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Parasitology

Development of a new TaqMan PCR assay for the detection of both *Entamoeba gingivalis* genotypes☆

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ABSTRACT

Entamoeba gingivalis is a parasitic protozoan found in the mouth of patients suffering from periodontitis, a widespread oral disease with an underestimated prevalence and major consequences on health. We present the development of the first TaqMan PCR assay targeting both *E. gingivalis* subtypes. This method has been evaluated on 50 samples from patients diagnosed with periodontitis in comparison with 2 different conventional PCRs, and a real-time SYBR Green PCR. Fifty percent of the samples were found positive for the *E. gingivalis* ST1 subtype with this new PCR, the SYBR Green PCR and one of the conventional PCRs. Among the 25 remaining samples, 12 (24%) were found positive for the *E. gingivalis* ST2 kamaktlii variant. This new TaqMan PCR could be used before and after periodontitis treatment to follow its efficacy and measure the parasite load in order to better understand the role of these parasites in oral diseases.

1. Introduction

Periodontitis is one of the most common oral diseases with an estimated prevalence of 50% between the ages of 35 and 44 in the WHO regions (Demmer and Papapanou 2000; Bonner et al. 2018). Between 2009 and 2014, in the USA, 42% of adults aged 30 years or older suffered from periodontitis, including 7.8% with severe periodontitis (Eke et al. 2018). This oral pathology represents an important public health concern, whose impact may be underestimated (Rashidi Maybodi et al. 2016). Characterized by host-mediated inflammation, periodontitis is frequently associated with gingival bleeding, bone resorption and even tooth loss (Tonetti et al. 2018). Its etiology remains unclear: the development of the disease may depend on interactions between microorganisms and neutrophils contained in periodontal pockets, host factors, and dysbiotic ecological changes in the oral microbiome (Garcia et al. 2018; Tonetti et al. 2018).

Among microorganisms found in the mouth *Entamoeba gingivalis* is a parasitic protozoan, first described in 1849 in patients with poor hygiene (Gros 1849; Gottlieb and Miller 1971; Lyons et al. 1983; Linke et al. 1989). Even if the natural niche of *E. gingivalis* is still unkown, this parasite is believed to be exclusively human and transmitted through contaminated food, mouth droplets and kissing. Moreover, this amoeba was also reported in the buccal cavity of dogs (Rousset et al. 1970) and in a Mexican pool (Rivera et al. 1983). According to some authors, this amoeba belongs solely to commensals inhabiting the mouth and thriving by feeding on bacteria and debris (Deng et al. 2017). For others, *E. gingivalis* would be one etiological agent of periodontitis having an indirect impact on the pathogenesis (Lyons et al. 1983; Wagner et al. 2006; Trim et al. 2011; Rashidi Maybodi et al. 2016).

In this context, prevalence data are important to better elucidate the role of *E. gingivalis* in periodontitis, and notably to promote better patient care. According to Feki and Molet ,(1990), *E. gingivalis* is present in almost 55% of patients with gingivitis and periodontitis and in 40% of patients with healthy gingival sulci (Feki and Molet ,1990). For Linke et al. (1989), *E. gingivalis* was found in 62% of patients with periodontitis (Linke et al. 1989). These variable prevalence data are due to different sampling or diagnostic methodologies and patient selection criteria.

The microscopic identification of *E. gingivalis* described in most studies is limited by the lack of sensitivity of direct examination, the fragility of the parasite as well as the confusion with macrophages (Dao 1985). Therefore, in-house molecular techniques have been developed to facilitate the detection and identification of theses parasites (Kikuta et al. 1996; Trim et al. 2011; Bonner et al. 2014). Compared to conventional

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PCR, real-time PCR has the advantages of being highly sensitive, performed in a closed system, avoiding post-PCR handling and saving time. However, until now, the only real-time PCR that has been developed has used the SYBR Green technology (Trim et al. 2011). SYBR Green has the disadvantage of binding to each existing double strand DNA, requiring the subsequent analysis of a melt curve to determine the specificity of the amplified product. Recently, Garcia et al. described a new *E. gingivalis* subtype, ST2, called the kamaktli variant, which differs from the only subtype previously reported (*E. gingivalis* ST1), by nucleotide polymorphisms in the ITS1-18S-ITS2 region (García et al. 2018a; García et al. 2018). All other available PCRs developed prior to this discovery did not take into account the existence of both subtypes, which makes their specificity imprecise, as well as all prevalence studies.

