

***Campylobacter* SPECIES IN CHILEAN PATIENTS WITH CHRONIC PERIODONTITIS**

ESPÉCIES DE *Campylobacter* EM PACIENTES CHILENOS COM PERIODONTITE CRÔNICA

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ABSTRACT: The purpose of this study is to identify the presence of *Campylobacter* species (*C. rectus*, *C. concisus*, *C. gracilis* and *C. ureolyticus*) in Chilean patients with chronic periodontitis, and to establish the relationship of these microorganisms with the periodontal conditions of smoker and non-smoker patients. Subgingival plaque samples were collected from four periodontal-affected tissue sites of 15 smoker and 15 non-smoker patients with chronic periodontitis. A sample per quadrant was obtained, with a probing depth of ≥ 6 mm and an insertion loss of >3 mm in each patient. Polymerase chain reaction (PCR) was performed with the specific 16S rDNA primers for the molecular detection of *C. rectus* and *C. gracilis*, specific *cpn60* primers for *C. concisus* and *hsp60* primers gene for *C. ureolyticus*. *Campylobacter* species showed an overall prevalence of 93.3% in periodontal patients, while *C. rectus* was the most frequent one (80%), followed by *C. concisus* (66.7%), *C. gracilis* (33.3%), and *C. ureolyticus* (10%). Only *C. gracilis* showed a statistically significant ($p = 0.002$) association with chronic periodontitis among samples from smoker and non-smoker patients. A high prevalence of the *Campylobacter* genus in the analyzed populations (93.3%) was found, being *C. rectus* the most frequent (80%) species. Besides, *C. gracilis* showed a statistically significant association between smoker state and chronic periodontitis.

KEYWORDS: *Campylobacter*. Chronic periodontitis. Smokers. PCR.

INTRODUCTION

Periodontitis is characterized by the presence of inflammatory lesions in the gingiva, with the formation of a periodontal pocket that leads to the loss of the alveolar bone and, eventually, affecting the tooth itself. The origin of infection of dental tissue is attributed to the accumulation of plaque (PEREZ-CHAPARRO et al., 2014; HENNE et al., 2014). Periodontal diseases are the second most prevalent diseases in the oral cavity. According to the Index of Periodontal Treatment Needs of the Community (GAMONAL et al., 1998), 20 years ago in Chile its prevalence was 90.9% among the population between 35 to 45 years of age, reaching 100% in 65 to 74-years-old people. Also, a recent study conducted in 2016, revealed a high prevalence of periodontitis, reaching more than 80% among Chilean patients over 18 years old (CARVAJAL, 2016).

The etiology of periodontal diseases is multifactorial, where various factors such as host, environment and infectious agents are involved (PEREZ-CHAPARRO et al., 2014; HENNE et al., 2014; VARGAS SEGURA et al., 2015; SRINIVASAN, 2016; TOMAS et al., 2016; MEURIC et al., 2017). *Campylobacter* species have

been isolated from humans and animals, being also associated with this disease (LASTOVICA, 2016). In humans, several species have been implicated as etiologic agents of periodontal disease (PEREZ-CHAPARRO et al., 2014; HENNE et al., 2014; VARGAS SEGURA et al., 2015). The *Campylobacter* species found in the oral cavity associated to oral pathologies are *C. rectus*, *C. concisus*, *C. gracilis*, *C. ureolyticus*, *C. curvus*, *C. showae* and *C. sputorum* (HENNE et al., 2014; VARGAS SEGURA et al., 2015).

C. rectus and *C. gracilis* are the most studied species in periodontal samples and considered to be the most prevalent *Campylobacter* species in periodontal tissue during disease (HENNE et al., 2014; VARGAS SEGURA et al., 2015). *C. concisus* was also identified in the oral cavity, being prevalent in biopsies of intestine and feces from patients with chronic gastrointestinal disorders (MUKHOPADHYA et al., 2011; MAHENDRAN et al., 2011; GEMMELL et al., 2017). *C. ureolyticus* has been detected in the subgingival bacterial plaque. However, it is rarely studied in periodontal disease (DUERDEN et al. 1987; DAHLÉN et al., 2018).

