UNIVERSIDADE DE TAUBATÉ
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Ocorrência de patógenos periodontais na cavidade bucal humana: relação com status periodontal, idade e tabagismo

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Ocorrência de patógenos periodontais na cavidade bucal humana: relação com status periodontal, idade e tabagismo

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Orientadora: Profa. Dra. Sheila Cavalca Cortelli
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2009

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Dedico este trabalho à minha esposa **Juliana**:

Eterna companheira;

Maior incentivadora;

Amor da minha vida.

Aos Nossos filhos **Vinícius** e **Joaquim**:

Razão de nossa existência.

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RESUMO

Hipótese do estudo: Esse estudo hipotetizou que o fator idade e a condição periodontal pessoal e/ou materna favorecem a ocorrência de patógenos periodontais, enquanto o tabagismo não a influencia. **Objetivo:** Avaliar a relação do fator idade, status periodontal e tabagismo sobre a presença de *A. actinomycetemcomitans, P. gingivalis, P. intermedia, T. forsythia* e *C. rectus*. E, o impacto do status periodontal materno sobre a ocorrência destas mesmas espécies em recém-nascidos. **Metodologia:** Para responder à questão relativa à idade, na primeira fase do estudo, independente da condição periodontal foram incluídos 330 indivíduos alocados em seis faixas etárias: recém-nascidos, crianças de 2,5 a 5 anos, crianças de 6 a 12 anos, adolescentes de 13 a 18 anos, adultos de 19 a 44 anos e adultos acima de 55 anos. Na segunda fase do estudo, na qual os fatores status periodontal e tabagismo foram considerados, selecionaram-se mais 76 pares mães/recém-nascidos (33 filhos de mães com periodontite e 43 de mães sem periodontite), adicionando-se ainda 112 crianças periodontalmente saudáveis, 109 crianças com gengivite, 40 adultos periodontalmente saudável (21 fumantes e 19 não fumantes) e 111 indivíduos com periodontite (53 fumantes e 58 não fumantes). Os parâmetros clínicos periodontais avaliados foram profundidade de sondagem e nível clínico de inserção (adolescentes e adultos), e, índice de placa e sangramento gengival (adultos, adolescentes e crianças). Amostras subgengivais foram coletadas dos participantes dentados enquanto amostras não dentárias foram coletadas de todos os grupos e todas processadas por PCR. **Resultados:** Considerando-se todos os grupos *C. rectus* foi a bactéria mais freqüente chegando a atingir prevalências de 98%. A ocorrência de *P. gingivalis, P.intermedia, A. actinomycetemcomitans* e *T. forsythia* foi mais elevada dentre os recém-nascidos filhos de mães com periodontite. Crianças com gengivite alocaram mais *P. gingivalis* no sulco (99,1%) e mucosa (97,2%) do que crianças periodontalmente saudáveis (67,5% e 64,8%, respectivamente). A análise intra-grupo de adultos periodontalmente saudáveis ou doentes não revelou diferenças estatisticamente significativas em relação a presença bacteriana comparando-se fumantes e não fumantes. **Conclusões:** A colonização bucal por patógenos periodontais ocorre precocemente e se altera ao longo da vida, sendo muitas vezes direcionada pela presença dos elementos dentários que no presente estudo influenciou sobretudo a ocorrência de *P. gingivalis*, *P. intermedia* e *T. forsythia*. Outra observação importante foi a de que a condição periodontal materna influenciou a colonização por patógenos periodontais em recém-nascidos. Similarmente, o status periodontal infantil acarretou aumento nas freqüências bacterianas. Finalmente, o tabagismo não alterou os perfis microbiológicos observados.

Palavras-chave: Bactéria, PCR, Gengivite, Periodontite, Grupos Etários, Tabagismo.

AQUINO DR. Occurrence of periodontal pathogens in the human oral cavity: association with periodontal status, age and tobacco use [Tese de doutorado]. Taubaté: Universidade de Taubaté, Faculdade de Odontologia, 2009. 77p.

ABSTRACT

Study hypothesis: The current study hypothesized that age and personal and/or maternal periodontal status favor the occurrence of periodontal pathogens, whereas tobacco use does not. **Aim:** To evaluate the relation between age, periodontal status and smoking and the presence of *A. actinomycetemcomitans, P. gingivalis, P. intermedia, T. forsythia* and *C. rectus*. Also, the impact of the mother's periodontal status on the occurrence of the same species in newborns. **Methodology:** In order to elucidate what's the age contribution, in the first stage of the study, regardless of periodontal status, 330 individuals were allocated in six age groups: newborns, children from 2.5 to 5 years, children from 6 to 12 years, adolescents from 13 to 18 years, adults from 19 to 44 years and adults over 55 years. In the second stage of the study, in which periodontal status and smoking were considered, another 76 mother/newborn pairs were chosen (33 sons of periodontitis mothers and 43 of no periodontitis mothers), another 112 periodontally healthy children, 109 gingivitis children, 40 periodontally healthy adults (21 smokers and 19 non-smokers) and 111 periodontitis subjects (53 smokers and 58 non-smokers) were added. The periodontal clinical parameters evaluated were probing depth and clinical attachment level (adolescents and adults), and, plaque index and gingival bleeding index (adults, adolescents and children). Subgingival samples were collected from dentate participants whereas non dental samples were collected from each group and were PCR-processed. **Results:** *C. rectus* was the most frequent bacterium for all groups, reaching 98% prevalence. The occurrence of *P. gingivalis, P. intermedia, A. actinomycetemcomitans* and *T. forsythia* was higher among newborns that are sons of mothers with periodontitis. Children with gingivitis allocated more *P. gingivalis* in the sulcus (99.1%) and mucosa (97.2%) than periodontally healthy children (67.5% and 64.8%, respectively). The intra-group analysis of periodontally healthy or diseased adults did not reveal statistically significant differences, when smokers and non-smokers were compared. **Conclusions:** Oral colonization by periodontal pathogens happens early in life and alters overtime, being many times guided tooth presence that, in the current study, influenced, above all, the occurrence of *P. gingivalis*, *P. intermedia* and *T. forsythia*. Another relevant finding was that the maternal periodontal status did influence the occurrence of periodontal pathogens in newborns. Similarly, the childhood periodontal status allowed increased bacterial frequencies. Finally, tobacco use did not alter the observed microbiological profiles.

Keywords: Age Groups, Gingivitis, Periodontitis, Bacteria, Polymerase Chain Reaction, Smoking.

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1 INTRODUÇÃO

A doença periodontal (DP) é uma doença de etiologia multifatorial, de origem infecciosa, deste modo iniciada pelo acúmulo de biofilme dentário, que acomete os tecidos de proteção e sustentação dos dentes. Conforme revisado por Nunn (2003), sua manifestação, gravidade e progressão são determinadas por diversos fatores, tais como: condições sociais, fatores sistêmicos, genéticos e demais fatores de risco atuantes (Nunn, 2003), além é claro da própria composição microbiana do biofilme. Assim, pode-se ter noção da complexidade da periodontia que tem buscado não apenas descrever cada um desses fatores como também avaliar suas possíveis associações.

Considerando-se a aceitação do biofilme dentário como agente etiológico primário das formas clássicas de gengivite e periodontite poder-se-ia supor que o conhecimento a seu respeito se encontrasse em um estágio mais avançado. Todavia, muitas questões relacionadas a microbiota periodontal e sua interação com o sistema imune do hospedeiro precisam ser elucidadas. E, interessantemente, por mais que se busque analisar e entender a resposta imuno-inflamatória em um número elevado de casos atuar sobre ou modificar o biofilme representa uma medida de saúde eficaz justamente por ser uma interferência no seu agente desencadeador. Adicionalmente, contribuir com o conhecimento sobre o componente microbiano bucal transcende as questões odontológicas pelo fato de paralelamente favorecer a saúde sistêmica e adentrar questões mais abrangentes como a percepção de saúde e qualidade de vida das pessoas.

Estimulados por esse interessante contexto, a presente tese se alicerçou em dois grandes estudos. No primeiro deles objetivamos esclarecer os momentos mais propícios para a colonização bacteriana intra-bucal que cada indivíduo pode

encontrar ao longo de sua vida. Essa proposta surgiu, sobretudo, da carência de dados e divergências encontradas na literatura, como abordaremos a seguir.

Cerca de 600 espécies bacterianas podem ser detectadas em amostras de cavidade bucal humana. Normalmente, 30 a 40 gêneros bacterianos podem ser isoladas de uma única amostra subgengival (Paster et al., 2002; Socransky, Haffajee, 2002; Kazor et al., 2003). Logo após o nascimento, as primeiras bactérias que colonizam a cavidade bucal do bebê derivam do trato geniturinário, pele ou cavidade bucal materna. Na ausência de dentes, os primeiros colonizadores tem que apresentar fatores de aderência bem desenvolvidos (Long & Swenson 1976). Embora as criança apresentem uma microbiota predominantemente Gram-positiva (Könönem et al, 1992), os anaeróbios também podem ser isolados.

Kimura et al. (2002), avaliaram a prevalência de 10 patógenos periodontais em 144 crianças de 2 a 13 anos de idade (12 crianças de cada idade). Após processamento por PCR, foi observada prevalência de *A. actinomycetemcomitans*, *C. rectus, E. corrodens, C. ochracea* e *C. sputigena* em torno de 50% em todas as idades avaliadas, demonstrando uma colonização precoce de alguns periodontopatógenos em crianças. Porém, *P. gingivalis* e *T. denticola* não foram detectadas em nenhuma criança avaliada. Sakai et al. (2007), avaliaram a prevalência de 4 patógenos periodontais (*A. actinomycetemcomitans, P. gingivalis, P. nigrescens* e *T. denticola*) na saliva de crianças com dentição mista em dois tempos diferentes com intervalo de um ano. Foram observadas prevalências de 4,7 e 1,7% para *A. actinomycetemcomitans,* 6,3 e 8,3% para *P. gingivalis*, 23,4 e 48,3 para *P. nigrescens* e 71,9 e 50% para *T. denticola* nos anos de 2003 e 2004 respectivamente. Diante dos resultados encontrados, os autores destacaram a elevada prevalência dos patógenos periodontais pesquisados em crianças com

condições de saúde dos tecidos periodontais. Kulekci et al. (2008) avaliaram a prevalência de *A. actinomycetemcomitans*, *P. gingivalis, T. forsythia, P. intermedia, P. nigrescens* e *T. denticola* em amostras de saliva estimulada de 41 crianças clinicamente saudáveis. *P. nigrescens* foi o patógeno periodontal mais prevalente (80%), seguido por *T. denticola* (32%), *A. actinomycetemcomitans* (24%) e *P. gingivalis* (12%). Já *P. intermedia* e *T. forsythia* não foram detectadas em nenhum indivíduo. Os autores destacaram a importância da detecção de alguns patógenos periodontais em crianças periodontalmente saudáveis, justificando uma possibilidade de transmissão por via salivar.

Similarmente, tanto na adolescência (Hamlet et al, 2004) quanto na vida adulta (Avila-Campos & Velasquez-Melendez 2002) patógenos periodontais verdadeiros podem ser isolados de indivíduos periodontalmente saudáveis. Com o avançar da idade nota-se que a perda dentária é acompanhada pelo desaparecimento de sítios especializados o que acarreta novamente redução na ocorrência de patógenos periodontais.

Cortelli et al (2008), investigaram a colonização de *C. rectus, P. gingivalis, A. actinomycetemcomitans, P. intermedia* e *T.forsythia* em recém-nascidos e adultos desdentados. Após processamento por PCR de amostras de dorso da língua e mucosa jugal de 74 indivíduos, os autores observaram que *C. rectus* foi o patógeno periodontal mais prevalente em ambos os grupos (20,9% na mucosa jugal de recém-nascidos e 77,4% na língua de desdentados). *P. gingivalis* e *P. intermedia* não foram detectados em nenhum recém-nascido, porém observou-se *P. gingivalis* na língua e mucosa dos desdentados (3,2%) assim como *P. intermedia* (9,6% na língua e 3,2% na mucosa). *T. forsythia* foi detectada tanto em recém-nascidos quanto em adultos desdentados, porém, sua maior prevalência foi observada na língua de recém-

nascidos (6,9%) e adultos desdentados (9,6%). A. *actinomycetemcomitans* foi isolado da mucosa de recém-nascidos (2,3%) e língua (12,9%) e mucosa (6,4%) de adultos desdentados. Diante dos resultados os autores destacaram a importância da detecção de patógenos periodontais em indivíduos desdentados.

A partir dos resultados da primeira etapa, que incluiu um grande número de indivíduos com saúde periodontal, apresentados no capítulo 1, outros questionamentos surgiram. Em termos gerais, a proposta central do segundo estudo foi confrontar os perfis microbianos encontrados na saúde e doença dos tecidos periodontais em faixas etárias específicas.

O potencial de transmissão intra-familiar exibido pelos periodontopatógenos tem sido alvo de alguns estudos (Tamura *et el.*, 2006) os quais enfocaram principalmente *A. actinomycetemcomitans* e *P. gingivalis* (Greenstein & Lamster, 1997) e apenas mais recentemente ampliaram o número de patógenos avaliados (Tamura *et al.*, 2006). Como o momento de aquisição destes periodontopatógenos no período pós-parto é ainda incerto (Frisken *et al.*, 1990) as pesquisas envolvendo a transmissão de mães a recém-nascidos têm justamente procurado esclarecer essa questão (Lee *et al.*, 2006).