In this study, first we developed a TaqMan PCR assay in order to accurately detect the presence of both *E. gingivalis* variants in clinical samples. Secondly, this PCR was used to evaluate the occurrence of *E. gingivalis* in 50 diseased sites in patients with periodontitis, in comparison to the two different conventional PCRs (Kikuta et al. 1996; Bonner et al. 2014) and the SYBR Green PCR (Trim et al. 2011). The specificity of these existing PCRs was therefore checked for both variants, for the first time.

2. Material and methods

2.1. Sampling methodology

Fifty samples were collected from patients suffering from periodontitis and consulting at the department of periodontology of Nancy University Hospital for two 6-month periods (September 2014–March 2015; and April 2016–September 2017).

Patients were examined by a trained specialist dentist and selected when they were diagnosed with chronic periodontitis according to the Armitage's classification (Armitage 2000; Caton et al. 2018).They should be adult and not have received any treatment that could potentially modify their oral microbiota in the last 3 months nor have undergone scaling or root planing in the last 6 months. One part of the samples came from a collection (DC_2016–2623) and the other samples were issued from patients who accepted to give their subgingival biofilm. Prior to the sampling procedure, the patients who agreed to the use of their personal clinical and microbiological information signed an informed consent form (approved by decree law Huriet no. 91-73).

Subgingival microbiota from periodontal pockets of 5 mm or more were collected into micro-centrifuge tubes with 2 sterile paper points after isolation of the tooth and cleaning of the supragingival biofilm with a cotton pellet. The samples were stored at −20 °C until DNA extraction.

2.2. DNA extraction

DNA samples were extracted by using the QIAamp DNA Mini Kit (Qiagen, France), in accordance with the manufacturer's instructions, including slight modifications. In summary, 400 μL of the Buffer AL was added to microcentrifuge tubes, each containing 2 or 3 paper points from 1 pathological site. An incubation step was conducted (lysis) at 56 °C for 30 min with 20 μL of Proteinase K followed by several washing steps. Finally, the DNA were re-suspended in 75 μL of elution buffer. The extracted DNA was quantified by spectrophotometry (NanoDrop 2000c Thermo Scientific) and either used immediately or stored at −20 °C until use.

2.3. Primer and probe design

In order to design the primers and probes, a bioinformatics analysis was conducted with the 30 *Entamoeba* sp. SSU rDNA following sequences available on GenBank (https://www.ncbi.nlm.nih.gov/genbank/) and corresponding to the species that are most found in human: 5 *E. gingivalis* subtype 1 (KF250435; KF250436; KF250434; KF250433; D28490), 7 *E. gingivalis* subtype 2 (kamaktli variant) (KX027286; KX027287; KX027288; KX027289; KX027294; KX027295; KX027296), 4 *E. coli* (FR686364; AB444953), 1 *E. moshkovskii* (AF149906), 4 *E. histolytica* (GQ423750; GQ423749; GQ423748; AB426549), 1 *E. dispar* (AB282661), and 8 *E. polecki* (FR686400; FR686398; FR686397; FR686395; FR686394; FR686393; FR686392; FR686357). Firstly alignments were made within each group of sequences for a same species, with the CLUSTAL Omega multiple sequence alignment program (https://www.ebi.ac.uk/Tools/msa/clustalo/) in order to select one representative sequence after controlling their similarity. Secondly, an alignment was made with the 7 selected sequences: *E. gingivalis* ST1 D28490, *E. gingivalis* ST2 KX027296; *E. histolytica* AB426549, *E. moshkovkii* AF149906, *E. dispar* Z49256, *E. coli* AB444953, *E. polecki* AF149913. The sequences of *Trichomonas tenax* D49495; *T. vaginalis* JX943583, *T. foetus* M81842, *Candida albicans* M60302 and *C. tropicalis* M60308 were added to this alignment.

EG1F and EG2R primers were designed to amplify a 153 bp fragment inside the SSU rDNA of *E. gingivalis.* The EG12P and EG12PK TaqMan probes were respectively chosen to be specific to the *E. gingivalis* subtype 1 and *E. gingivalis* subtype 2 var. kamaktli. The bioinformatics specificity of the EG1F - EG2R primer pair and the EG12P or EG12PK probes were controlled with a systematic search of the corresponding sequences on the Nucleotide Basic Local Alignment Search Tool (BLAST: https://blast.ncbi.nlm.nih.gov/Blast.cgi) (Tables 1 and 2).

