette, paper point	Multiplex PCR (DNA) AGE	LMP2	25 MiP + 20 MdP + 47 SvP / 0 HC	Significant association between EBV and CP, and the severity of the disease.
[54]	AgP	17 AgP / 17 HC	SbgP	Paper point	LAMP (DNA) AGE + TA	BamHI W	64.7% AgP / 47.1% HC	No significant association between EBV1 and AgP. Highest risk of AgP when *A. actinomycetemcomitans* and EBV1/HCMV are together.

Study	Periodontitis Type	Sample Size	Sample Type	Sampling Type	Amplification Type	Target	EBV Occurrence	Main Findings
[59]	GAP	165 GAP: 165 AS + 165 n-AS	SbgP	Paper point	qPCR (DNA) AGE	EBNA1	23 AS + NA n-AS	EBV association with *A. actinomycetemcomitans*. Although the presence of EBV (herpesvirus in general) is not necessary for the progression of GAP, it can facilitate it, possibly by promoting pathogenicity and virulence of periodontopathic bacteria in a virus and bacterial species-dependent manner.
[60]	AgP, CP [32]	18 AgP +12 CP 30 HC	SbgP	Curette	TaqMan [33][34][35][36] (DNA)	NA	19 (AgP + CP) 3 HC	Significant presence of EBV in periodontitis sites as compared to healthy sites. Positive correlation of EBV with *P. gingivalis* and *T. forsythia*. [37][38][39][40][41]
[61]	AgP, ApP	22 AgP + 3 ApP 25 HC	SbgP	Paper point	TaqMan qPCR (DNA)	EBNA1 [42]	16 AgP + 3 ApP 4 HC	Prevalence and copy number of EBV significantly higher in periodontitis patients than in healthy controls.
[62] [43]	CP (MdP, SvP)	20 patients: 9 MdP + 11 SvP	SbgP	Curette	TaqMan EBV R-GENE qPCR (DNA)	BXLF1	0–9861.14 × 10^2 copies/µg	Different levels of EBV occurrence in CP patients.
[41]	AgP, CP	[44] 57 AgP 59 Cp 43 HC	Tissue	Surgery	Nested PCR (DNA) AGE SYBER Green qPCR (DNA)	EBNA2 BALF5	25 AgP: 4.41–7.01 $\log_{10}$ copies/g AgP 28 CP: 5.06–7.31 $\log_{10}$ copies/g CP 5 HC: 4.57–5.[45]	Significant occurrence of EBV in the AgP and CP groups compared to the [45] HC. Significant association between EBV load and periodontitis.

NA2 [31] several CR, was n by the nally, the ese PCR phoresis followed agments, vith AGE sociation low-level antitative iminates y around r (FRET) quantify (gDNA)

In the TaqMan system, in addition to the two amplification primers used in conventional PCR, a dual-labeled fluorogenic hybridization probe is used. The probe hybridizes specifically in the DNA target region between the two PCR primers. One fluorescent dye serves as a reporter and its emission is quenched by the second fluorescent dye. Nuclease degradation of the hybridization probe by Taq DNA polymerase releases quenching of the reporter fluorescence, resulting in an increase in peak fluorescence [44][46]. The principle of FRET hybridization is based on the hybridization of two single-stranded, sequence-specific, fluorescent-labeled oligonucleotides (with donor and acceptor dyes) to the target sequence in close proximity in a head-to-tail orientation. The energy absorbed by the

Study	Periodontitis Type	Sample Size	Sample Type	Sampling Type	Amplification Type	Target	EBV Occurrence	Main Findings
			[45][48]				log_{10} copies/g HC	

Abbreviations: Periodontitis type: AgP—aggressive periodontitis; ApP—apical periodontitis; CP—chronic periodontitis; HC—healthy control; GAP—generalized aggressive periodontitis; MdP—moderate periodontitis; MiP—mild periodontitis; SvP—severe periodontitis. Sample type: GCF—gingival crevicular (periodontal pocket) fluid; GS—gingival sulcus; IDPT—interdental papilla tissue; PAnT—periapical tissue [49]; PaIECs—palatal epithelial cells; PP—periodontal pocket; PT—pulp tissue; SbgP—subgingival plaque. Site type: AgPS—aggressive periodontitis site; AS—active site; CPS—chronic periodontitis site; DS—deep site; HS—healthy site; nAS—non active site; PaIS—palatal site; SS—shallow site. Amplification type: FRET—fluorescence resonance energy transfer; PCR—polymerase chain reaction; qPCR—real time quantitative PCR; RT—reverse transcription reaction; RT-qPCR—reverse transcription qPCR. Amplicon detection type: AGE—agarose gel electrophoresis; PAGE—polyacrylamide gel electrophoresis; TA—turbidity assay. NA—not available.