Scientific evidences show that smoking is a risk factor for periodontitis, being active smokers

more likely to develop more severe periodontal diseases, with higher levels of some of the periodontal microorganisms (BERGSTRÖM, 2003; SHCHIPKOVA et al., 2010; CORETTI et al., 2016).

Having in mind the great diversity and different structure-composition of the subgingival microbiota in chronic periodontitis (TOMAS et al., 2016), the high prevalence of this infectious condition and the scarce information about the different preperiodontopathogens in our country (CARVAJAL, 2016), it seems to be necessary to explore the association of *Campylobacter* species with chronic periodontitis.

Different molecular methodologies have been used to detect the presence of periodontal bacterial species, which are difficult or even impossible to culture (ERICK; PFISTER, 2002; SRINIVASAN, 2016; MIRA et al., 2017). Using PCR assay, we have attempted to determine the presence of *C. rectus*, *C. concisus*, *C. gracilis* and *C. ureolyticus* in periodontal pockets of Chilean patients with chronic periodontitis, establishing the relationship of the presence of these microorganisms with the pathological conditions of smokers and non-smokers.

MATERIAL AND METHODS

This is a descriptive cross sectional study, whose target population was adult patients diagnosed with moderate to severe chronic periodontitis at Valdivia city, Chile (39° 46' Southern latitude). Patient selection and sampling, and bacterial identification were carried out at the Dental Clinics and the Institute of Clinical Microbiology of the Universidad Austral de Chile, respectively. The sample size was calculated with the stratified probabilistic method, using the EpiDat 4.2 software, assuming a reference population of 250 patients, an expected frequency of the event under study of 90%, a confidence level of 95% and an error of 7% (GAMONAL et al, 1998). This study was approved by the Ethics Committee of the Universidad Austral de Chile.

The participants were adult smokers and non-smokers patients between 35 and 74 years old, who had been clinically and radiographically diagnosed with moderate to severe chronic periodontitis, with no underlying diseases or controlled-underlying diseases. The smoker status was determined in patients who smoked at least 5 cigarettes a day and had been smoking for a minimum of 10 years. Non-smoker patients were those who had never smoked in their lives. This information was gathered through a standardized

questionnaire. Any patients who have undergone an antimicrobial therapy, immunosuppressant therapy, treatments with bisphosphonates, steroidal and non-steroidal anti-inflammatory drugs in the last 6 months were not enrolled in the study, as well as pregnant patients with acute periodontal cases and with less than 20 teeth in the mouth.

After recording clinical measurements, 4 periodontal affected sites were selected in each patient of both groups, one per quadrant, with a probing depth ≥ 6 mm and insertion loss > 3 mm in each patient. The area was isolated with cotton rolls, gently dried with air. If the patient had supragingival deposits, they were carefully removed with curettes. Samples of subgingival material were obtained by inserting two sterile absorbent paper cones standard No. 30 in the deepest part of the periodontal pocket for 20 seconds. Immediately after removing the sample, it was placed into a 1.5 ml Eppendorf tube with sterile TE buffer solution (10 mM Tris-HCl, 1 mM EDTA; pH 8). The samples were transported at 4°C to the clinical microbiology laboratory for being immediately processed.

The tubes containing the samples were vortexed for 30 seconds and then subjected to boil for 5 minutes. Later, DNA extraction was performed using the Tissue DNA Kit (D3396-02 by E.Z.N.A <http://goo.gl/BRuv>) according to manufacturer's protocol.