2.4. PCR assays

Primers and probes used in this study were listed in Table 2. Each PCR was performed in duplicate, whatever the primers and probes sets. The quality of the DNA extraction and the absence of DNA degradation were assessed for all samples by the detection of a 151-bp human sequence (Accession number NT_032977.9) with the HF (CAATGCCTCCTGCACCAC) and HR (CCATCACGCCACAGTTTCC) primers (Bonner et al. 2014).

The TaqMan PCR assays were performed by using the QuantiTect Probe PCR kit (Qiagen, France) with the newly designed EG1F-EG2R primers and the EG12P or EG12PK hydrolysis probes. Each 50 μL reaction consisted of 25 μL 2× QuantiTect Probe PCR Master Mix, 1 μL of each primer (20 μM; 0.4 μM in final concentration), 0.5 μL of probe (20 μM in total; 0.2 μM in final concentration), and 17.5 μl of RNase-free water. Five microliters of patient DNA was added to each reaction. For all PCRs, an iCycler thermocycler equipped with a MyiQ2 Optics Module was used (version 2.1) under the following conditions: 95 °C for 15 min of Taq polymerase activation; 45 cycles of a denaturation step at 94 °C for 15 s, followed by a combined hybridization and elongation at 60 °C for 60 s.

For both conventional PCR assays, EGO-1-EGO-2 and EGF-EGR primer sets were used (Kikuta et al. 1996; Bonner et al. 2014). PCRs were performed in a final volume of 50 μL: 25 μL of Taq Purple mix (Ozyme, France) was added to 0.5 μL of each primer (20 μM initial concentration), 2 μL of 25 mM $MgCl_2$ and 17 μl of RNase-free water. Five microliters of patient DNA was added. The PCR program was: 95 °C for 2 min (activation); 30 cycles of 95 °C for 30 s (denaturation), 55 °C for 30 s(hybridization), 72 °C for 1 min (elongation); and 5 min at 72 °C (final extension). PCR products (5 μL) were resolved on 1.5% agarose gel.

The real-time PCR assay using SYBR Green was performed with the LightCycler FastStart DNA Master SYBR Green I kit (Roche, France), in a final volume of 20 μL, under these conditions: 1 μL of each primer EGHF and EGHR (Trim et al. 2011) (0.5 μM in final concentration), 2 μL of mix (1×), 2.4 μL of 25 mM $MgCl_2$ (3 mM in final concentration), 11 μl of Rnase-free water, 5 μl of patient DNA for each tube reaction. The amplification conditions were: 95 °C for 10 min, 45 cycles of 95 °C for 10 s, 62 °C for 10 s, 72 °C for 10 s. The melt curve began at 95 °C for 15 s followed by a 0.1 °C decrease in temperature every 6 s to 65 °C. A cooling step at 40 °C for 30 s was finally added.

Table 1
Specificity of the primers and probes used in this study.