Tem sido sugerido que mães com doença periodontal atuam como potenciais reservatórios de patógenos capazes de colonizar a cavidade bucal de seus filhos (Watson, 1994; Tamura *et al.*, 2006). Todavia, os estudos ainda são inconclusivos em determinar se essa primeira colonização é apenas transitória ou se ao contrário, pode aumentar o risco futuro de desenvolvimento para a doença periodontal (Ooshima *et al.*, 2003; Sakai *et al.*, 2007). Supondo que as mães pudessem atuar fonte de patógenos, Rosa e colaboradores (2002) avaliaram a influência materna sobre a condição bacteriológica e clínica de seus filhos com idades entre 5 e 6 anos.

Interessantemente, as crianças em idade escolar apresentaram padrões de higiene oral e perfis bacterianos semelhantes ao de suas mães. A transmissão intra-familiar foi confirmada por Kimura et al. (2002). E mais tarde esse mesmo autor e seus colaboradores (Tamura et al., 2006), concluíram que as mães deveriam se preocupar com a condição periodontal dos seus filhos, além é claro da sua própria condição periodontal.

Embora a associação bacteriana nos pares mães-filhos venha sendo avaliada, a detecção de patógenos periodontais pode ser tecnicamente inviável na comunidade geral, em especial em países em desenvolvimento como o Brasil. Mas, o exame clínico periodontal é de fácil execução e de baixo custo. Dessa forma, se a condição clínica materna influenciar a ocorrência precoce de patógenos em seus filhos teremos no diagnóstico clínico um método de aplicação ampla que poderá beneficiar a ambos. Deste modo, essa foi a primeira questão avaliada na segunda fase do estudo (Capítulo 2).

Os estudos conduzidos com a população infantil têm incluído, sobretudo crianças periodontalmente saudáveis (Farsi et al, 2008; Kulekci et al, 2008) buscando por meio de raros estudos longitudinais estabelecer o risco de periodontite agressiva (Fine et al, 2007; Haubek et al, 2008). A fim de avaliar a ocorrência bacteriana, poucas pesquisas têm enfocado simultaneamente grupos populacionais infantis com periodonto saudável e gengivite (Gafan et al, 2004; Tanaka et al, 2008). No estudo de 2004, Gafan et al, estimaram por PCR a prevalência bacteriana no biofilme de crianças na presença (n = 53) ou ausência de gengivite (n = 65). Detectou-se *P. gingivalis, A. actinomycetemcomitans* e *T. forsythensis* em ambos os grupos, independente da condição gengival tendo sido a última bactéria associada a sítios saudáveis. Assim, no capítulo 3 comparamos a ocorrência de *A.*

actinomycetemcomitans, P. intermedia, P. gingivalis, *T. forsythia* e *C. rectus* em crianças periodontalmente saudáveis e com gengivite.

Em relação a população adulta, parece haver aumento na prevalência e nas contagens bacterianas associado ao desenvolvimento e gravidade da doença periodontal (Ximenez-Fyvie *et al.*, 2000; Tanaka *et al.*, 2002; Cortelli *et al.*, 2005; Bodet *et al.*, 2007). No entanto, o predomínio de alguns gêneros bacterianos predispõe o indivíduo a desenvolver lesões periodontais específicas, como gengivite, periodontite crônica e agressiva (Potera, 1999). Söder *et al.*, (1993) destacaram a relação entre a infecção polimicrobiana que ocorre em casos de periodontite com o alto grau de destruição tecidual observada enquanto Slots *et al.* (1995) destacaram a elevada prevalência de *P. gingivalis* e *P. intermedia* em indivíduos com periodontite avançada. Moter et al. (2006) sugeriram o papel dos treponemas como indicadores de periodontite. Já Takeshita et al. (2009) demonstraram relação entre a ocorrência salivar de microrganismos comensais com saúde peridontal. Outros achados incitam novos questionamentos sobre a microbiota periodontal, por exemplo, a relevância anteriormente atribuída aos níveis de leveduras (Farsi et al. 2008) assim como a relação de espécies bacterianas menos freqüentes, como *S. aureus* e *S. mutans*, com a diferenciação de sítios periodontias entre indivíduos com periodontite crônica ou agressiva (Fritschi et al., 2008). O que não se sabe ao certo é se esse mesmo perfil microbiano se aplica à população brasileira adulta em virtude da influência geográfica sobre a microbiota ter sido previamente apontada pela literatura (Haffajee et al, 2004). No capítulo 4 avaliou-se a influência da condição clínica periodontal sobre a presença de patógenos periodontais em adultos. Adicionalmente, nesse mesmo capítulo objetivou-se verificar uma possível influência do tabagismo sobre a microbiota bucal.

Apesar de reconhecido fator de risco para a periodontite a influência do tabagismo sobre a microbiota periodontal ainda não foi esclarecida (Rivera-Hidalgo, 2003). Van Winkelhoff et al. (2001) propuseram uma análise comparativa da microbiota subgengival após terapia periodontal entre pacientes fumantes e não fumantes e concluíram que o hábito de fumar pode ser um importante fator na determinação da microbiota subgengival. Eggert *et al.* (2001) reportaram um dado interessante ao sugerir que em fumantes, mesmo as bolsas rasas (< 5mm de PS) representam um *habitat* favorável para *A. actinomycetemcomitans, P.gingivalis* e *P. intermedia.* Porém, neste mesmo ano, Boström *et al.* (2001) não encontraram diferenças significativas entre a microbiota de fumantes e não fumantes com periodontite avançada.

Mais tarde, em 2005, Apatzidou et al. falharam ao demonstrar interferência do tabagismo sobre a prevalência de patógenos periodontais Gomes et al. (2006) relacionaram os perfis periodontais clínico e microbiológico com o hábito de fumar. Os resultados advindos de análise por PCR em tempo real mostraram prevalência similar de *P. gingivalis* entre os grupos enquanto *M. micros* e *D. pneumosintes* foram mais prevalentes dentre os fumantes. Em 2007, Könönen et al. observaram maior prevalência de *P. intermedia* e *T. denticola* e menor prevalência de *A. actinomycetemcomitans* nos indivíduos que fumavam diariamente em comparação aos nunca fumantes.

Pelo exposto, a presente tese tentou contribuir com o conhecimento relativo à microbiota bucal de indivíduos em diferentes faixas etárias e de como ela pode ser influenciada por fatores específicos. Adicionalmente, procurou-se verificar se determinados padrões ocorrem também na nossa população.

2 CAPÍTULOS

2.1 CAPÍTULO 1

Título do projeto: Detecção de microrganismos periodonto patogênicos, avaliação de fatores e indicadores de risco e suas associações com a condição bucal e periodontal desde recém-nascidos à idosos

Delineamento: estudo transversal

População total: 330 indivíduos alocados em sete grupos: (A) recém-nascidos, (B) crianças de 2,5 a cinco anos, (C) crianças de seis a 12 anos, (D) adolescentes de 13 a 18 anos, (E) adultos de 19 a 44 anos, (F) adultos dentados com mais de 55 anos e (G) adultos desdentados com mais de 55 anos.

Objetivo: Elucidar as faixas etárias nas quais ocorre a colonização inicial por periodontopatógenos na cavidade bucal humana.

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Etiological Analysis of Initial Colonization of Periodontal Pathogens in Oral Cavity[▽]

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It is unclear when the initial colonization by periodontal pathogens occurs in the oral cavity. Therefore, we report here the association between specific age groups and the time when the initial colonization by periodontal pathogens occurs in the oral cavity in such groups. Findings are based on an epidemiological analysis of the prevalence of five periodontal pathogens in the oral cavities of a wide range of age populations, from newborn to elderly, who were randomly selected in a geographic region of Brazil. These periodontal pathogens include *Campylobacter rectus*, *Porphyromonas gingivalis*, *Aggregatibacter actinomycetemcomitans*, *Prevotella intermedia*, and *Tannerella forsythia* and were analyzed in the bacterial samples isolated from gingival sulcus, the dorsum of the tongue, and cheek mucosa of diverse age groups, using a bacterial DNA-specific PCR method. Results indicated that there are distinct age-related groups where initial colonization by the five periodontal pathogens examined in this study can be detected and that the presence of teeth is a permissive factor for colonization by *P. gingivalis*, *P. intermedia*, and *T. forsythia*. Although it remains unclear exactly how or when target pathogens colonize healthy subjects, an understanding of age-related groups does provide a potentially useful tool in the early detection and prevention of periodontitis in healthy individuals.

Recent studies demonstrated that not only are periodontal pathogens, such as *Porphyromonas gingivalis*, *Prevotella intermedia*, and *Tannerella forsythia*, present in the subgingival pockets of periodontally diseased patients but they also colonize other oral sites, including, for example, the supragingival plaques of the same patients. This indicates that these gram-negative anaerobic bacteria are also present in an aerobic atmosphere (23). In addition, these pathogens were found in both sub- and supragingival plaques of periodontally healthy subjects (25). These findings imply that colonization by these pathogens may occur (i) in the aerobic environment of periodontally healthy subjects and (ii) in the oral cavity of healthy subjects as commensal bacteria. However, when commensal bacteria convert to a pathogenic state, a further significant implication is that such conversion may be causal for the onset of periodontal disease (10).

Based on the findings noted above, a novel, although still hypothetical, paradigm is emerging to explain the properties of bacteria associated with periodontal disease. These bacterial pathogens, including *Porphyromonas gingivalis*, *Prevotella intermedia*, and *Tannerella forsythia*, are opportunistic pathogens and, therefore, do not follow Koch's postulates (29). Opportunistic pathogens are infectious agents that usually do not induce disease in a healthy host, but they can affect people with a poorly functioning or suppressed immune defense system. Under these conditions, it would be useful to gain (i) an understanding of when initial colonization by these periodontal pathogens occurs in the oral cavity of healthy subjects and (ii) an ability to establish such colonization in site-specific tissue. This would lead to the development of relevant information for the development of diagnostic and prevention strategies for periodontal disease, even though the mechanisms underlying the onset and progression of periodontal disease remain unclear. *Streptococcus mutans* is a causal microorganism for dental caries, and it occurs in the oral cavity of infants aged between 3 and 24 months (21). As such, *S. mutans* provides a classic example illustrating a temporal gate for colonization by a bacterium, since it has been reported that early colonization by *S. mutans* occurs in approximately 80% of infants. To date, however, it has been unclear whether such age-related groups for the initial colonization by periodontal pathogens exist. Furthermore, it has not yet been reported what, if any, site-specific tissues may be associated with such initial colonization.

To address these questions, the present study conducted an epidemiological analysis of the prevalence of periodontal pathogens in the oral cavities of a wide range of age populations from newborn to elderly who were randomly selected in a geographic region of Brazil. In particular, study parameters dictated that the randomly selected subject population should have an incidence of periodontal diseases proportional to the whole population in the region. Since some anaerobic pathogens may require a deep (pathogenic) periodontal sulcus (>3 mm) for their initial colonization, both healthy and periodontally diseased subjects were recruited in this study. More specifically, the five species of periodontal pathogens, *Campylobacter rectus*, *P. gingivalis*, *Aggregatibacter actinomycetemcomitans*, *P. intermedia*, and *T. forsythia*, were screened from the bacteria sampled from subgingival sulcus, cheek mucosa, and the dorsum of the tongue by a bacterium-specific DNA

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TABLE 1. Subject classification by age-related groups and clinical periodontal profiles in each group[a]

Group	Age (yr [mo for group A])		No. of subjects		Plaque index [mean ± SD (range)]	Gingival index [mean ± SD (range)]	PPD (mm) [mean ± SD (range)]	CAL (mm) [mean ± SD (range)]
	Range	Mean ± SD	Per group	Male/female				
A	0–4	2.84 ± 1.60	43	25/18	ND[b]	ND	ND	ND
B	2.5–5	3.88 ± 1.02	36	15/21	ND	ND	ND	ND
C	6–12	9.33 ± 1.99	33	18/15	0.40 ± 0.16 (0.04–0.76)	0.02 ± 0.03 (0.0–0.1)	ND	ND
D	13–18	14.73 ± 1.34	30	22/8	0.53 ± 0.31 (0.00–1.00)	0.22 ± 0.27 (0.00–1.00)	1.91 ± 0.85 (0.95–4.63)	0.93 ± 0.76 (0.04–3.11)
E	19–44	34.60 ± 7.49	63	12/51	0.65 ± 0.29 (0.00–1.05)	0.51 ± 0.30 (0.00–1.05)	2.90 ± 0.79 (1.53–4.69)	2.94 ± 1.29 (0.03–5.95)
F	>55 (with teeth)	59.75 ± 6.28	60	33/27	0.49 ± 0.36 (0.00–1.00)	0.40 ± 0.29 (0.00–1.00)	2.99 ± 0.83 (1.26–4.85)	3.36 ± 1.55 (0.30–6.86)
G	>55 (without teeth)	64.75 ± 8.49	65	33/32	ND	ND	ND	ND

[a] Plaque and gingival index data represent mean values per age-related group determined on the basis of all clinical scores. A plaque index score of 0 represents the absence of supragingival plaque, and a score of 3 represents a large amount of plaque. A gingival index score of 0 represents the absence of gingival inflammation, and a score of 3 indicates severe gingival inflammation.

[b] ND, not determined.

sequence method using PCR. The overall aim of this study was to elucidate the age ranges when initial colonization by periodontal pathogens occurs in the oral cavity.

MATERIALS AND METHODS

Human subjects. Participants included in the present epidemiological study were recruited from the region of Vale do Paraíba, São Paulo, Brazil. The eligible sample population was recruited from a total of 1,839 subjects who visited the Department of Dentistry, University of Taubaté, from August 2004 to July 2006. After screening for the exclusion criteria, which are described below, 330 Brazilian subjects representing multiple ethnic groups were finally recruited for the study. These subjects were allocated among seven different generational groups, as follows: (A) newborns with no teeth, (B) children aged 2.5 to 5 years, (C) children aged 6 to 12 years, (D) adolescents aged 13 to 18 years, (E) adults aged 19 to 44 years, (F) elderly over 55 years of age with teeth, and (G) elderly over 55 years with no teeth. A summary of the human subject data, including the number in each age-generational group, age range, and number of males and females, as well as smoking habits, is shown in Table 1.