Real-time qPCR is considered a sensitive, reliable, stringent, simple, specific, precise and fast method [11]. Since nucleic acid amplification and detection occurs in the same sealed tube, the risk of amplicon contamination is negligent compared with conventional PCR methods. Due to advanced instrumentation, the real-time qPCR testing is much simpler to perform and the test results are acquired much faster [12]. Currently, several real-time qPCR-based EBV detection and quantification kits are commercially available, such as EBV R-GENE (bioMerieux), EBV ELITe MGB (ELITechGroup), artus EBV PCR Kits (QIAGEN), etc. However, attention should be paid when comparing the data of different studies [50]. The source of deviation could be the units of measurement (copies per milliliter, copies per microgram of DNA, copies per positive cell) of the EBV targets (LMP2, BKRF1 or BamHI W (EBNA1), BNRF1 (membrane protein), BXLF1 (thymidine kinase), BZLF1, BALF5 or BFRF1 (transmembrane protein), etc.).

EBV DNA can be detected with high specificity, sensitivity and rapidity on par with the real-time qPCR method utilizing the loop-mediated isothermal amplification (LAMP) method. The LAMP reaction requires a DNA polymerase with strand displacement activity and a set of four specially designed inner and outer primers that recognize a total of six distinct sequences within the target DNA. Iwata et al. (2006) designed primers for the EBV LAMP assay based on BamHI W gene sequences [51]; Liu et al. (2013) later designed an extended set of LAMP primers for latent (EBNA1, EBNA2, LMP1 and LMP2A) and lytic (BZLF1) transcripts [52]. During LAMP reaction specific DNA targets are amplified at 63–65 °C, without thermocycling, accumulating 10^9 copies of the target in less than an hour. The final products of LAMP are stem-loop DNAs with several inverted repeats of the target DNA and cauliflower-like structures with multiple loops. LAMP amplicon product is further detected by turbidity assay (TA) of the white precipitate of magnesium pyrophosphate and/or AGE. The reaction is described in detail in [51][53]. Elamin et al. used this technique to assess the presence of putative periodontopathic bacteria (*Aggregatibacter actinomycetemcomitans*, *Porphyromonas gingivalis*, *Tannerella forsythia* and *Treponema denticola*) and two periodontal herpesviruses (EBV1 and HCMV) in individuals with aggressive periodontitis [54]. Though they reported no significant association between EBV1 and the disease, the highest risk of aggressive periodontitis was observed when *A. actinomycetemcomitans* was detected together with EBV1 and/or HCMV.

Using a sensitive and reproducible reverse transcription qPCR (RT-qPCR) method different transcripts of EBV can be detected and quantified to distinguish distinct states of latent or lytic EBV infection or closely monitor reactivation of EBV. In theory, RT-qPCR differs from qPCR only by the addition of a preliminary step, the initial complementary DNA (cDNA) synthesis from an RNA template by an RNA-dependent DNA polymerase (reverse transcriptase). After the RT reaction, suitable detection chemistry to report the presence of PCR amplicons, an instrument to monitor the amplification in real-time and appropriate software for quantitative analysis are required [55]. From the list of recent studies (Table 1), Hernádi et al. used the RT reaction to convert the EBNA2 messenger RNA into cDNA followed by nested PCR to detect that EBNA2 expression was significantly more frequent in apical

periodontitis lesions as compared to healthy controls [38]. They concluded that EBV infection was a frequent event in apical periodontitis and that symptoms were likely to occur if the lesion is aggravated with active EBV infection. Vincent-Bugnas and coauthors used the sensitive RT-qPCR technique and observed that EBV latent (EBNA1, EBNA2, LMP1 and LMP2) transcripts were detectable in all PP samples of chronic periodontitis (CP) patients, which were within the range expressed by EBV-infected cell lines [27]. EBNA1 was expressed at the highest and very similar levels to those measured in EBV-infected cell lines. Moreover, the EBNA1 expression level was correlated with the severity of the CP. On the other side, the IE viral transactivator BZLF1, known to induce the EBV lytic cycle, was also expressed in CP samples but at a level lower than that observed in the EBV-producing cell line. Overall, their conclusions derived from RT-qPCR analysis were that EBV-infected periodontal cells were likely in a state of latent EBV infection and that the level of EBV infection correlated with disease severity.