Name	GenBank accession number	Location	Sequences
			EG1F
E. gingivalis ST1	D28490	104	TAG**TACCATACAAGGAATAGCT-TTG**TGAAT
E. gingivalis ST2	KX027296	82	TAG**TACGATACACGGAATAGCT-TTG**TGAAT
E. histolytica	AB426549	128	TAGTAAAATACAAGGA-TAGCT-TTGTGAAT
E. moshkovskii	AF149906	128	TAGTAAAGTACAAGGA-TAGCT-TTGTGAAT
E. dispar	Z49256	128	TAGTAAAGTACAAGGA-TAGCT-TTGTGAAT
E. coli	AB444953	128	GAAATACGTACAAGGA-TATCT-TTGAGAAT
E. polecki	AF149913	125	TA-GTAAAAAGAAGGA-TAACTCTTGTTAAT
T. tenax	D49495	120	-GGCGACCAA-CAGGT----CTTAAATGGAT
T. vaginalis	JX943583	84	-GGCGACCAA-CAGGT----CTTAAATGGAT
T. fœtus	M81842	178	-GGCGACCTTTCAGGT----CTTATTTGGAT
C. albicans	M60302	131	TACCTTACTACTTGGA-TAACC-GTGGTAAT
C. tropicalis	M60308	131	TACCTTACTACTTGGA-TAACC-GTGGTAAT
			EG12P probe; **EG12PK probe**
E. gingivalis ST1	D28490	158	ATCCTGTTCTATT---------ACTAGAATAGGCGCAT---
E. gingivalis ST2	KX027296	136	ATCCC**AGTTGTTT---------GTACAAGTGGGCGCAT**---
E. histolytica	AB426549	181	ATCCAGTTTGTATT-------AGTACAAAATGGCCAAT---
E. moshkovskii	AF149906	181	ATCCGGTTTGTATT-------AGTACAAGTCGGCCACT---
E. dispar	Z49256	181	ATCCAATTTGTATT-------AGTACAAAGTGGCCAAT---
E. coli	AB444953	192	GTCGAGTGTGTTCC-TCCGGGAGCATAATCTACTGAGGAGG
E. polecki	AF149913	185	ATCTATATGATTAC-------AGCGTAATAAAGCAA-----
T. tenax	D49495	170	ATACATGCGATTG----------TTTCTCCAGATGT-----
T. vaginalis	JX943583	134	ATACATGCGATTG----------TTTCTCCAGATGT-----
T. fœtus	M81842	229	ATACATGCGATTG----------TTTCACTGAGT-T-----
C. albicans	M60302	185	ATCCCGACTGTTTGGAAGGGATGTATTTATTAGATAAA---
C. tropicalis	M60308	185	ATCCCGACTGTTTGGAAGGGATGTATTTATTAGATAAA---
			EG12P probe; **EG12PK probe**
E. gingivalis ST1	D28490	169	ACTAGAATAGGCGCAT----------TTCGAACAGGAATGT
E. gingivalis ST2	KX027296	147	**GTACAAGTGGGCGCAT**----------TCCCG--AGGAATGC
E. histolytica	AB426549	194	GTACAAAATGGCCAAT---------TTATTTAA--ATGAATTG
E. moshkovskii	AF149906	194	GTACAAGTCGGCCACT---------CTCTTCAC--GGGGAGTG
E. dispar	Z49256	194	GTACAAAGTGGCCAAT---------TTATGTAA--GTAAATTG
E. coli	AB444953	211	GCATAATCTACTGAGGAGGGGAGGATCCTTATGGTCCTTTCAA
E. polecki	AF149913	198	GCGTAATAAAGCAA----------TTTATTA
T. tenax	D49495	180	-TTTCTCCAGATGT----------
T. vaginalis	JX943583	144	-TTTCTCCAGATGT----------
T. fœtus	M81842	239	-TTTCACTGAGT-T----------
C. albicans	M60302	205	GTATTTATTAGATAAA----------AAATCAA
C. tropicalis	M60308	205	GTATTTATTAGATAAA----------AAATCAA
			EG2R
E. gingivalis ST1	D28490	257	TTT**GACAAGGAATCAATGAAAATATC**TGATC
E. gingivalis ST2	KX027296	232	TT-**GACAAGGAATCAATGAGAATATC**TGATC
E. histolytica	AB426549	281	TT-AACAAGTAACCAATGAGAATTTCTGATC
E. moshkovskii	AF149906	281	TT-AACAAGTAACCAATGAGAATTTCTGATC
E. dispar	Z49256	281	TT-AACAAGTAACCAATGAGAATTTCTGATC
E. coli	AB444953	320	TTTCACAAGTCATCATTAAGAATATCTGACC
E. polecki	AF149913	275	TTTTACAAGTAACTGTTTTAAATATCTGACC
T. tenax	D49495	218	-TCAGAGGCACGCCATTCG-ACTGAGTGACC
T. vaginalis	JX943583	183	ATCAGAGGCACGCCATTCG-ACTGAGTGACC
T. fœtus	M81842	276	-CCGG--GCACACCAATCG-ATTGAGCGACC
C. albicans	M60302	282	GCTGGCGATGGTTCATTCA-AATTTCTGCCC
C. tropicalis	M60308	282	GCTGGCGATGGTTCATTCA-AATTTCTGCCC

2.5. Validation experiments

In order to assess the validity of the protocol used for the TaqMan PCR assays, several criteria were studied: specificity, repeatability, reproducibility, cross-contamination risk, efficiency and analytical sensitivity (LOD = limit of detection) (Bustin 2010).