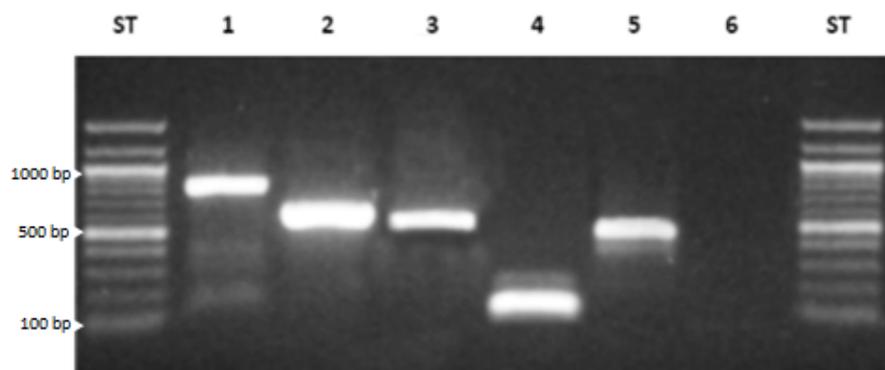
The reference DNAs were obtained from the following strains: *C. jejuni* ATCC 33560, *C. coli* NLEP 1726, *C. rectus* ATCC 33238, *C. concisus* ATCC 33237, *C. gracilis* ATCC 33236 and *C. ureolyticus* NCTC 10941. DNA extraction was performed following the above-mentioned protocol.

Specific oligonucleotides were designed using bibliographic references and databases published in NCBI GenBank (Table 1). For each primer, a search was performed using the BLAST (Basic Local Alignment Search Tool) program in order to verify their specificity and rule-out possible crossed reactions with other oral species by comparing primer sequences (ALTSCHUL et al., 1997).

The molecular detection of *Campylobacter* species was performed using the KOD Hot Start DNA Polymerase kit (TB341) (Novagen, Germany, 2003 (<http://goo.gl/L8zp0>) following the instructions of the manufacturer. PCR amplicons were analyzed by 2% agarose gel electrophoresis in TBE 1X buffer. The gel was stained with 0.5µg/ml ethidium bromide and visualized under ultraviolet light. Positive reactions were determined by the presence of bands of molecular size corresponding to species (Figure 1).

Table 1. Oligonucleotides sequence, amplicon size, and specific gene target and reference used for selecting each primer.

Oligonucleotides (5'-3')	Amplicon size (pb)	Target gen	Reference
<i>Campylobacter spp.</i> GGATGACACTTTTCGGAGC CATTGTAGCACGTGTGTC	816	16S rRNA	LINTON et al., 1996
<i>Campylobacter rectus</i> TTTCGGAGCGTAAACTCCTTTTC TTTCTGCAAGCAGACACTCTT	598	16S DNA	ASHIMOTO et al., 1996
<i>Campylobacter concisus</i> GGCTCAAAAGAGATCGCTCA CCCTCAACAACGCTTAGCTC	555	Cpn60 gene	CHABAN et al., 2009
<i>Campylobacter gracilis</i> AACGGAATTTAAGAGAGCTT CTTTCCCGATTATCTTATG	147	16S DNA	SIQUEIRA; ROÇAS, 2003
<i>Campylobacter ureolyticus</i> GAAGTAAAAAGAGGAATGGATAAAGAAGC CTTCACCTTCAATATCCTCAGCAATAATTTAAAAGA	540	hsp60 gene	VANDAMME et al., 2010

**Figure 1. PCR electrophoretic analysis of positive samples.** ST: standard molecular size; Lane 1: *Campylobacter spp.*; Lane 2: *Campylobacter rectus*; Lane 3: *Campylobacter concisus*; Lane 4: *Campylobacter gracilis*; Lane 5: *Campylobacter ureolyticus*; Lane 6: negative control.

Data were analyzed using descriptive and association statistics tests through the software SPSS Statistics 18 (IBM, Chicago, USA).

RESULTS

Subgingival crevicular fluid samples were taken from 30 patients (19 women and 11 men) with a mean age of 51.5 years, of whom 15 were smokers and 15 non-smokers. Demographic information and clinical parameters of the patients evaluated are summarized in Tables 2 and 3, respectively.