Data and personal information related to medical and dental histories of the subjects were obtained from responses by individuals or their parents to our questionnaire. Either subjects themselves, or their legal guardians, signed an informed consent form, which was previously approved by the Institutional Committee on Research Involving Human Subjects of the University of Taubaté (protocol 362/03).

Exclusion criteria for subject recruitment. Subjects presenting any of the following conditions were excluded from the study: (i) antibiotic prophylaxis for dental treatment, (ii) uncontrolled systemic diseases, (iii) immunological compromise, and (iv) pregnancy or women currently breast-feeding. Also excluded were subjects (i) who were wearing orthodontic devices, (ii) who had been undergoing periodontal treatment 12 months before the beginning of the study or had been taking antibiotics within 6 months prior to the clinical and microbial examination, or (iii) whose first and second molars or mesial and lateral incisors were missing or who had fewer than three molars or only one incisor remaining. As discussed in "Human subjects," the age limit for the older subjects was not specifically set in this study; rather, both elderly subjects over 55 years with teeth (F) and those without teeth (G) were included.

Clinical measurements and diagnosis. One trained and certified examiner conducted all clinical measurements and collected the microbial samples. The calibration protocol for the optimization of intraexaminer difference followed methods similar to those published by Araujo et al. (2). Baseline data analysis was performed to determine if the intraexaminer reliability was calibrated. Using kappa statistics (K) for the categorical clinical measurement variables, such as periodontal probing depth (PPD) and clinical attachment level (CAL), the standard error of these measurements was monitored. The examiner's clinical measurement technique was considered calibrated if the standard error for the measurements was ≤0.8 and a K value ranged between 0.8 and 0.95. The reproducibility of the intraexaminer measurements was recalculated every 6 months.

The periodontal examinations were performed to determine periodontal status of all subjects with teeth. Subject groups representing individuals older than 2.5 years (i.e., those subjects in groups B, C, D, E, and F, as noted above) received radiographic examination to evaluate the presence of periodontal bone resorption. More specifically, a bite-wing X-ray examination was conducted for groups B and C, whereas a periapical radiograph was taken from groups D, E, and F. However, for subjects in group A, newborns (0 to 4 months old), and group G, elderly over 55 years of age with no teeth, only visual clinical examination was conducted without X-ray examination. For children from 6 to 12 years of age (group C), the clinical examination included plaque index (28) and gingival index (22). For adolescents from 13 to 18 years of age (group D) and adults and elderly with teeth (groups E and F), we performed plaque and gingival index measurements, radiographic examinations, and complete periodontal screening, including PPD and CAL measured in six sites per tooth, using a manual periodontal probe (PCPUNC15BR; Hu-Friedy).

A diagnosis of periodontal condition followed the criteria defined by Tanner et al. (31).

Participants in group B were all diagnosed as periodontally healthy subjects because they showed no evidence of periodontal bone resorption (as evaluated by bite-wing X ray) and showed no clinical signs of periodontal inflammation upon visual examination. All participants in group C were also considered periodontally healthy based on the lack of periodontal bone resorption and a gingival index of less than 1. For groups D, E, and F, healthy participants had a mean periodontal attachment level of <1.5 mm and no sites with >2-mm attachment loss. For early periodontitis 1 (EP1), patients in these groups also had a mean periodontal attachment level of <1.5 mm but at least one site with 2-mm attachment loss. For EP2, patients had >1.5-mm to <2.0-mm mean periodontal attachment loss. In addition, the subjects either who were healthy or who did not fit Tanner's criteria were classified according to the mean of attachment loss: chronic periodontitis 1 (CP1; CAL of >2 mm and <3 mm), CP2 (CAL of ≥3 mm and <5 mm), and CP3 (CAL of ≥5 mm).

Sampling of microorganisms. A pooled subgingival sample was collected from each subject with teeth (153 subjects) from the mesiobuccal aspect of all first molars (n = 4 molars/subject) and mesial incisors (n = 2 incisors/subject, right maxillary or left mandibular) using sterile paper points inserted to the depth of the gingival sulcus after removal of supragingival plaque using sterile curettes. For subjects missing those teeth (first molars and mesial incisors), microbial samples were obtained from second molars and/or lateral incisors (see also "Exclusion criteria for subject recruitment"). After being placed in the sulcus for 60 seconds, paper points were removed and immediately transferred into a microtube containing reduced Ringer's solution (Oxoid Ltd., Basingstoke, Hampshire, United Kingdom). In addition, microbial samples taken from the left side of the cheek and the dorsum of the tongue were obtained from all subjects included in the present study. These samples were taken from areas of approximately 1 cm^2, using a swab with reduced Ringer's solution, rotated six times. Each swab was transferred into a microtube also containing reduced Ringer's solution (1 ml).

Bacterium-specific PCR. The bacterial cells in the microtube were dispersed using a vortex mixer at maximal setting for 1 min and then maintained at −80°C until laboratory processing. The presence of *C. rectus*, *P. gingivalis*, *A. actinomycetemcomitans*, *P. intermedia*, and *T. forsythia* was determined by PCR, as described below.

In the Laboratory of Molecular Biology at the University of Taubaté (Sao Paulo State, Brazil), the bacterial suspensions were thawed and centrifuged at 12,000 × *g* for 3 min, and the DNA was extracted from the bacterial pellet using a DNA isolation kit, following the manufacturer's instructions (InstaGene purification matrix; Bio-Rad Laboratories, Hercules, CA). Briefly, 5 μl of the sample DNA was added to 45 μl of reaction mixture containing 5 μl of 10× PCR buffer (Promega, Madison, WI), 1.25 units of *Taq* DNA polymerase (Promega), and 0.2 mM of deoxyribonucleotide mixture (Pharmacia LKB, Piscataway, NJ). PCR

amplification was performed using a thermal cycler (Perkin-Elmer, Wellesley, MA). The bacterium-specific primer (5'-3') sequences used in this study are as follows: *C. rectus*, sense, 5'-TTTCGGAGCGTAAACTCCTTTTC-3', and antisense, 5'-TTTCTGCAAGCAGACACTCTT-3' (PCR product size 598 bp); *A. actinomycetemcomitans*, sense, 5'-AAACCCATCTCTGAGTTCTTCTTC-3', and antisense, 5'-ATGCCAACTTGACGTTAAAT-3' (550 bp); *P. intermedia*, sense, 5'-TTTGTTGGGGAGTAAAGCGGG-3', and antisense, 5'-TCAACAT CTCTGTATCCTGCGT-3' (575 bp); *P. gingivalis*, sense, 5'-AGGCAGCTTGC CATACTGCGG-3', and antisense, 5'-ACTGTTAGCAACTACCGATGT-3' (404 bp); *T. forsythia*, sense, 5'-GCGTATGTAACCTGCCCGCA-3', and antisense, 5'-TGCTTCAGTGTCAGTTATACCT-3' (641 bp).

The detailed protocol for PCR-mediated DNA amplification was previously published by this group (Cortelli et al. [8]). PCR products were separated in a 1.5% agarose gel (Sigma, Dorset, United Kingdom) by an electrophoresis performed at 4 V/cm in Tris-acetate-EDTA buffer (Promega). The DNA bands present in the gel were stained with 0.5 μg/ml ethidium bromide (Amersham, Arlington Heights, IL) and photographed under 300-nm UV light illumination.

Statistical analysis. The association between bacterial occurrence and genders and smoking habits was analyzed within each age range using the chi-squared test. Results were determined to be statistically significant at $P < 0.05$. All tests were performed using statistical software (SPSS for Windows Release 12.0; SPSS Inc., Chicago, IL).

RESULTS

The prevalence of bacteria detected in the gingival sulcus, tongue, and cheek mucosa was analyzed for the subjects with teeth who were classified on the basis of their periodontal condition (Fig. 1). More specifically, subjects older than 2.5 years of age were grouped as follows: (i) healthy, (ii) EP1, (iii) EP2, (iv) CP1, (v) CP2, and (vi) CP3 (see the criteria for diagnosis described under "Clinical measurements and diagnosis" in Materials and Methods). Among the bacteria tested, *C. rectus* showed the highest prevalence (more than 80% in sulcus) throughout all subject groups. While the prevalence of *C. rectus* in the cheek mucosa was always slightly lower than that found in sulcus or tongue, at least 60% of subjects (healthy group) possessed *C. rectus* in the cheek mucosal surface. Therefore, *C. rectus* appeared to colonize ubiquitously in the oral cavity without preference to environmental oxygen represented by anaerobic (sulcus) or aerobic (tongue and cheek mucosa) atmosphere. *P. gingivalis* was prominently found in the subjects with periodontitis, while incidence of this bacterium in the healthy subjects was extremely low (2% in sulcus, 0% in tongue and cheek mucosa). Very interestingly, the incidence of *P. gingivalis* in the sulcus demonstrated an increasing trend, along with the severity of periodontitis in the subject groups (from EP1 to CP3). The prevalence of *P. gingivalis* in the tongue or cheek mucosa was always lower than that in the sulcus. Especially, *P. gingivalis* was not found in the cheek mucosa of EP1 and EP2 groups, whereas *P. gingivalis* was found at low prevalence (less than 16%) in the cheek mucosa of the CP1, CP2, and CP3 groups.

The prevalence of *P. intermedia* and *T. forsythia* showed similar profiles of bacterial recovery from all groups examined. These two bacteria were detected from patient groups as well as healthy groups. On average, the incidence of *T. forsythia* in the sulcus was slightly higher than that of *P. intermedia* for all groups examined. The incidences of *P. intermedia* and *T. forsythia* in the tongue and cheek mucosa were always lower than those in sulcus, and the cheek mucosa demonstrated the lowest prevalence. *A. actinomycetemcomitans*, a putative pathogen for localized aggressive periodontitis, was found in sulcus, tongue, and cheek mucosa in all healthy and patient groups at a lower frequency than that of the other four species of bacteria examined (less than 28% in sulcus). The prevalence of *A. actinomycetemcomitans* found in tongue and cheek mucosa was equal to, or lower than, its prevalence in sulcus. These data indicated that colonization by *P. gingivalis* is tightly associated with periodontitis and that the colonization by the other four bacteria can also be found in the healthy oral cavity. However, while these data do conform to the conventional format for etiological studies involving periodontal bacterial infection, they do not offer any clues which would concretely establish the time of onset of initial bacterial colonization. Therefore, to achieve the primary goal of this study, i.e., to examine an age-related group which corresponds to the detection of initial bacterial colonization, additional epidemiological analysis was conducted using these patient data with the addition of periodontally healthy groups (A, B, and G in Table 1).

Subjects recruited for this study were grouped on the basis of their age (Fig. 2). In particular, two groups, 0 to 4 months old and elderly subjects (>55 years) without any teeth (tongue and cheek mucosa data only), were additionally included in the population shown in Fig. 1. According to these statistics, the prevalence of *C. rectus* was still the highest among the bacteria examined in the present study, irrespective of the age groups. However, the prevalence of *C. rectus* in tongue and cheek mucosa was lower in the 0- to 4-month newborn group than in the rest of the groups (tongue: 0 to 4 months, 18%; >2.5 years, intergroup average, 78.3% ± 9.4% [standard deviation]; cheek: 0 to 4 months, 21.5%; >2.5 years, intergroup average, 66.9% ± 8.3% [standard deviation]), indicating that colonization by *C. rectus* starts in the oral cavity as early as 0 to 4 months. Very interestingly, in the edentulous elderly subjects (group G), the prevalence of *C. rectus* remained high (79% in tongue, 63% in cheek mucosa) compared to the other four species of bacteria, which showed a prevalence of less than 10% in both tongue and cheek mucosa, indicating that the presence of teeth (or sulcus) is not permissive for colonization by *C. rectus*.

The incidence of *P. gingivalis* in group C (Fig. 2) was sporadic (13 to 18 years old: sulcus, 9%; tongue, 0%; and cheek mucosa, 0%), while a prominent emergence of *P. gingivalis* was found in subjects older than 19 years (groups E and F). The prevalence of *P. gingivalis* in the edentulous elderly subjects (group G) was remarkably low compared to that in the same age group with teeth (group F), suggesting that colonization by *P. gingivalis* requires the presence of teeth (or sulcus).

A. actinomycetemcomitans was found sporadically at an incidence of less than 20% through all age groups (Fig. 2). Although colonization by *A. actinomycetemcomitans* was found in younger age groups (groups A to D), it is unclear if such colonization at younger ages leads to localized aggressive periodontitis. Only a longitudinal cohort study will elucidate this question. It is noteworthy that none of the subjects in this study was diagnosed with localized aggressive periodontitis, in which *A. actinomycetemcomitans* is accepted as a major pathogen.