For the specificity, TaqMan PCR were performed on several bacterial, fungal or protozoan DNA either isolated in the mouth, the digestive tract or as environmental contaminants. Consequently, the DNA of 3 fungal species cultures (*Aspergillus fumigatus*, *Geotrichum capitatum*, *Candida albicans* ATCC 90028) and 5 oral bacteria (*Prevotella intermedia* ATCC 25611, *Porphyromonas gingivalis* ATCC 33277, *Fusobacterium nucleatum* spp. vincentii ATCC 49256, *Aggregatibacter actinomycetemcomitans* ATCC 29522, *Streptococcus B* Baly A) was extracted with the Qiagen QIAamp DNA Mini kit, quantified and amplified. In addition, a *Trichomonas tenax* DNA and a mixture of DNA from the 4 parasites *i.e. Giardia lamblia*, *Cryptosporidium parvum*, *Entamoeba histolytica* and *Dientamoeba fragilis* (RIDA GENE Stool Panel kit) were tested under the same conditions.

For both PCR assays, the analytical sensitivity (expressed by the Limit of Detection) corresponding to the lowest detected DNA concentration, was assessed using 10-fold serial dilutions of a DNA pool extracted from positive patients. The tested range was as follows: from 1 (pool not diluted) to 1/10 000, each dilution being tested in duplicate.

PCR efficiency was estimated through the linear regression of the dilution curve.

The repeatability (intra-assay variance) was evaluated together with the contamination risk by testing a positive DNA sample alternately with a negative sample, both in triplicate, in three repetitions in the same reaction.

Table 2
Primers and probes used for this study.

Primers and probes	Sequence	Size (bp)	PCR assay	Ref.
EGO-1	5′-GAATAGGCGCATTTCGAACAGG-3′	1400	conventional	Kikuta et al. 1996
EGO-2	5′-TCCCACTAGTAAGGTACTTACTC-3′			
EGF	5′-AGGAATGAACG GAACGTACA-3′	203	conventional	Bonner et al. 2014
EGR	5′-CCATTTCCTTCTTCTATTGTTTCAC-3′			
EGHF	5′-TACCATACAAGGAATAGCTTTGTGAATAA-3′	135	SYBR green	Trim et al. 2011
EGHR	5′-ACAATTGTAAATTTGTTCTTTTTCT-3′			
EG1F	5′-TACCATACAAGGAATAGCTTTG-3′	153	Taq Man	This publication
EG2R	5′-GATATTTTCATTGATTCCTTGTC-3′			
EG12P	5′-FAM-AGAATAGGCGCATTTCGAACAGGA-BHQ1			
EG12PK	5′-HEX-AGTTGTTTGTACAAGTGGGCGCAT-BHQ1			

The reproducibility (inter-assay variability) was assessed on a same DNA control sample amplified in five separate reactions.

The presence of inhibitors in samples without amplification was controlled by spiking these DNA samples with a positive *E. gingivalis* DNA control. For each sample, the Ct obtained from *E. gingivalis* DNA amplification was independently compared to the positive Ct control by using an equation taking into account the variability of the Ct values: M +/− 3 × SD, where M is the mean Ct value calculated from the repeatability mean (29.47 or 30.57 respectively for the EG12P and EG12PK assay) and SD the corresponding standard deviation (0.35 or 0.33 respectively for the EG12P and EG12PK assay). If the Ct values were not comprised in the range determined by the previous equation: M − 3SD; M + 3SD corresponding to 28.42; 30.52 or 29.58; 31.56, according to EG12P or EG12PK assays, respectively, the presence of inhibitors in the sample might be suspected. The samples should then be diluted 10-fold, the spiking test resumed and the values recalculated.

3. Results

3.1. Limit of detection and efficiency, specificity, repeatability, reproducibility

During the analytical sensitivity tests, the lowest amplifications were obtained with the dilution of 1/1000 (Ct = 39.36 or 40.21, respectively for ST1 and ST2).Thus, the limit of detection of both primer sets and probes was fixed at a Ct = 39. Amplifications were performed in duplicate and in cases where the Ct value was greater than 39 in one tube, the analysis was conducted a second time. Subsequently, if Ct > 39 in both samples the result was considered negative. If only one sample of the duplicate still had a Ct > 39 the analysis was reconsidered by increasing the DNA amount. One reason for different outcomes might be proximity to the detection limit. The PCR efficiencies estimated through the linear regression of the dilution curves made it possible to determine coefficients for both ST1 and ST2 assays: r2 = 0.99 for both assays, with a slope of the standard curve respectively of −3.23 and −3.46 corresponding to efficiencies of 103% and 94.4%. The ST1 TaqMan assay is thus a slightly more efficient than the ST2 one.

Among all the tested microorganisms, no cross amplification was obtained ensuring the specificity of the primers and probes, with respect of each *E. gingivalis* ST1 and ST2 genotypes.