4. Immunohistochemistry (IHC)

To identify the precise cellular location of EBV, morphology-based techniques are used. IHC may be applied to confirm the presence, distribution, localization of EBV in the cells/tissues and distinguish latent from lytic infection based on protein expression profiles. IHC for EBV detection involves the staining of key EBV proteins such as EBNA1, EBNA2, LMP1, LMP2A and BZLF1 [63]. Commercial antibodies to EBV for IHC assays are available. There are also automated and standardized procedures routinely and widely used in pathology laboratories to detect EBV proteins in tissue specimens, such as FLEX monoclonal mouse anti-Epstein–Barr virus, LMP, Clones CS.1–4 (DAKO), which are used together with Autostainer Link instruments.

IHC procedures are performed on formalin-fixed, paraffin-embedded (FFPE) tissue sections of periodontal biopsies and cytological preparations from the periodontal environment. A standard IHC protocol is a multistep procedure involving deparaffinization/rehydration, heat- or proteolytic-induced antigen retrieval, blocking of non-specific staining, permeabilization, immunostaining with a primary antibody specific to a target antigen, incubation with labeled secondary antibody and detection. IHC allows for chromogenic (chromogenic immunohistochemistry—CIHC) and fluorescent (immunofluorescent—IF) detection types. For CIHC detection, the antibody is conjugated to an enzyme (such as horseradish peroxidase (HRP) or alkaline phosphatase (AP)), which converts a substrate (such as 3,3′-diaminobenzidine (DAB) or 3-amino-9-ethylcarbazole (AEC)) into a colored precipitate at the antigen site. For IF detection, the fluorophore (such as Alexa Fluor family dyes or fluorescein isothiocyanate (FITC)) conjugated antibody is excited by and emits light at specific wavelengths. Following the immunostaining, counterstaining with hematoxylin (chromogenic detection) or with DAPI (fluorescence detection) is performed to contextualize the antigen of interest. After the completion of all staining, the tissue is mounted and visualized by a bright-field (CIHC) or fluorescence/confocal (IF) microscope.

Multiplex chromogenic and fluorescence immunohistochemistry has recently emerged as a potent tool for the simultaneous detection of multiple biological markers on a single tissue section using a consecutive or simultaneous staining approach [64]. Multiplexed strategies allow compiling maximal information per tissue section of a limited sample and to understand coexpression and colocalization of multiple targets within tissue architecture.

Though IHC is a sensitive, versatile technique with many applications, careful control selection and proper optimization of the protocol is required. Besides, because the evaluation of the staining intensity of IHC is subjective, the ambiguity in the evaluation of the results and inter- or intraobserver variability may be problematic [65].

IHC, multiplex IHC and combined IHC techniques were employed for EBV analysis in periodontitis research (Table 2). In this context, using the CIHC approach for LMP1 protein immunostaining Saboia-Dantas et al. observed EBV in 31% of apical periodontitis lesions obtained after teeth extraction [66]. Vincent-Bugnas et al. applied IF costaining of viral latent proteins LMP1 and LMP2, and junctional EC marker cytokeratin 19 (CK19) to detect latent EBV-infected periodontal ECs (pECs) in non-surgical liquid-based cytological samples derived from PPs of CP patients (Figure 2 [27]). They estimated that around 32% of the $CK19^+$ cells were infected with EBV ($LMP2^+$).

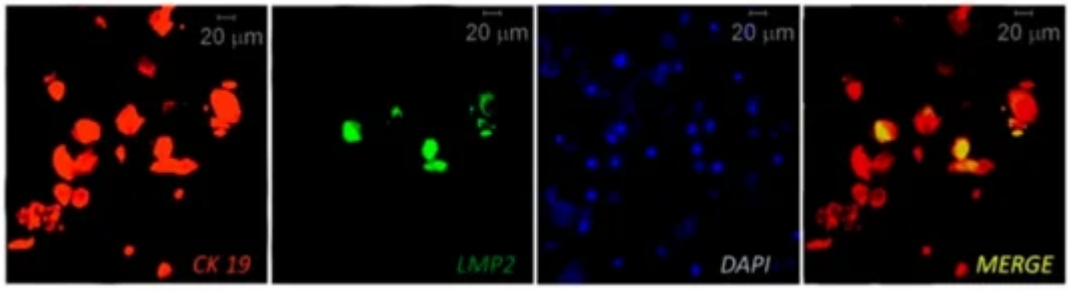

Figure 2. Immunofluorescent (IF) costaining of CK19 (junctional epithelial cell marker cytokeratin 19) and LMP2 (EBV latent membrane protein 2) to detect EBV-infected epithelial cells in samples taken from a periodontitis patient. The cell nuclei are counterstained with DAPI. Reprinted from [27].