The initial colonization by *P. intermedia* appeared to occur from the ages of 6 to 12 years (Fig. 2). While only sporadic colonization by *T. forsythia* was found in groups A and B (ages 0 to 4 months and 2.5 to 5 years, respectively), a major increase in *T. forsythia* colonization was detected in group C (6 to 12

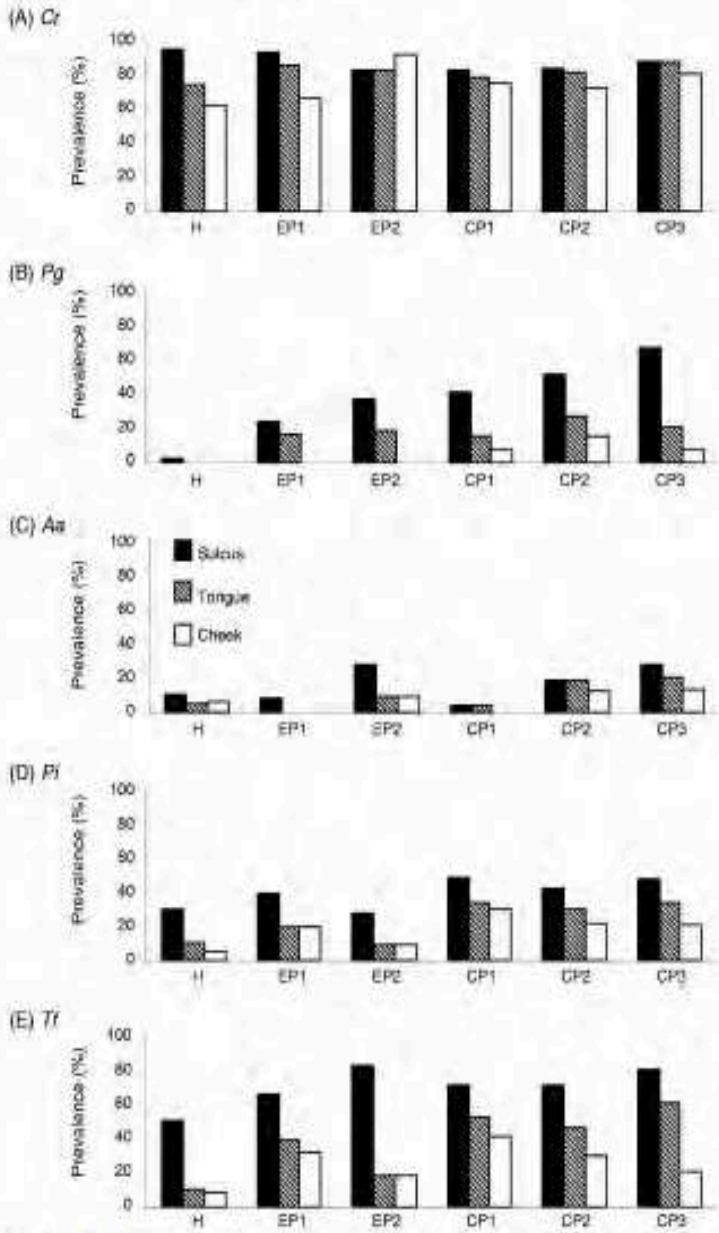

FIG. 1. Prevalence of periodontal pathogens in gingival sulcus, tongue, and cheek mucosa of different subject groups classified on the basis of their periodontal disease status. The subject groups B to F shown in Table 1 (aged >2.5 years, a total of 222 subjects who have teeth) were employed in the analysis. The presence of DNA specific to *C. rectus* (A), *P. gingivalis* (B), *A. actinomycetemcomitans* (C), *P. intermedia* (D), and *T. forsythia* (E) in the samples isolated from subgingival sulcus, the dorsum of the tongue, and cheek mucosa of each subject was determined by PCR, following the method described in Materials and Methods.

years). The prevalence of *P. intermedia* and *T. forsythia* in the older age groups with teeth (13 to 18 years, 19 to 44 years, and >55 years) remains at a level similar to that in group C (6 to 12 years). However, the prevalence of these two bacteria, *P. intermedia* and *T. forsythia*, in group G (>55 years old without teeth) remarkably decreased in the tongue and cheek mucosa, indicating that teeth (or sulcus) is a permissive factor for colonization by *P. intermedia* and *T. forsythia*.

Smoking is known as a risk factor promoting the incidence of periodontitis. Therefore, we have further classified the subjects into smokers and nonsmokers (Table 2). Especially, in the group of subjects 19 to 44 years old (E) and those subjects aged >55 years (F), nearly one-half of recruited subjects were identified as smokers. However, when the association between smoking and the presence of all bacteria was evaluated, there was no statistically significant difference observed in the sulcus, tongue, or cheek mucosa in either group E or group F (chi-squared test; data not given). Therefore, smoking did not ap-

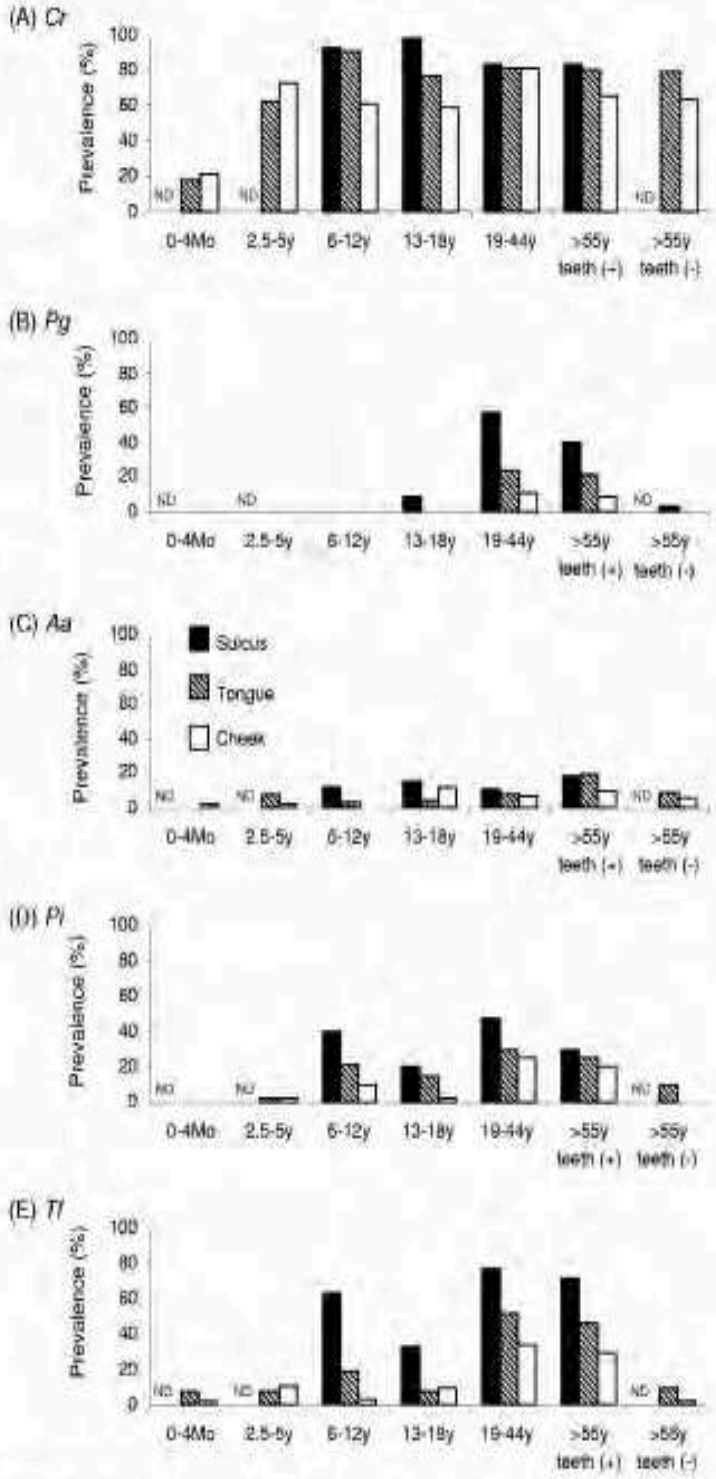

FIG. 2. Prevalence of periodontal pathogens in gingival sulcus, tongue, and cheek mucosa of different subject groups classified on the basis of their age and presence of teeth. The subject groups A to G shown in Table 1 (aged >0 months, a total of 330 subjects with or without teeth) were employed in this analysis. The presence of DNA specific to *C. rectus* (A), *P. gingivalis* (B), *A. actinomycetemcomitans* (C), *P. intermedia* (D), and *T. forsythia* (E) in the samples isolated from subgingival sulcus, the dorsum of the tongue, and cheek mucosa of each subject was determined by PCR, following the method described in Materials and Methods. ND, not determined.

TABLE 2. Distribution of different statuses of periodontitis in the groups of subjects with teeth

Periodontal status	No. of subjects by group[a] and smoking status with periodontal status						
	B (2.5–5 yr; never smoked)	C (6–12 yr; never smoked)	D (13–18 yr; never smoked)	E (19–44 yr)		F (>55 yr; with teeth)	
				Smoker[b]	Never smoked	Smoker[b]	Never smoked
Healthy	36[c]	33[c]	15	2	0	0	0
EP1	0	0	9	3	4	4	6
EP2	0	0	2	5	2	2	0
CP1: CAL, >2 mm and <3 mm	0	0	1	5	10	4	7
CP2: CAL, >3 mm and <5 mm	0	0	3	12	14	16	12
CP3: CAL, ≥5 mm	0	0	0	3	3	4	5
Total	36	33	30	30	33	30	30

[a] Groups of subjects who have teeth are shown. Groups B to F correspond to the age-related groups shown in Table 1. Newborn and edentulous elderly groups are not shown because of the lack of periodontal tissues.

[b] No significant difference in bacterial prevalence was detected between the smokers and nonsmokers in group E and group F.

[c] Participants in group B were all diagnosed as periodontally healthy subjects because they showed no evidence of periodontal bone resorption (as evaluated by bite-wing X ray) and showed no clinical signs of periodontal inflammation upon visual examination. All participants in group C were also considered periodontally healthy based on the lack of periodontal bone resorption and a gingival index of less than 1.

pear to be a factor that influences the prevalence of bacteria tested in this study. The influence of gender difference on the prevalence of bacteria within each age-related group (all groups, A to G) also did not show any statistical difference as analyzed by chi-square test (data not shown).

DISCUSSION

Several studies have shown an association between the onset and progression of periodontal disease and the presence of periodontal pathogens in the periodontal sulcus of periodontally diseased patients in Brazil and many other countries, as well. Those pathogens found in Brazilian patients with periodontal diseases include *A. actinomycetemcomitans*, *P. gingivalis*, *P. intermedia*, *T. forsythia*, *C. rectus*, *Eikenella corrodens*, *Treponema denticola*, *Streptococcus* spp., and some others (3, 4, 6, 7, 8, 9, 24, 26, 32, 33, 34). In particular, these previous studies focused on the prevalence of periodontal pathogens in diseased subjects representing four different stages of clinical treatment, as follows: (i) before periodontal treatment, (ii) after nonsurgical mechanical therapy (scaling and root planing), (iii) after antibiotic therapy, and (iv) after surgical treatment. However, none of the studies using a Brazilian population has yet addressed the question of when initial colonization by periodontal pathogens occurs in the oral cavity. For this reason, the present study utilized an epidemiological approach to specifically examine the prevalence of five types of periodontal pathogens in the oral cavities of subjects in diverse age groups ranging from newborns to elderly, with or without teeth.

Data from this study were analyzed and used to achieve the major aim of this report: to establish a link between particular age-related groups and the time when the initial colonization by periodontal pathogens might occur in the oral cavities of subjects. These subjects were randomly chosen from a geographic region in Brazil in order to include both healthy and periodontally diseased individuals in the study. The data in the present study demonstrated that (i) initial colonization by *T. forsythia* was found in tongue and cheek mucosa of newborns (0 to 4 months), and more than 60% of healthy subjects possessed *T. forsythia* in gingival sulcus at ages 6 to 12 years; (ii) initial colonization by *C. rectus* was found as early as ages 0 to 4 months and remained high through all ages, irrespective of the presence of teeth; (iii) initial colonization by *P. intermedia* in the oral cavity appeared to occur in periodontal sulcus during ages 6 to 12 years; (iv) initial colonization by *P. gingivalis* seemed to start during the ages of 19 to 44 years; and (v) the presence of teeth in the oral cavity is a permissive factor for colonization by *P. intermedia*, *T. forsythia*, and *P. gingivalis*.

Previous studies showed that the incidence of *T. forsythia* in the periodontal sulcus positively correlates with the clinical signs of periodontal disease and can be regarded as a risk indicator for attachment and bone loss (15, 16, 17). According to the work of Tanner et al. (31), the incidence of *P. gingivalis* and *T. forsythia* in subgingival and tongue samples is associated with early periodontitis compared to the samples isolated from healthy subjects. For the latter, Tanner et al. found that *T. forsythia* is present in the subgingival samples isolated from healthy subjects, which agrees with the findings of the present study. However, they did not detect *T. forsythia* in the tongues of healthy adults (aged 20 to 40 years), which is contrary to our finding that *T. forsythia* can be found in the tongue of healthy subjects at low incidence, about 11% (Fig. 1). More importantly, the present study demonstrated that colonization by *T. forsythia* can be detected in the tongue as early as age 0 to 4 months, in individuals who obviously have no teeth (Fig. 2). Therefore, our data indicate that *T. forsythia* is an opportunistic pathogen whose colonization of the oral cavity does not necessarily result in the onset of periodontal disease.

Additional findings revealed in the present study demonstrated the significance of the association between age-related groups and the prevalence of periodontal bacteria colonizing particular affected tissues. To illustrate, the initial colonization by *C. rectus*, *P. gingivalis*, *A. actinomycetemcomitans*, *P. intermedia*, and *T. forsythia* was first noted in all ages of dentate subjects, but it was also observed that the prevalence of each bacterium remained at high levels in subgingival plaque, tongue, and cheek mucosa of elderly subjects (>55 years). However, a major difference between *C. rectus* and the other bacteria, including *P. gingivalis*, *A. actinomycetemcomitans*, *P. intermedia*, and *T. forsythia*, was found with respect to their

level of incidence in the elderly group without teeth. Specifically, the prevalence of *P. gingivalis*, *A. actinomycetemcomitans*, *P. intermedia*, and *T. forsythia* in the oral cavity (tongue and cheek) remarkably diminished in the edentulous elderly, whereas the prevalence of *C. rectus* in these edentulous elderly remained high. Considering the scarcity of data regarding the prevalence of periodontal pathogens in the edentulous elderly (30), we believe that the present study is the first to demonstrate that the presence of teeth appeared to be a permissive factor for colonization by species, such as *T. forsythia*, *A. actinomycetemcomitans*, *P. gingivalis*, and *P. intermedia* (Fig. 2, group F versus group G).