In order to reduce the cross-contamination risk, 3 negative samples alternated with 3 positive ones in the same run (mean SD = 0.265), and no cross-contamination was detected.

The intra-assay variance (repeatability) was tested with 9 repetitions of a same positive DNA aliquot. For each specific *E. gingivalis* ST1 and ST2 PCR, respectively, a global mean Ct value of 29.47 and 30.57 was obtained with coefficients of variation (CV) of 0.90% and 1.00%.

The mean Ct for inter-assay reproducibility was obtained from 5 separate amplifications of a positive control and corresponded respectively to 26.18 (ST1) and 30.29 (ST2) with a coefficient of variation (CV) of 0.5% (ST1) and 0.80% (ST2).

3.2. Clinical sample analyses

The 50 DNA isolated from clinical samples were tested simultaneously for the presence of *E. gingivalis* with 2 conventional PCRs (EGO1-EGO2; EGF-EGR) (Kikuta et al. 1996; Bonner et al. 2014), the only existing SYBR Green PCR (Trim et al. 2011) and the new TaqMan PCR targeting ST1 and ST2. The results are outlined in Table 3.

Twenty-five samples out of the 50 were found positive concomitantly with the TaqMan PCR assay specific to *E. gingivalis* ST1, the SYBR green real-time PCR and the EGF-EGR conventional PCR. Five of these 25 samples were found negative with the EGO1-EGO2 conventional PCR. In the case of these SYBR green PCR amplicons, the samples were characterized by obtaining a melting curve with a fusion temperature at 76 °C.

Of the 25 remaining samples, all were found negative either by the *E. gingivalis* ST1 new real-time PCR, or with conventional PCR, 12 (24%) were found positive for *E. gingivalis* ST2 kamaktlii. These 12 samples were also amplified with SYBR green PCR with a melting curve showing a 79 °C temperature.

Among all samples, 11 were only amplified by SYBR green PCR, with a Ct > 35 cycles (ranging between 37.63 and 42.36), melting curve temperatures at 72 °C for 9 of them and 76 °C for 2 of them. Because of the Ct > 35 cycles, as recommended by Trim et al., the samples were considered negative (Trim et al. 2011). For 2 samples no signal was obtained whatever the amplification method.

The absence of DNA degradation was assessed by the detection of a 151-bp human sequence for all tested samples for which a negative result was obtained regardless of the PCR used.

The spiking test was performed on 13 negative samples with the real-time ST1 and ST2 PCR. For all of samples, Ct was comprised in the range 28.42–30.52, and no inhibition was suspected.

Overall, in our study the global prevalence of *E. gingivalis* in patients suffering from periodontitis was 74% (37/50). More specifically, *E. gingivalis* ST1 and ST2 were respectively detected in 50% and 24% of the diseased samples. Both conventional PCRs can only detect *E. gingivalis* ST1 whereas the SYBR Green real-time PCR amplifies the ST1 and ST2 *E. gingivalis* variants. Nevertheless, of the 25 samples that were found positive for ST1 *E. gingivalis*, the EGO1-EGO2 PCR was positive for only 20 of them, whereas the EGF-EGR could detect all ST1 samples. EGF-EGR PCR seems more suited for the detection of ST1 *E. gingivalis* than EGO1-EGO2. With SYBR Green PCR, the 25 ST1 *E. gingivalis* could be differentiated from the 12 ST2 *E. gingivalis* by their fusion temperatures, respectively 76 °C or 79 °C.

4. Discussion

Periodontitis is a widespread oral disease, associated with other pathological conditions such as cardiovascular disease, diabetes, premature birth, justifying the interest in this pathology (Zhou et al. 2015; Hwang et al. 2018; Papapanou et al. 2018; Puertas et al. 2018). Even if some authors have suggested that *E. gingivalis* could synthesize

Table 3
Results of the PCR assays obtained from 50 clinical samples.