Table 2. Characteristics of the studies using tissue-based detection methods.

Study	Periodontitis Type	Sample Size	Sample Type	Sampling Type	Tissue-Based Detection Type	Target	EBV Occurrence	Main Findings
[66]	ApP	35 ApP	Apical lesion	Teeth extraction	CIHC	LMP1	11 ApP	EBV occurrence in about 31% of ApP samples.
[67]	ApP	20 ApP	Apical lesion	Submarginal incision	EBER-ISH	EBER	0 ApP	No signs of cells harboring EBV in 20 apical samples analyzed

Study	Periodontitis Type	Sample Size	Sample Type	Sampling Type	Tissue-Based Detection Type	Target	EBV Occurrence	Main Findings
								by EBER-ISH.
[39]	CP	41 SS + 56 DS	Gingival tissue	Flap surgery	EBER-ISH + CIHC	EBER CD19	EBER$^+$ CD19$^+$	Numerous CD19$^+$ B cells infiltrated in the connective tissue subjacent to the gingival epithelium; numerous cells in the same location were EBER$^+$.
[27]	CP	3 CP: 3 PP 3 CP: 3 PalS	SbgP PalECs	Curette, cytospin cuvette	IF costaining	LMP1, LMP2 CK19	3 PP 0 PalS	Around 32% of the CK19$^+$ epithelial cells infected with EBV (LMP2$^+$).
					EBER-ISH + CIHC	EBER CK19	EBER$^+$, CK19$^+$ PP EBER$^-$, CK19$^+$ PalS	EBER$^+$ periodontal epithelial cells (pECs) were detected only in PP samples.
		20 CP: 20 SS + 20 DS 10 HC: 10 HS	SbgP GS	Curette, cytospin cuvette	EBER-ISH + CIHC	EBER CK19	DS > SS > HS	Frequency of EBV$^+$ pECs higher in deep pockets than in shallow pockets

Study	Periodontitis Type	Sample Size		Sample Type	Sampling Type	Tissue-Based Detection Type		Target		EBV Occurrence		Main Findings
												and healthy sites. A positive correlation between EBV infection and disease severity.
[68]	PApP	9 PApP	6 PApP	PApG	Endodontic surgery	EBER-ISH	CIHC	EBER	LMP1	6 PApP	6PApP	EBER detected in the cytoplasm and nuclei of B cells and plasma cells (PC) in 66.7% of PApGs, but not in healthy gingival tissues. All EBER$^+$ PApGs positive for LMP1. LMP-1-expressing cells localized in the same areas as EBER-expressing cells.
		5 HC	5 HC	Gingival tissue	Teeth extraction					0 HC	0 HC	
[62]	CP (SvP)	5 SvP		Gingival tissue	Surgery	EBER-ISH +		EBER		EBER$^+$		Numerous EBV-infected cells, mostly overlapping with CD138$^+$ PCs. EBV-infected

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Study	Periodontitis Type	Sample Size	Sample Type	Sampling Type	Tissue-Based Detection Type	Target	EBV Occurrence	Main Findings
					Multiplex CIHC	CD3, CD20, CD138, Kappa	$CD3^+$, $CD20^+$, $CD138^+$, $Kappa^+$	PCs formed high-density clusters along the periodontal epithelium associated with $CD3^+$ T cells and $CD20^+$ B cells.

Abbreviations: Periodontitis type: ApP—apical periodontitis; CP—chronic periodontitis; PApP—periapical periodontitis; HC—healthy control; SvP—severe periodontitis. Sample type: GS—gingival sulcus; PApG—periapical granuloma; PalECs—palatal epithelial cells; SbgP—subgingival plaque. Site type: DS—deep site; HS—healthy site; PalS—palatal site; PP—periodontal pocket; SS—shallow site. Tissue-based analysis type: CIHC—chromogenic immunohistochemistry; EBER-ISH—EBV-encoded RNA in situ hybridization; IF—immunofluorescent staining.

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