On the other hand, while the presence of teeth appeared to be "permissive," it is not "requisite" for colonization by *T. forsythia*, *A. actinomycetemcomitans*, *P. gingivalis*, and *P. intermedia* because all these microorganisms were still present at a low prevalence in the dorsum of the tongue and in the cheek of newborn and edentulous elderly subjects. Therefore, our findings contradict the report by Danser et al. (11), who demonstrated that *A. actinomycetemcomitans* and *P. gingivalis* disappear from the oral cavity after the extraction of all teeth. Additionally, our data also disagree with the work of Könönen et al. (19), who did not detect *A. actinomycetemcomitans* in 51 edentulous subjects (44 to 92 years; mean age, 74 years) who used complete dentures. However, in the present study, *P. gingivalis* was not detected in any subject under 12 years of age, and the first occurrence of this microorganism was observed at a very low incidence (6.67%) in subgingival plaque, but not in tongue or cheek samples, of adolescents (13 to 18 years). It is noteworthy that this finding contradicts the report by Lamell et al. (20), who detected *P. gingivalis* DNA in the samples of tongue, buccal mucosa, or mesial sulcus of all teeth at rates of 36% (first examination) to 43% (second examination at recall after 1 to 3 years) between the ages of 0 and 18 years. They therefore concluded that *P. gingivalis* inhabits the oral cavities of child subjects of any age (0 to 18 years) and usually colonizes only transiently. It is conceivable that the difference between the present study and the report by Lamell et al. (20) in the ability to detect periodontal pathogens on mucosal surfaces may depend on the sensitivity of the method used for bacterial DNA detection. Nonetheless, there should be factors, other than the presence of teeth, which affect colonization by *P. gingivalis* or transitory habitation by *P. gingivalis*. Such factors may change the properties of *P. gingivalis* through the activation of a two-component system (13), which would allow the bacterium to survive in the aerobic environment of tongue and cheek surfaces.

The diversity of bacterial composition in the plaque isolated from different sites of the oral cavity has been well documented. These sites include supra- and subgingival sulcus, tongue, cheek, and other areas of the oral cavity. For example, Aas et al. (1), utilizing culture-independent molecular techniques, defined the bacterial diversity of the healthy human oral cavity. This study examined the bacterial composition in nine sites, including supra- and subgingival plaque, the lip vestibule, soft palate, hard palate, lateral side of the tongue, tongue dorsum, buccal epithelium, and tonsils. The results showed that some species, such as those of *Gemella*, *Granulicatella*, *Streptococcus*, and *Veillonella*, were common to all nine sites, whereas *Rothia dentocariosa*, *Actinomyces* spp., *Streptococcus sanguis*, *Streptococcus gordonii*, and *Abiotrophia defectiva* were detected only in supragingival plaque on the teeth. The results also demonstrated that, in general, *Streptococcus salivarius* was present on the tongue dorsum, indicating that certain strains of microorganisms detected in subgingival plaque could also be detected in other areas of the oral cavity.

The present study further addressed the question of whether microorganisms present in subgingival plaque could also be present in the cheek and dorsum of the tongue (Fig. 1 and 2). The subject population randomly recruited in this study showed a high prevalence of *C. rectus* through all age groups, as well as at the three sampling sites of periodontal sulcus, tongue, and cheek mucosa. Moreover, since the presence of *C. rectus* in the subgingival pocket appeared to be associated with its detection in the tongue and cheek, tongue sampling may offer a convenient, as well as efficient, means of detecting *C. rectus* in the oral cavity. Aside from *C. rectus*, the probability of simultaneous occurrence of the other bacteria used in this study in the subgingival plaque, tongue, and cheek was low (data not shown). Taken together, these findings suggested that sampling of microorganisms from different sites of the oral cavity is required to identify a particular microorganism in the oral cavity of human subjects.

While several studies have implicated the effects of smoking in the composition of the subgingival microbial flora (14, 18, 35, 36), others failed to show any differences in the prevalences of periodontal pathogens for either smokers or nonsmokers (5, 12). The present study found no correlation between smoking and the prevalence of the five tested bacteria. It is true that Shiloah et al. (27) showed that the prevalence of pathogenic bacteria in the periodontal sulcus of periodontitis-free individuals is related to their daily consumption of cigarettes and duration of cigarette smoking. However, it is not realistic to monitor the daily consumption of cigarettes by younger smokers, even if this factor may affect the early colonization by periodontal pathogens in the periodontal sulcus of those in the younger populations. Since it is known that environmental factors, such as nutrition and lifestyle, affect the onset and progression of periodontal disease, epidemiological studies, which involve more study arms of environmental factors in addition to smoking, will provide insight into the effect of smoking on colonization by periodontal pathogens.

To summarize, the prevalence of *C. rectus*, *P. gingivalis*, *A. actinomycetemcomitans*, *P. intermedia*, and *T. forsythia* demonstrated differences in their initial colonizations in both the types of tissues and temporal ranges for the diverse age groups examined in this study. *C. rectus* was found in subjects aged 0 to 4 months and remained at high levels in the oral cavity (tongue and cheek) of elderly subjects, irrespective of their possession of teeth. Initial colonization by *P. intermedia* and *T. forsythia* in subgingival sulcus was detected in a very young group (6 to 12 years), whereas *P. gingivalis* was first detected in a much older group (19 to 44 years). However, the prevalence of all three bacteria declined in the elderly individuals without teeth but not in elderly individuals with teeth. The prevalence of *P. gingivalis*, *P. intermedia*, and *T. forsythia* found in tongue and cheek mucosa was always lower than their prevalence detected in the subgingival sulcus. In conclusion, the findings related to the association between age-related groups and the time of initial colonization by the five major periodontal patho-

gens may provide a potentially useful set of markers for the early detection of opportunistic pathogens during their benign state. This could then ultimately provide new insights into the mechanisms underlying pathogenic conversion of these opportunistic pathogens, resulting in the identification of targets for novel preventive and therapeutic approaches.

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2.2 CAPÍTULO 2

Título do projeto: O impacto da condição periodontal e do tabagismo, pessoal e/ou materno, sobre a ocorrência de patógenos periodontais em recém-nascidos, crianças e adultos

Delineamento: Estudo transversal

População parcial: 76 recém-nascidos, distribuídos em dois grupos de acordo com a condição clínica periodontal de suas mães (presença ou ausência de periodontite).

Objetivo específico: O objetivo do presente estudo foi avaliar a influência da condição clínica periodontal materna sobre a ocorrência de bactérias periodontopatogênicas em recém-nascidos.

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Outorgada: Profa. Dra. Sheila Cavalca Cortelli

Co-orientação: Gilson C N Franco

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Fase: pesquisa concluída e artigo submetido

Artigo submetido: Aquino DR et al. The impact of the maternal periodontal status on the detection of periodontal pathogens in newborn children. Submetido a **International Journal of Paediatric Dentistry** (Anexo B).

The impact of the maternal periodontal status on the detection of periodontal pathogens in newborn children

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Summary

Background. Studies suggest that mothers can act as a reservoir of periodontal pathogens and contribute for the early colonization of their newborn children.

Aim. To evaluate the influence of the mother's periodontal clinical status on the prevalence of periodontal pathogens in newborns.

Design. 76 pairs of newborns and their biological mothers were selected. After a periodontal examination, the mother-child pairs were divided into two groups according to their maternal periodontal clinical status: mothers with (Group A, n=33) or without periodontitis (Group B, n=43). The oral colonization of newborns by five periodontopathogens (*A. actinomycetemcomitans, P. intermedia, P. gingivalis*, *T. forsythia* and *C. rectus*) was determined using a DNA-specific PCR method. Statistical analysis was performed using the Chi-square test considering any result as significant when $p<0.05$.

Results. *C. rectus* was the most prevalent species observed (23.25%) in Group B, while in Group A *P. gingivalis* (96.97%) had the highest detection rate, followed by *T. forsythia* (60.60%) and *P .intermedia* (39.39%). Between-groups comparisons showed a greater frequency of all study pathogens among newborns from Group A.

Conclusions. The maternal clinical periodontal status was a satisfactory indicator of the bacterial colonization pattern observed in their newborn children. Thus, longitudinal studies should be performed to confirm this relationship.

Key words: Infant, Periodontitis, Bacteria, Polymerase Chain Reaction.

Introduction

Among the several pathogens that are known to be related to periodontal disease, *Aggregatibacter actinomycetemcomitans, Porphyromonas gingivalis, Prevotella intermedia, Tannerella forsythia* and *Campylobacter rectus* can be found in individuals with different severities of periodontitis[1-3]. However, studies that evaluate the pattern of transmission have primarily focused on the prevalence of *A. actinomycetemcomitans* and *P. gingivalis*[4], while the acquisition of other key periodontal pathogens remains to be elucidated[5]. More recently, the potential for bacterial transmission has been focus of a number of studies[5-7] that attempted to investigate the precise moment when the first contact between the individual and the periodontal pathogen occurs[8]. Unfortunately, the precise moment when underlying the acquisition of the periodontal pathogen take place remains unclear[9]. Moreover, these studies have failed to conclusively determine whether or not this first colonization event is transitory or whether this event may function to increase the future risk for the establishment of the periodontal disease[7,10].

Umeda et al.[11] evaluated the distribution of periodontal pathogens in 56 children (from ages 1 to 15 years) and their parents. A PCR-based method was used to determine the prevalence of microbial organisms in saliva and dental plaque samples. A positive correlation was observed for the presence of oral pathogens between parents and their children. Thus, the authors suggested a possible vertical familiar transmission of those periodontal pathogens. In a separate study, Kobayashi *et al.*[12] also investigated the progress of infection by periodontal pathogens and the role of the child's mother in this transmission process. The results indicated that the colonization of periodontal bacterial in children increased with age and that the bacterial microbiota in children was very similar to that in their mothers. Nevertheless,

in both studies, information regarding the exact moment of this first colonization event was not elucidated due to the lack of conclusive data.

It is important to emphasize that transmission studies of children-parent pairs have primarily aimed to determine if the pathogen found in a given child is exactly the same microbial strain recovered from the mother or the father. As a diagnostic method, this kind of analysis is very limited, especially in developing countries, because this method is time-consuming and cost-ineffective. Comparisons between periodontally diseased or healthy patients have demonstrated that the first group possesses higher levels of bacteria[13]. Thus, as suggested by Tamura *et al.*[5], it seems reasonable that oral cavities from mothers with periodontal disease can act as potential reservoirs of pathogens that may procure an early oral bacterial colonization event in their children. However, the direct impact of the maternal periodontal status established by a cheaper and faster clinical examination technique has not been studied. Therefore, it was hypothesized that even after a fairly short period of time following birth, newborns whose mothers had periodontal disease may present a higher prevalence of pathogens than newborns whose mothers are periodontally healthy. In order to test this hypothesis, this cross-sectional study aimed to evaluate whether or not the maternal periodontal clinical status influences the prevalence of periodontal pathogens in newborn children.

Matherials and methods

Population

The protocol was approved by the ethical research committee (University of Taubaté Ethics Committee, protocol #0317/07). The mothers of the children received detailed information regarding the nature and the procedures involved in the study

and signed informed consent forms. In order to establish the sample size of the present study, a pilot project was performed with four newborns (Chi-square-X^2, α significance level from 1 to 5% and a test power equivalent to 80%).

Seventy-six newborns (maximum age of three months) and their mothers were included in this cross-sectional study. A complete periodontal examination was performed at the dental clinic by the same trained and calibrated examiner immediately before microbial samples were collected to measure the probing pocket depth, the clinical attachment level, the plaque and bleeding upon probing scores. Based on the obtained periograms, all of the mothers were classified as periodontitis patients according to López's criteria, which indicate that they have at least four periodontal sites with a PD≥4mm and a CAL≥3mm distributed in different teeth[14]. Therefore, according to the maternal clinical periodontal status, the pairs were divided into two groups, specifically newborns whose mothers were diagnosed with periodontal disease (Group A, n=33) and newborns whose mothers were diagnosed as not possessing periodontal disease (Group B, n=43). All of the clinical and microbial examinations were performed between July 2007 and December 2007. The medical and dental information from additional pairs was obtained using a questionnaire, and only newborns who had close contact with their mothers were included. Therefore, newborns who spent a significant amount of time with nurses, nannies or relatives were excluded from the study. In addition, in order to minimize the time of contact with siblings and other members of their respective families, only newborns with a birth date occurring after the beginning of the academic school year were accepted for this study.

Microbial procedures

Microbial samples were taken from the dorsum of the tongue of all newborns according to method described by Cortelli et al.[2]. Briefly, the samples were taken from areas of approximately 1cm^2 using a swab with reduced Ringer's solution that was rotated six times. Each swab was transferred into a microtube also containing reduced Ringer's solution (1ml) and frozen (-80°C) until the time of the analysis.

The bacterial cells in the microtube were dispersed using a Vortex and centrifuged for 3 minutes at 12,000rpm. From the cellular bacteria pellet, genomic DNA was extracted using a commercial DNA purification Kit (InstaGene®, Bio-Rad laboratories, Hercules, CA, USA) according to the manufacturer's instructions.

The presence of *C. rectus*, *P. gingivalis*, *A. actinomycetemcomitans*, *P. intermedia* and *T. forsythia* was determined by polymerase chain reaction (PCR). A total volume of 25µL of the PCR mixture contained 10µL of the DNA sample, 2.5µL of a 10x PCR buffer (Invitrogen, Carlsbad, CA, USA), 1.25 units of Taq DNA polymerase (Invitrogen), 0.2mM of each deoxyribonucleotide (Invitrogen, Carlsbad, CA, USA), 1.5mM of MgCl and 1.0µM of each primer.