Samples		conventional PCR		qPCR SYBR Green			TaqMan EG12P probe			TaqMan EG12PK probe		
n°	Total concentration (ng/μL)	EGF/EGR	EGO1/EGO2	+/−	Ct	Melt Curve	+/−	Ct	spiking	+/−	Ct	spiking
1	4.2	+	−	+	28.56	76	+	34.09	NA	−	NA	NA
2	4.6	+	−	+	28.15	76	+	33.78	NA	−	NA	NA
3	7.7	+	-	+	28.26	76	+	33.48	NA	−	NA	NA
4	5.2	+	−	+	27.09	76	+	32.34	NA	−	NA	NA
5	5	+	−	+	26.95	76	+	31.9	NA	−	NA	NA
6	9	+	+	+	26.11	76	+	31.31	NA	−	NA	NA
7	6.9	+	+	+	25.98	76	+	30.82	NA	−	NA	NA
8	9.8	+	+	+	24.49	76	+	30.43	NA	−	NA	NA
9	9.4	+	+	+	25.17	76	+	30.41	NA	−	NA	NA
10	9.7	+	+	+	25.01	76	+	30	NA	−	NA	NA
11	14.8	+	+	+	23.4	76	+	29.7	NA	−	NA	NA
12	15	+	+	+	24.01	76	+	29.43	NA	−	NA	NA
13	10.3	+	+	+	24.83	76	+	29.22	NA	−	NA	NA
14	7.4	+	+	+	24.17	76	+	29.01	NA	−	NA	NA
15	8.8	+	+	+	23.66	76	+	28.75	NA	−	NA	NA
16	13.2	+	+	+	24.39	76	+	28.68	NA	−	NA	NA
17	45	+	+	+	22.34	76	+	28.24	NA	−	NA	NA
18	7	+	+	+	22.91	76	+	28.18	NA	−	NA	NA
19	17.9	+	+	+	23.47	76	+	27.75	NA	−	NA	NA
20	12.2	+	+	+	22.99	76	+	27.63	NA	−	NA	NA
21	24.8	+	+	+	22.58	76	+	27.51	NA	−	NA	NA
22	16.5	+	+	+	21.13	76	+	26.89	NA	−	NA	NA
23	36.9	+	+	+	22.73	76	+	26.45	NA	−	NA	NA
24	27.4	+	+	+	22.55	76	+	26.31	NA	−	NA	NA
25	120.3	+	+	+	24.04	76	+	24.72	NA	−	NA	NA
26	68.6	−	−	+	20.89	79	−	NA	NA	+	25.68	NA
27	6.6	−	−	+	27.86	79	−	NA	NA	+	33.89	NA
28	4.1	−	−	+	31.62	79	−	NA	NA	+	38.37	NA
29	18	−	−	+	22.64	79	−	NA	NA	+	29.79	NA
30	21.4	−	−	+	23.16	79	−	NA	NA	+	28.44	NA
31	1.7	−	−	+	31.74	79	−	NA	NA	+	38.22	NA
32	9.1	−	−	+	23.35	79	−	NA	NA	+	29.32	NA
33	6.3	−	−	+	27.06	79	−	NA	NA	+	31.69	NA
34	14.2	−	−	+	23.65	79	−	NA	NA	+	29.56	NA
35	31	−	−	+	23.67	79	−	NA	NA	+	28.93	NA
36	8.2	−	−	+	26.11	79	−	NA	NA	+	32.02	NA
37	13	−	−	+	24.44	79	−	NA	NA	+	29.73	NA
38	14.9	−	−	+	39.75	76	−	NA	30.06	−	NA	31.33
39	4.8	−	−	+	38.61	76	−	NA	29.61	−	NA	30.41
40	8.3	−	−	+	39.3	72	−	NA	29.47	−	NA	30.43
41	84	−	−	+	37.63	72	−	NA	29.64	−	NA	30.68
42	13.6	−	−	+	39.1	72	−	NA	29.57	−	NA	30.23
43	4	−	−	+	42.36	72	−	NA	29.46	−	NA	30.56
44	6.2	−	−	+	39.45	72	−	NA	29.58	−	NA	30.92
45	5.3	−	−	+	41.33	72	−	NA	29.8	−	NA	31.05
46	5.2	−	−	+	41.15	72	−	NA	29.87	−	NA	30.34
47	9	−	−	+	39.74	72	−	NA	29.91	−	NA	30.19
48	4.9	−	−	+	41.39	72	−	NA	29.84	−	NA	29.99
49	4.2	−	−	−	NA	/	−	NA	29.57	−	NA	29.71
50	94.9	−	−	−	NA	/	−	NA	29.94	−	NA	30.26

proteolytic enzymes, its role in pathogenesis of periodontitis remains unclear (Bonner et al. 2018).

Some investigators suggest that *E. gingivalis* could possess genes similar to those of *E. histolytica*, involved in the expression of cysteine proteinases or virulence factors (Trim et al. 2011). Therefore, some authors have speculated that the parasite is correlated to periodontitis but has not been formally demonstrated to be involved in its etiology, according to some criteria for causation (for instance, with respects to Koch's postulates), and a greater understanding of its role could open perspectives for more accurate therapies of this disease (Bonner et al. 2018).