Campylobacter spp. frequency was high (93.3%) being *C. rectus* to be the most prevalent

species (80%) followed by *C. concisus* (66.6%), *C. gracilis* (33.3%) and *C. ureolyticus* (10%).

Campylobacter spp. showed a high prevalence in smoker patients with moderate to severe chronic periodontitis. Among these patients, the species *C. rectus*, *C. concisus*, *C. gracilis* and *C. ureolyticus* were found. In non-smoker patients, where *C. gracilis* was the least frequent one but *C. ureolyticus* was identified more frequently in non-smoker patients with chronic periodontitis than in smokers, while *C. gracilis* showed statistically significant differences associated with the smoking habit (Table 4).

Table 2. Patients distribution according to age and gender

	Age ^a	Male n (%)	Female n (%)	Total n (%)
Smokers	52 ± 7.6*	7 (46.7)**	8 (53.3)**	15 (100)
Non-smokers	51 ± 5.9*	4 (26.7)***	11 (73.3)***	15 (100)

^aMean ± standard deviation* Not statistically significant $p > 0.05$ (p value 0.69); ** not statistically significant $p > 0.05$ (p value 0.26); *** not statistically significant $p > 0.05$ (p value 0.26)**Table 3.** Clinical parameters found in the evaluated patients

Clinical parameters	Smoker	Non-smoker	p value
Bleeding on probing	59 ± 27.41	60.8 ± 28.29	0.444
Pocket probing depth	5.5 ± 0.90	5.4 ± 0.49	0.367
Clinical attachment level	8.44 ± 3.05	9 ± 3.08	0.352
Pocket probing depth	76.26 ± 22.83	70.89 ± 24.83	0.319

Statistically significant $p < 0.05$ **Table 4.** Campylobacter species found among smoker and non-smoker patients

	Frequency		Value of p (χ^2)
	Smokers (%)	Non-smokers (%)	Smokers v/s Non-smokers
Campylobacter spp.	100	86.7	0.143
<i>C. rectus</i>	93.3	66.7	0.068
<i>C. concisus</i>	73.3	60	0.439
<i>C. gracilis</i>	60	6.7	0.002 ^a
<i>C. ureolyticus</i>	6.7	13.3	0.543

^aStatistically significant ($p = <0.05$)

DISCUSSION

The results of this study indicated a high prevalence of *Campylobacter* spp. in the population under study. PCR analysis revealed the presence of the species *C. rectus*, *C. gracilis*, *C. concisus* and *C. ureolyticus*, while the presence of *C. gracilis* was found to be statistically significant in smoker patients with moderate to severe chronic periodontitis.

The clinical parameters of the patients did not show significant differences; smokers and non-smokers had similar characteristics although a slight difference caused a plaque index with a lower rate in non-smokers. However, the evidence showed that there are no differences of plaque among smokers and non-smokers, suggesting that there is no association between the accumulation of plaque and oral hygiene of the patient (BERGSTRÖM, 2003; APATZIDOU et al., 2005).

The prevalence of *C. rectus* (80%) was high and similar to the findings of RAMS et al., (1993). However, it exceeds the frequency reported by other authors (MACUCH; TANNER, 2000; KAMMA et

al., 1999; GAJARDO et al., 2004). Nevertheless, all the studies mentioned, agree on that *Campylobacter* is the most frequent microorganism in chronic periodontitis.

C. concisus showed a prevalence of 66.6%, which is higher than the one reported by KAMMA et al. (1999) and MACUCH and TANNER (2000). However, the detection of *C. gracilis* was low (6.7%), but differed from the study of MACUCH and TANNER (2000), who reported a smaller occurrence of this pathogen. DUERDEN et al. (1987), found a prevalence of 6.7% of *Bacteroides ureolyticus*, later reclassified as *C. ureolyticus* (VANDAMME et al., 2010). These results are lower than those obtained in our investigation. Significantly, the prevalence of periodontal pathogens differs from one geographical area to another (HAFFAJEE et al., 2004; HERRERA et al., 2008), explaining the differences between the various studies.