The PCR amplification was performed in a Mastercycler Gradient thermal cycler (Eppendorf, Westbury, NY, USA) using a standard protocol that includes an initial denaturation step at 95ºC for 5 minutes, followed by 35 cycles of denaturation at 95ºC for 30 seconds, an annealing step at 55ºC for 30 seconds, and an extension step at 72ºC for 1 minute, with a final extension period of 72ºC for 5 minutes.

The bacteria-specific primer (5'-3') sequences used in this study include *C. rectus,* sense (5'-TTTCGGAGCGTAAACTCCTTTTC-3') and antisense (5'-TTTCTGCAAGCAGACACTCTT-3'), generating a PCR product of 598bp amplicon; *A. actinomycetemcomitans,* sense (5'-

AAACCCATCTCTGAGTTCTTCTTC-3) and anti-sense (5'-ATGCCAACTTGACGTTAAAT-3'), generating a 550bp amplicon; *P. intermedia,* sense (5'-TTTGTTGGGGAGTAAAGCGGG-3'), and antisense (5'-TCAACATCTCTGTATCCTGCGT-3'), generating a 575bp amplicon; P. gingivalis, sense (5'-AGGCAGCTTGCCATACTGCGG-3') and antisense (5'-ACTGTTAGCAACTACCGATGT-3'), generating a 404bp amplicon; and *T. forsythia,* sense (5'GCGTATGTAACCTGCCCGCA3') and antisense (5'-TGCTTCAGTGTCAGTTATACCT-3'), generating a 641bp amplicon.

The final products were separated on 1.5% agarose gel, stained with ethidium bromide (0.5µg/mL) and photographed under ultraviolet light to confirm the existence of the target oral bacterial species. A 100bp DNA ladder (Invitrogen, Carlsbad, CA, USA) was used as the molecular weight marker. Both positive and negative controls were included for the PCR reaction in order to verify the primer specificity and identify and DNA contamination.

Statistical analysis

The frequencies of the periodontal pathogens were analyzed using the Chi-squared test (X^2). Statistical analyses were performed using statistical software (Bio Estat 5.0 and SPSS11.0) where the statistical significance was established at 5%.

Results

In the present study the 76 newborns who were analyzed were composed of 34 males and 42 females, where 43 of the children had mothers without periodontal disease and the remaining 33 children had mothers with periodontal disease (Table 1).

In newborns of the mothers without periodontal disease, *C. rectus* was observed more often ($p<0.05$) than other bacterial species (Figure 1). On the other hand, in the group of children whose mothers were affected by periodontal disease, *P. gingivalis* was the most prevalent pathogen observed ($p<0.05$), followed by *P. intermedia*, *T. forshytia*, *C. rectus*, and *A. actinomycetemcomitans* (Figure 2).

With the exception of *C. rectus*, the between-groups comparison regarding the prevalence of the studied pathogens revealed a higher prevalence of bacteria ($p<0.05$) among newborns from Group A when compared to the newborns from Group B (Table 2).

Finally, the estimated odds ratio for the relative risk of the acquisition of periodontal pathogens in the different groups was determined. The results indicated no significant difference between the maternal periodontal status and the acquisition of periodontal pathogens.

Discussion

Arriving at an early diagnosis for any pathology must always be the aim of every health care professional. However, for periodontal disease, the diagnosis has been achieved in most cases only after the manifestation of the disease's clinical symptoms.

Periodontal disease is one of the most common chronic infections in adults, affecting approximately 4 to 19% of the population of Central and South America[15]. Once periodontal disease presents an infectious etiology[16] initiating from the colonization of specific pathogens, its early diagnosis is likely to be directly related to the detection of those pathogens in the oral cavity. Despite several studies that have shown the occurrence of the same bacterial species between mothers and their children with the aim of verifying a possible mechanism for the transmission, it is

known that such expensive and high technology methodology has limited application in various populations worldwide, especially in developing and underdeveloped countries. In order to address this issue, the present study attempted to verify whether or not the motherly periodontal status, as assessed through a clinical examination, had the potential to predict the early oral occurrence of periodontal pathogens in their newborn children. Because any method used that may indicate a possible early acquisition of suspected periodontal pathogens is relevant and can contribute to prevent periodontal diseases[17, 18].

In various parts of the world, children have been affected by gingivitis, and, as indicated by reports from adult populations, a small proportion of children are also affected by periodontitis probably with similar etiological microbial factors. In this cross-sectional study, we have compared the frequencies of specific bacteria between newborns and their mothers who were either diagnosed or not diagnosed with periodontal disease and the process clearly revealed a difference in the characterization of the bacterial colonization. The intra-group analysis indicated that in the maternal group that was not diagnosed with periodontitis, the most prevalent bacterium was *C. rectus* (23.25%), followed by other pathogens including *T. forshytia* (9.30%), *A. actinomycetemcomitans* (2.32%) whereas *P. intermedia* and *P. gingivalis* were not detected (Figure 1). According to previous studies[19-21], our findings seem compatible with the mothers' periodontal clinical status, in principle, due to the remarkable relationship of *C. rectus* with periodontally healthy subjects. On the other hand, the intra-group analysis of newborns from the group of mothers who were diagnosed with periodontitis revealed a notable increase in the detection of species that are considered true periodontal pathogens[21, 22] including *P. gingivalis* (96.97%), *T. forshytia* (60.60%), and *A. actinomycetemcomitans* (18.18%) in addition to the

prevalence of *P. intermedia* (39.39%), which is known as a moderately common periodontal pathogen (Figure 2).

The present study further analyzed an inter-group relationship. Aside from *C. rectus*, the other pathogens mentioned above were shown to be more prevalent among newborns from mothers who were diagnosed with periodontal disease. Although the purpose of this study was to verify a possible association between the maternal clinical periodontal status and the oral presence of specific bacterial species, our results appear to corroborate previous observations from studies that compared the oral microbiota of mothers and their sons[5,8].

Interestingly, our findings suggest that the presence of periodontal disease in the mother favors at least a transitory early appearance of periodontal pathogens in the newborn. Although this study is unable to state the precise moment of this transmission event and, above all, whether or not there will be a permanent colonization that could potentially lead to an infectious event, we must emphasize the importance of incorporating preventive strategies primarily for mothers since this would undoubtedly procure health benefits for their children. Simple procedures such as clinical periodontal examination performed during the pregnancy and during the latescent period can contribute to an increase in oral health. Of course, factors involving such procedures must be discussed with the obstetrician so that the adopted preventive and therapeutic decision will be in the best interest of both the mother and the child.

Pre-natal evaluation is currently a very rigorous and specific process involving several evaluations and laboratory examinations in order to bring about a thorough accompaniment of fetal development and analysis of the mother's clinical condition.

This context appears to be an adequate opportunity to include a routine dental and periodontal evaluation on which the periodontics could determine what preventive and therapeutic procedures should be adopted in order to minimize this potential transmission.

Further studies are required to determine if the clinical periodontal status of the mother can serve as a potential risk factor that negatively impacts the child's oral health.

Bullet points:

What this paper adds

- This study suggests that the mothers' periodontal clinical status can be a strong indicator of the periodontal pathogens colonization of their newborns.
- The inclusion of a routine dental and periodontal during the pre-natal evaluation can minimize the potential of periodontal pathogens transmission for newborns.

Why this paper is important to paediatric dentists

- This study supports the establishment of an appropriate dental preventive program by paediatric dentists in newborn based on the mothers' periodontal clinical status.

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Figure legends

Figure 1 – The oral distribution of periodontal pathogens within the newborns from mothers without periodontal disease.

Figure 2 – The oral prevalence of periodontal pathogens within newborns from mothers with periodontal disease.

Tables:

Table 1 – Distribution of the population.

	Maternal periodontal clinical status		Total
	Group A Periodontitis +	Group B Periodontitis -	
Male	9	25	34
Female	24	18	42
Total	33	43	76
(MI±SD)	(1.57 ± 0.86)	(2.83 ± 1.60)	(2.28 ± 1.46)

MI – Mean age in months; SD – Standard deviation.

Table 2 – The oral frequencies (F) of periodontal pathogens in newborns according to the maternal clinical periodontal status.

			Cr	Pg	Aa	Pi	Tf
Maternal periodontal clinical status	Periodontitis -	Bacteria +	10	0	1	0	4
		Bacteria -	33	43	42	43	39
		F	23.25%	0.00%	2.32%	0.00%	9.30%
	Periodontitis +	Bacteria +	6	32	6	13	20
		Bacteria -	27	1	27	20	13
		F	18.18%	96.97%	18.18%	39.39%	60.60%
p valor			0.5907	**0.0000**	**0.0489**	**0.0000**	**0.0018**

Bacteria +: number of newborns showing detectable levels of the tested bacterial species

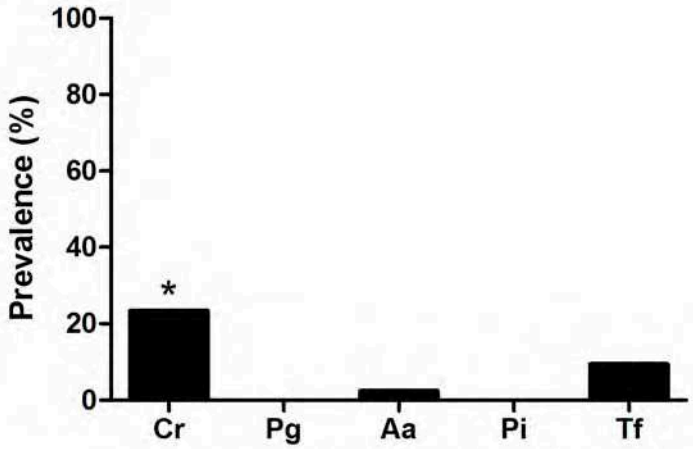
Bacteria -: number of newborns showing undetectable levels of the tested bacterial species

F= Frequency

Chi-square Test – inter-groups evaluation.

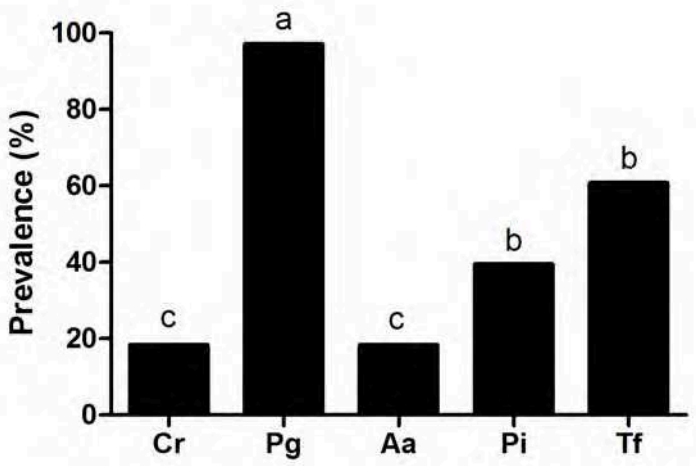
Figures:

Figure 1



* - Inter-species statistically significant difference ($p<0.05$). Chi-square test.

Figure 2



Different lower case letters (a,b,c) indicate a statistically significant difference ($p<0.05$). Chi-square test.

2.3 CAPÍTULO 3

Título do projeto: O impacto da condição periodontal e do tabagismo, pessoal e/ou materno, sobre a ocorrência de patógenos periodontais em recém-nascidos, crianças e adultos

Delineamento: Estudo transversal

População parcial: 254 crianças, dispostas em dois grupos de acordo com a condição gengival: 145 crianças com no máximo 30% dos sítios periodontais apresentando sangramento gengival e 109 crianças com mais de 30% dos sítios periodontais com sangramento gengival.

Objetivo específico: O objetivo do presente estudo foi avaliar a influência da condição clínica periodontal sobre a ocorrência de bactérias periodontopatogênicas em crianças.

Financiamento: Fapesp/ Auxílio à Pesquisa para o Doutorando Davi Romeiro Aquino (processo número 07/58340-0)

Outorgada: Profa. Dra. Sheila Cavalca Cortelli

Co-orientação: Gilson C N Franco

Equipe: Davi Romeiro Aquino, Gilson César Nobre Franco, José Roberto Cortelli, Fernando de Oliveira Costa, Juliana Guimarães Santos e Sheila Cavalca Cortelli

Fase: pesquisa concluída e artigo em elaboração

Artigo: Influência da condição clínica periodontal infantil sobre a ocorrência de bactérias periodontopatogênicas.

Metodologia:

O presente estudo foi aprovado pelo Comitê de Ética em Pesquisa da Universidade de Taubaté sob o protocolo nº 0317/07 (Anexo A).

Considerações éticas e caracterização geral da população:

Os indivíduos incluídos no presente estudo foram incluídos no período compreendido entre Junho de 2007 e Agosto de 2008. Todos os responsáveis legais receberam na semana que antecedeu a coleta de dados um Termo de Consentimento Livre e Esclarecido (TCLE) contendo informações sobre os objetivos e metodologia empregados na pesquisa. Esclarecimentos adicionais foram fornecidos verbalmente pelos pesquisadores. Após leitura e esclarecimentos das dúvidas, os responsáveis assinaram o TCLE e as crianças foram submetidas ao exame clínico e obtenção das amostras. Para obtenção de informações pessoais e caracterização da condição de saúde geral, os responsáveis também receberam um questionário auto-aplicável que retornou à professora após o preenchimento.