In the oral cavity, amoebae feed on bacteria multiplying inside them instead of being destroyed (Trim et al. 2011). These bacteria could thus be protected from the immune system and antibiotic periodontal therapy. Without treatment, bacteria could then re-invade the tissue and cause a refractory case, explaining why periodontitis appears as a cyclical disease, with dynamic exacerbation and regression states (Linke et al. 1989; Trim et al. 2011). A commercial kit has been developed to detect bacteria found in the gingival sulcus or periodontal pocket (micro-Ident plus 11, Lifesciences) while it is not the case for *E. gingivalis*, which explains the use of in-house PCR (Santigli et al. 2016).

In Bonner's study, a category of samples was characterized by the microscopic observation of amoebae in accordance with the clinical dental aspects of patients but a negative PCR result. This could be due either to a lower amount of amoebae in samples, or confusion between the parasite and amoeba-like cells such as neutrophils (Bonner et al. 2014), or presence of the new variant *E. gingivalis* ST2 (kamaktlii) (Garcia, 2018 a; b). In our study, we didn't use microscopic observation of the samples because of its many limitations. As a result, the molecular detection of *E. gingivalis* seems relevant for this non-cultivable parasite. Here, the TaqMan PCR has the advantage of selectively detecting both *E. gingivalis* subtypes depending on the probe used.

Here, 2 conventional PCRs and 1 SYBR Green PCR were used simultaneously with both TaqMan assays on 50 clinical samples. Five DNA samples failed detection by the conventional EGO1-EGO2 PCR, whereas they

were detected with the 3 other techniques, assessing its lesser sensitivity. For these samples, the obtained Ct ranged between 31.9 and 34.09 corresponding to a low PCR amount, for a total DNA concentration of between 5 and 7.7 ng/μL. The amplification of these samples with conventional EGF-EGR PCR successfully assessed the interest of these primers. Regarding the SYBR Green PCR results, for 12 samples, an amplification was obtained, with a Ct < 38, and a fusion temperature at 79 °C, leading to the discovery that both 76 °C and 79 °C fusion temperatures allow to differentiate the variants (Trim et al. 2011).

Different *E. gingivalis* incidence values were found in the literature, probably reflecting various sample collection methods. In Trim's publication, *E. gingivalis* was detected in 27% of diseased gingival pockets by conventional PCR assay and in 69% of them by real-time PCR (SYBR Green). As a result, the latter technique was more sensitive. Nevertheless, the obtained fusion temperature and the distribution between ST1 and ST2 variants were not detailed. Moreover, in this study, *E. gingivalis* was not detected in any of the healthy gingival pocket sites regardless of the type of PCR (Trim et al. 2011).

In our study, the spiking method was used to eliminate the hypothesis of inhibitors in samples. In previous publications, this technique was described with conventional PCR; results were therefore visually assessed, a fainter band indicating the probable presence of inhibitors in samples (Bonner et al. 2014). Here, we used a quantitative method (real-time PCR) to interpret the results and an equation based on the normal rule as the best approach to suggest an inhibition. This adaptation renders this test more precise than conventional PCR.

Many studies have identified the amoeba in periodontal samples using different sampling protocols: biofilm was collected with only 1 paper point, curette or probe (Kikuta et al. 1996; Trim et al. 2011; Bonner et al. 2014). In Trim et al. (2011), only 1 paper point was inserted at the base of the sulcus for sample recovery, whereas in Kikuta et al. (1996), a subgingival plaque was sampled with a curette dispensed in 100 μl (Kikuta et al. 1996; Trim et al. 2011). In Bonner et al. (2014), 1 site was used for each patient; dental plaque was sampled with a probe, saliva-mounted, microscopically observed, and used for PCR if the amount was sufficient. In our study, subgingival microbiota from periodontal pockets of 5 mm or more were collected with 2 sterile paper points after isolating the tooth and cleaning the supragingival biofilm. Consequently, comparing results between studies seems difficult because methodologies are always variable (Bonner et al. 2014).

This new PCR assay, experimentally validated, is the first real-time system specifically targeting both *E. gingivalis* variants. The application of this new quantitative PCR could help in assessing whether usual non-surgical periodontal treatments targeting microbiota and including amoebae are effective, by measuring parasite loads before and after treatment.

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