We found a higher occurrence of *Campylobacter* spp. in smoker patients with chronic periodontitis than in non-smokers. *C. gracilis* showed a statistically significant difference in

smokers, which was also observed by KAMMA et al. (1999) for *C. concisus*, *C. rectus* and *C. gracilis*. However, in our study, *C. rectus* and *C. concisus* showed no statistically significant differences, although they had a higher detection values in smokers than in non-smokers.

Reported evidences show that the smoker patients displayed a decrease of oxygen tension in the oral cavity, creating favorable environmental conditions in the periodontal pocket for the colonization of certain periodontopathogens (ABUSLEME et al., 2013; NICOLIĆ-JAKOBA et al., 2012). Therefore, it can be suggested that the oxygen conditions are important for *C. gracilis*, *C. rectus* and *C. concisus* and, in this oxygen reduced subgingival environment, they could increase the prevalence of *C. gracilis* for being a strict microaerobic microorganism (LASTOVICA, 2016).

C. ureolyticus was more frequently found in non-smokers patients and, even though there was no statistical difference, it is necessary to perform further studies to determine its prevalence in other geographical centers to contrast values and describe environmental conditions that could favor the colonization of this agent in the periodontal pocket.

Although the PCR provides a faster and more accurate molecular detection of microorganisms compared to the traditional culture methods of cultivation (ERICK; PFISTER, 2002), it fails to determine other characteristics of the

identified species. Therefore, the combined use of traditional microbiological methods with molecular methods would allow performing additional monitoring of microbiological diagnosis for patients with periodontal diseases (LINTON et al., 1996).

This is the first study carried out in Chile, showing the prevalence of species of *Campylobacter* associated to chronic periodontitis. These results represent an advance and a contribution to the best knowledge about the agents of periodontitis in this geographical region, opening new paths and perspectives of association between the clinic and the laboratory. The association between the clinic and the laboratory, in addition to improving the etiological diagnosis of chronic periodontitis, will make it possible to establish programs for the control and prevention of this disease.

Our results demonstrated a high prevalence of *Campylobacter* in the analyzed population, showing an association between species of the genus *Campylobacter* and periodontal disease, suggesting that smoking is a risk factor for the association of *C. gracilis* with moderate and severe chronic periodontitis.

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RESUMO: O objetivo deste estudo é identificar a presença de espécies de *Campylobacter* (*C. rectus*, *C. concisus*, *C. gracilis* e *C. ureolyticus*) em pacientes chilenos com periodontite crônica, e estabelecer a relação desses microrganismos com as condições periodontais de pacientes fumantes e não fumantes. Amostras de placa subgingival foram coletadas de quatro locais de tecido periodontal afetado de 15 pacientes fumantes e 15 não fumantes com periodontite crônica. Obteve-se uma amostra por quadrante, com profundidade de sondagem ≥ 6 mm e perda de inserção > 3 mm em cada paciente. A reação em cadeia da polimerase (PCR) foi realizada com os primers específicos 16S rDNA para a detecção molecular de *C. rectus* e *C. gracilis*, primers específicos de *cpn60* para *C. concisus* e gene primers de *hsp60* para *C. ureolyticus*. As espécies de *Campylobacter* apresentaram uma prevalência geral de 93,3% nos pacientes periodontais, enquanto *C. rectus* foi o mais frequente (80%), seguido por *C. concisus* (66,7%), *C. gracilis* (33,3%) e *C. ureolyticus* (10%). Apenas *C. gracilis* apresentou associação estatisticamente significativa ($p = 0,002$) com a periodontite crônica entre as amostras de pacientes fumantes e não fumantes. Observou-se alta prevalência do gênero *Campylobacter* nas populações analisadas (93,3%), sendo *C. rectus* as espécies mais frequentes (80%). Além disso, *C. gracilis* mostrou associação estatisticamente significativa entre estado de fumante e periodontite crônica.

PALAVRAS-CHAVE: *Campylobacter*. Periodontite crônica. Fumantes. PCR.

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