Cálculo amostral

O número de indivíduos incluídos no presente estudo foi determinado por um teste estatístico para cálculo amostral a partir de um projeto piloto. Foram obtidas amostras microbiológicas de 10 crianças (8,87±1,65 anos de idade), as quais foram processadas por PCR para as bactérias *A. actinomycetemcomitans, P. intermedia, P. gingivalis*, *T. forsythia e C. rectus*. Após obtenção dos resultados microbiológicos, foram confeccionadas tabelas de contingência 2X2. O teste estatístico Qui-quadrado

(X^2) para cálculo de amostra foi aplicado com auxílio do software *Bio Estat* 4.0 (Mamirauá Institute, Belém, PA, Brasil). Considerou-se um poder do teste de 80% e nível α de significância de 5%. Após aplicação do teste, o número mínimo de indivíduos a serem incluídos no presente estudo foi estabelecido em 200 crianças, sendo 100 em cada um dos grupos experimentais.

Exame clínico periodontal

Foram obtidas as medidas de Índice de Placa (IP) e Índice de Sangramento Gengival (ISG) dicotômicas de acordo com Ainamo & Bay (1975).

Para a realização do exame clínico periodontal, foram utilizados os seguintes instrumentos: espelho plano nº5 (Duflex®), pinça clínica (Duflex®) e sonda periodontal milimetrada tipo Carolina do Norte PCPUNC15BR (Hu-Friedy®) esterilizados.

Dois examinadores treinados e calibrados realizaram o exame periodontal. Para calibração intra e inter-examinadores, foi aplicado o índice estatístico Kappa (K). Os examinadores foram considerados calibrados quando da obtenção de um índice de concordância igual ou superior a 90%.

Critérios de inclusão e determinação dos grupos:

Foram incluídas no presente estudo 254 crianças cujos pais responderam adequadamente ao questionário e autorizaram sua participação. Essas crianças deveriam apresentar dentição mista e foram dispostas em dois grupos de acordo com a condição gengival. O primeiro grupo (145 crianças) foi composto de crianças

apresentando no máximo 30% dos sítios periodontais com sangramento gengival, i.e., grupo saudável. O segundo grupo foi composto de 109 crianças apresentando mais de 30% dos sítios periodontais com sangramento gengival, i.e. grupo gengivite.

Critérios de exclusão

Além dos indivíduos que não atenderam aos critérios de inclusão, foram excluídas crianças submetidas à antibioticoterapia sistêmica nos últimos seis meses. Indivíduos portadores de aparelho ortodôntico fixo ou removível também foram excluídos do estudo. Assim como aqueles com cáries extensas ou desordens sistêmicas indicadas pelos responsáveis.

Obtenção das amostras microbiológicas

Foram realizadas coletas de dorso da língua com *swab* tamponado de algodão previamente umedecido com tampão Tris-EDTA (TE) (Bio-Rad®). Para esta coleta, o *swab* foi rotacionado 6X na área central da língua para obtenção de uma única amostra extra-sulcular por criança. Adicionalmente, foram coletadas amostras microbiológicas intra-sulculares dos sítios mesio-vestibulares dos dentes 16, 11, 26, 36, 31 e 46, empregando para isto, cone de papel autoclavado n°30 (Dentsply®). Nos casos de ausência dos dentes pré-determinados, foram obtidas amostras intra-sulculares dos dentes imediatamente adjacentes (17, 21, 27, 37, 41 e 47).

Neste procedimento, cada sítio periodontal selecionado foi isolado com roletes de gaze esterilizada e o biofilme dental supragengival foi removido com algodão esterilizado. Um cone de papel foi inserido na porção mais apical do sulco

periodontal e mantido em posição por sessenta segundos (Cortelli et al., 2005). Em seguida, os cones de papel foram colocados em um único microtubo tipo Eppendorf® contendo 1,5mL de solução de Ringer reduzida e transportados ao laboratório de Biologia Molecular da UNITAU. As amostras foram mantidas a temperatura de -80ºC até o seu processamento.

Análise microbiológica pela Reação em Cadeia da Polimerase (PCR).

Os microtubos foram homogeneizados em agitador mecânico (Vortex®, Phoenix, AP56) e então 300µL foram centrifugados por dez minutos (4690 X *g)*. Após remoção do sobrenadante, 200µL de matriz comercial de extração e purificação de DNA (Instagene, Bio-Rad®) foram adicionados ao *pellet* formado. Após homogeneização por dez segundos, o material foi mantido em banho-maria por trinta minutos a 56°C. O material foi então novamente homogeneizado por trinta segundos e mantido por mais oito minutos em banho-maria a 100ºC. A conclusão do processo de extração e purificação deu-se pela homogeneização por trinta segundos e centrifugação por quatro minutos.

A reação em cadeia da polimerase (PCR) foi realizada em termociclador tipo Mastercycler Gradient (Eppendorf®) na seguinte condição: Um ciclo inicial a 95ºC/5min., 35 ciclos 95ºC/30seg., 55ºC/30seg., 72ºC/1min., e um ciclo final de 72ºC/5min.

Com a finalidade de verificar o sucesso do processo de extração de DNA, todas as amostras envolvidas no presente estudo foram processadas inicialmente utilizando *primer* específico para o gene da actina humana. As amostras negativas

para o gene da actina humana foram novamente submetidas ao processo de extração e posteriormente amplificação. A partir de DNA extraído de todas as amostras, a presença de *A. actinomycetemcomitans, P. intermedia, P. gingivalis*, *T. forsythia e C. rectus* foi avaliada empregando primers específicos (Figura 1).

Bactéria	Primers	Produto esperado (PB)
A.actinomycetemcomitans	5'AAACCCATCTCTGAGTTCTTCTTC3' 5'ATGCCAACTTGACGTTAAAT3'	550
P. gingivalis	5'-AGGCAGCTTGCCATACTGCG-3' 5'-ACTGTTAGCAACTACCGATGT-3'	404
P. intermedia	5'-TTTGTTGGGGAGTAAAGCGGG-3' 5'-TCAACATCTCTGTATCTGCGT-3'	575
T. forsythensis	5'-GCGTATGTAACCTGCCCGCA-3' 5'-TGCTTCAGTGTCAGTTATACCT-3'	641
C. rectus	5'-TTTCGGAGCGTAAACTCCTTTT-3' 5'-TTTCTGCAAGCAGACACTCTT-3'	598

PB – Pares de base

Figura 1 – Descrição dos *primers* utilizados no presente estudo

Para a análise dos produtos amplificados pela PCR foi empregada eletroforese conduzida a 10V/cm^2 em solução tamponada (TBE) por uma hora empregando-se gel de agarose a 1,5% corados com Brometo de Etídio. A visualização foi realizada em câmara de irradiação ultravioleta (UV). Marcador de peso molecular (Ladder 100 – Invitrogen®), bem como, controles positivos e negativos foram empregados em todos os géis, os quais foram fotografados e

comparados com os produtos amplificados a partir de cepas padrão, cedidas pelo Instituto Fio Cruz, RJ.

Resultados Preliminares:

Foram incluídos no presente estudo 254 crianças, sendo 122 do gênero masculino e 132 do feminino (Tabela 1).

Inicialmente, em uma avaliação intra-grupo, no grupo de crianças periodontalmente saudáveis e em amostras intra-sulculares, *P. gingivalis* foi o patógeno periodontal mais prevalente. Para as amostras extra-sulculares, *P. gingivalis* e *C. rectus* foram as bactérias mais prevalentes (Figura 2). Para as crianças com gengivite, *P.gingivalis* foi o patógeno periodontal mais prevalente independente do local de coleta (Figura 3).

Quando da análise intra-grupos, *C. rectus* apresentou-se mais prevalente no sulco das crianças periodontalmente saudáveis em comparação as com gengivite. *P. gingivalis* esteve mais freqüente no grupo de crianças com gengivite independente do local de coleta (Tabela 2).

Tabela 1– Distribuição da população estudada

	Saudável	Gengivite	Total
Masculino	73	49	122
Feminino	72	60	132
Total (MI±DP)	145 (8.11±2.59)	109 (8.54±1.32)	254 (8.41±1.79)

MI – Média de Idade; DP – Desvio Padrão

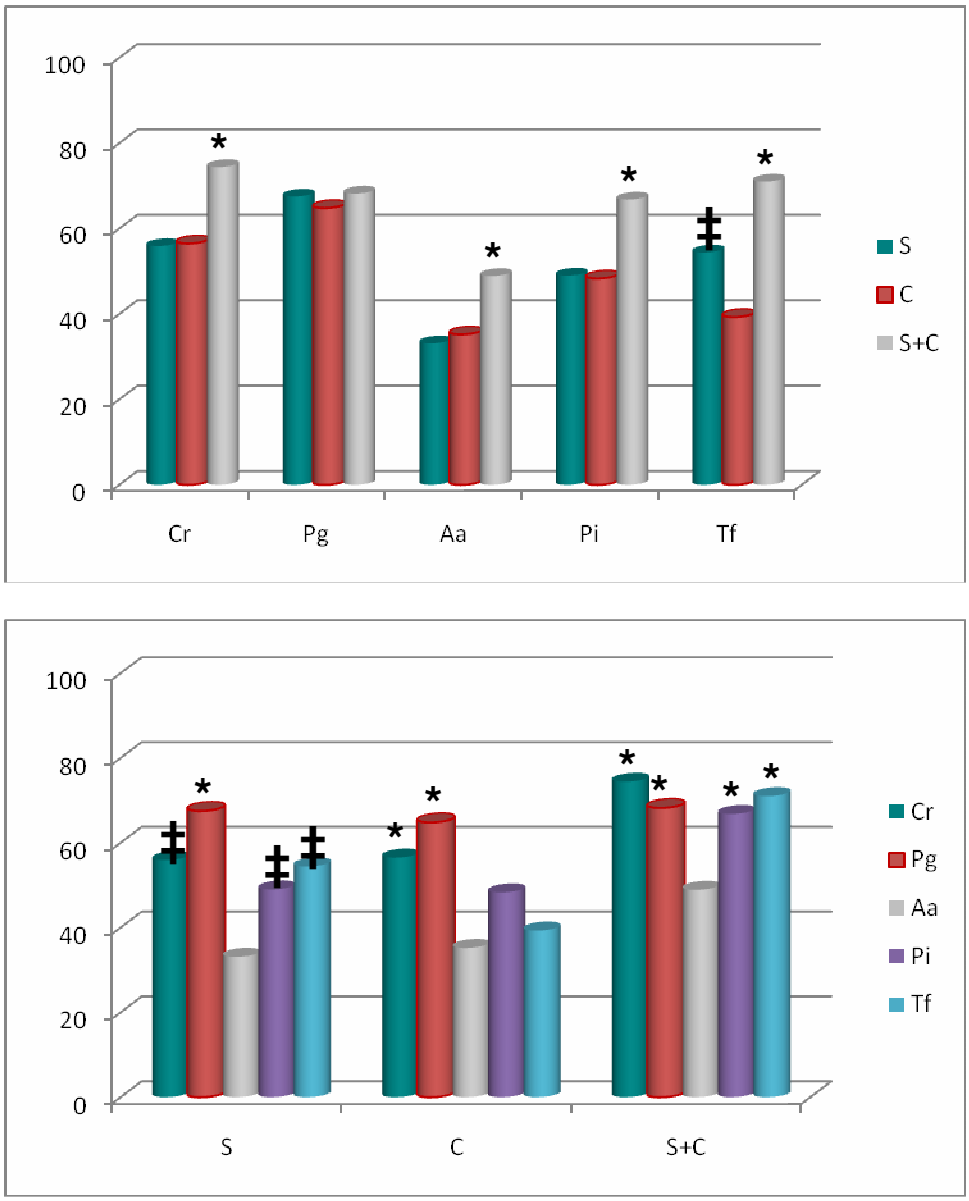


Figura 2 – Distribuição da freqüência dos patógenos periodontais nos indivíduos do grupo saudável

S – Amostras intra-sulculares; C – Amostras extra-sulculares; S+C – Avaliação conjunta das amostras intra e extra-sulculares; *, ‡ - Diferença estatisticamente significativa, $p<0,05$, teste Qui-quadrado

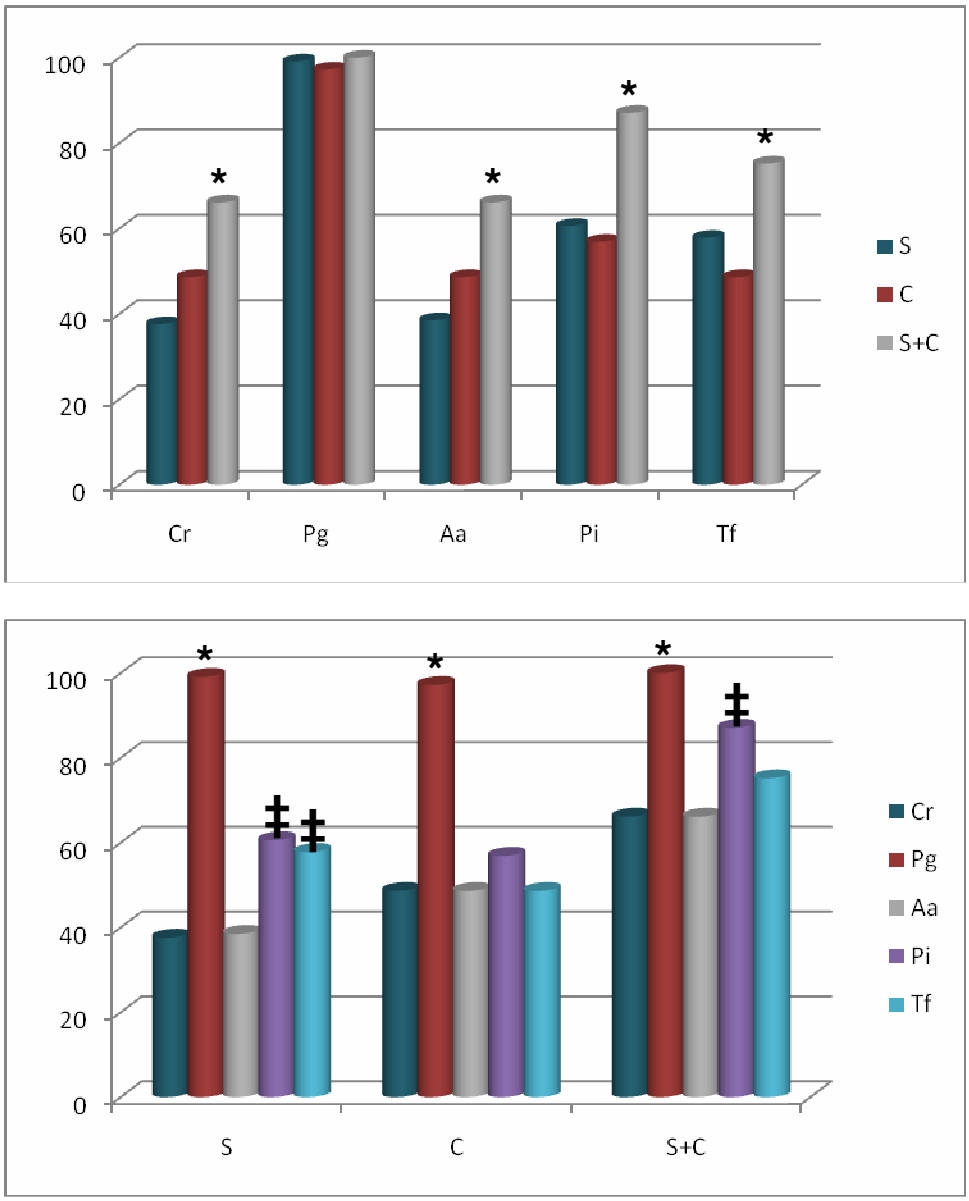


Figura 3 – Distribuição da freqüência dos patógenos periodontais nos indivíduos do grupo gengivite

S – Amostras intra-sulculares; C – Amostras extra-sulculares; S+C – Avaliação conjunta das amostras intra e extra-sulculares; *,‡ - Diferença estatisticamente significativa, $p<0,05$, teste Qui-quadrado

Tabela 2 – Freqüência dos patógenos periodontais nos grupos avaliados

		Cr			Pg			Aa			Pi			Tf		
		S	C	S+C	S	C	S+C	S	C	S+C	S	C	S+C	S	C	S+C
SG	+	81	82	108	98	94	99	48	51	71	71	70	97	79	57	103
	-	64	63	37	47	51	46	97	94	74	74	75	48	66	88	42
	f(%)	55.9	56.5	74.4	67.5	64.8	68.2	33.1	35.2	48.9	49.0	48.3	66.8	54.4	39.3	71.0
G	+	41	53	72	108	106	109	42	53	72	66	62	95	63	53	82
	-	68	56	37	1	3	0	67	56	37	43	47	14	46	56	27
	f(%)	37.6	48.6	66.1	99.1	97.2	100.0	38.5	48.6	66.1	60.6	56.9	87.1	57.8	48.6	75.2
p valor		**0.0059**	0.2601	0.1856	**0.0001**	**0.0001**	**0.0001**	0.4456	**0.0425**	**0.0096**	0.0880	0.2180	**0.0004**	0.6898	0.1755	0.5476

S – Amostras intra-sulculares; C – Amostras extra-sulculares; S+C – Avaliação conjunta das amostras intra e extra-sulculares; SG – Grupo saúde gengival; G – Grupo gengivite; f - Freqüência

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2.4 CAPÍTULO 4

Título do projeto: O impacto da condição periodontal e do tabagismo, pessoal e/ou materno, sobre a ocorrência de patógenos periodontais em recém-nascidos, crianças e adultos

Delineamento: Estudo transversal

População parcial: 214 indivíduos adultos distribuídos em quatro grupos de acordo com a condição periodontal e o hábito de fumar, sendo um grupo periodontalmente saudável [fumante (n=51) e não fumante (n=52)] e outro com periodontite [fumante (n=53) e não fumante (n=58)].

Objetivo específico: O objetivo do presente estudo foi avaliar a influência da condição clínica periodontal e do tabagismo sobre a presença patógenos periodontais em adultos.

Financiamento: Fapesp/ Auxílio à Pesquisa para o Doutorando Davi Romeiro Aquino (processo número 07/58340-0)

Outorgada: Profa. Dra. Sheila Cavalca Cortelli

Co-orientação: Gilson C N Franco

Equipe: Davi Romeiro Aquino, Gilson Cesar Nobre Franco, José Roberto Cortelli, Fernando de Oliveira Costa, Juliana Guimarães Santos e Sheila Cavalca Cortelli

Fase: pesquisa concluída e análise estatística em elaboração

Artigo: Influência da condição clínica periodontal e tabagismo sobre a presença de patógenos periodontais em adultos.

Metodologia:

O presente estudo foi aprovado pelo Comitê de Ética em Pesquisa da Universidade de Taubaté sob o protocolo nº 0317/07 (Anexo A).

População do estudo:

Todos os indivíduos receberam um Termo de Consentimento Livre e Esclarecido (TCLE) contendo informações sobre os objetivos e metodologia empregados na pesquisa. Esclarecimentos adicionais foram fornecidos verbalmente pelos pesquisadores. Após leitura e esclarecimentos das dúvidas, os participantes assinaram o TCLE e foram submetidos à anamnese para obtenção de informações pessoais e caracterização da condição de saúde geral.

Cálculo amostral

O número de indivíduos incluídos foi determinado por teste estatístico para cálculo amostral a partir de um projeto piloto. Foram obtidas amostras microbiológicas de 10 adultos, as quais foram processadas no Laboratório de Biologia Molecular da UNITAU. Após obtenção dos resultados microbiológicos, foram confeccionadas tabelas de contingência 2X2. O teste estatístico Qui-quadrado (X^2) para cálculo de amostra foi aplicado com auxílio do software *Bio Estat* 4.0 (Mamirauá Institute, Belém, PA, Brasil). Considerou-se um poder do teste de 80% e nível α de significância de 5%. Após aplicação do teste, o número mínimo de indivíduos a serem incluídos no presente estudo foi 50 por grupo experimental.

Critérios de inclusão de determinação dos grupos:

Foram incluídos no presente estudo, no período compreendido entre Julho e Dezembro de 2008, 214 indivíduos adultos de acordo com a condição periodontal e o hábito de fumar, sendo um grupo periodontalmente saudável [fumante (n=51) e não fumante (n=52)] e outro grupo com periodontite [fumante (n=53) e não fumante (n=58)], segundo os critérios de López et al. (2002), onde para o indivíduo ser considerado portador de periodontite, deveria apresentar no mínino 4 sítios periodontais com PS ≥ 4mm e NCI ≥ 3mm, distribuídos em dentes diferentes.

Foram considerados fumantes indivíduos que relataram fumar no mínimo dez cigarros/dia (Ward & Klesges, 2001) há pelo menos três anos. Ex-fumantes, que pararam de fumar há pelo menos cinco anos foram incluídos como não fumantes. Os demais ex-fumantes não foram incluídos.

Critérios de exclusão

Além dos indivíduos que não atenderam aos critérios de inclusão, foram excluídos indivíduos submetidos à antibioticoterapia local e/ou sistêmica nos últimos seis meses e/ou tratamento periodontal nos 12 meses que antecederam o início do estudo. Pacientes diabéticos descompensados, imunossuprimidos, gestantes, portadores de alterações hormonais, bem como, usuários de aparelho ortodôntico e próteses fixas extensas foram excluídos do estudo. Finalmente, participantes com menos de dez dentes bem como aqueles que requeriam cobertura antibiótica para exame periodontal foram excluídos.

Exame clínico periodontal

Foram mensurados os valores de Profundidade de sondagem (PS), Nível clínico de Inserção (NCI), Índice de Placa (IP) e Índice de Sangramento Gengival (ISG) dicotômicos de Ainamo & Bay (1975) . Todos os parâmetros clínicos foram observados em seis pontos por dente (Fetner, 1994), em todos os dentes presentes, exceto os terceiros molares. Para a realização do exame clínico periodontal, foram utilizados os seguintes instrumentos: espelho plano nº5 (Duflex®), pinça clínica (Duflex®) e sonda periodontal milimetrada tipo Carolina do Norte PCPUNC15BR (Hu-Friedy®) esterilizados.

Dois examinadores treinados e calibrados realizaram o exame periodontal. Para calibração intra e inter-examinadores, foi aplicado o método Erro Padrão da Medida (EPM) para as variáveis contínuas, PS e NCI, e o índice estatístico Kappa (K) para as variáveis categóricas, IP e ISG (Araujo et al*.,* 2003). Os examinadores foram considerados calibrados quando da obtenção de um índice de concordância igual ou superior a 90% para ambos os testes.

Obtenção das amostras microbiológicas

Foram realizadas coletas do dorso da língua com *swab* tamponado de algodão previamente umedecido com tampão Tris-EDTA (TE) (Bio-Rad®). Para esta coleta, o *swab* foi rotacionado 6X na área central da língua para obtenção de uma única amostra extra-sulcular por indivíduo e coletas intra-sulculares, com cone de papel autoclavado n°30 (Dentsply®), dos cinco sítios que apresentaram os maiores valores de PS. Estas amostras foram obtidas em dentes diferentes e

preferencialmente de faces dentárias não contíguas e de hemi-arcos distintos. No grupo de indivíduos portadores de periodontite, além da PS foi considerado o NCI para seleção dos dentes. O grau de inflamação gengival foi utilizado como critério de desempate entre valores iguais de PS e NCI.

Para o grupo saudável foram coletadas amostras subgengivais dos sítios mésio-vestibulares dos dentes 16, 11, 26, 36, 31 e 46. Quando os mesmos não estiveram presentes, foram utilizados os dentes imediatamente adjacentes (17, 21, 27, 37, 41 e 47).

Análise microbiológica pela Reação em Cadeia da Polimerase (PCR).

Os microtubos foram homogeneizados em agitador mecânico (Vortex®, Phoenix, AP56) e então 300µL foram centrifugados por 10 minutos (4690 X *g)*. Após remoção do sobrenadante, 200µL de matriz comercial de extração e purificação de DNA (Instagene, Bio-Rad®) foram adicionados ao *pellet* formado. Após homogeneização por dez segundos, o material foi mantido em banho-maria por trinta minutos a 56°C. O material foi então novamente homogeneizado por trinta segundos e mantido por mais oito minutos em banho-maria a 100ºC. A conclusão do processo de extração e purificação deu-se pela homogeneização por trinta segundos e centrifugação por quatro minutos.

A reação em cadeia da polimerase (PCR) foi realizada em termociclador tipo Mastercycler Gradient (Eppendorf®) na seguinte condição: Um ciclo inicial a 95ºC/5min., 35 ciclos 95ºC/30seg., 55ºC/30 seg., 72ºC/1min., e um ciclo final de 72ºC/5min.

Com a finalidade de verificar o sucesso do processo de extração de DNA, todas as amostras envolvidas no presente estudo foram processadas inicialmente utilizando *primer* específico para o gene da actina humana. As amostras negativas para o gene da actina humana foram novamente submetidas ao processo de extração e posteriormente amplificação. A partir de DNA extraído de todas as amostras, a presença de *A. actinomycetemcomitans, P. intermedia, P. gingivalis*, *T. forsythia e C. rectus* foi avaliada empregando primers específicos (Figura 1).

Bactéria	Primers	Produto esperado (PB)
A.actinomycetemcomitans	5'AAACCCATCTCTGAGTTCTTCTTC3' 5'ATGCCAACTTGACGTTAAAT3'	550
P. gingivalis	5'-AGGCAGCTTGCCATACTGCG-3' 5'-ACTGTTAGCAACTACCGATGT-3'	404
P. intermedia	5'-TTTGTTGGGGAGTAAAGCGGG-3' 5'-TCAACATCTCTGTATCTGCGT-3'	575
T. forsythensis	5'-GCGTATGTAACCTGCCCGCA-3' 5'-TGCTTCAGTGTCAGTTATACCT-3'	641
C. rectus	5'-TTTCGGAGCGTAAACTCCTTTT-3' 5'-TTTCTGCAAGCAGACACTCTT-3'	598

PB – Pares de base

Figura 1 – Descrição dos *primers que* utilizados no presente estudo

Para a análise dos produtos amplificados pela PCR foi empregada eletroforese conduzida a 10V/cm^2 em solução tamponada (TBE) por uma hora empregando-se gel de agarose a 1,5% corados com Brometo de Etídio. A

visualização foi realizada em câmara de irradiação ultravioleta (UV). Marcador de peso molecular (Ladder 100 – Invitrogen®), bem como, controles positivos e negativos foram empregados em todos os géis, os quais foram fotografados e comparados com os produtos amplificados a partir de cepas padrão, cedidas pelo Instituto Fio Cruz, RJ.

Resultados preliminares:

Foram incluídos no presente estudo 214 indivíduos, sendo 103 periodontalmente saudáveis e 111 portadores de doença periodonrtal (Tabelas 1 e 2).

Foi proposta inicialmente uma comparação intra-grupo da freqüência dos patógenos periodontais em função do hábito de fumar. Não foi observada diferença estatisticamente significativa quando da avaliação isolada dos grupos saúde (Figura 2) e doença periodontal (Figura 3).

Tabela 1– Distribuição da população estudada

	Saúde periodontal		Total
	Fumante	Não-fumante	
Masculino	18	22	40
Feminino	33	30	63
Total (MI±DP)	51 (32,53±8,29)	52 (31,13±7,97)	103 (31,81±8,09)
	Doença periodontal		Total
	Fumante	Não-fumante	
Masculino	26	24	50
Feminino	27	34	61
Total (MI±DP)	53 (34,98±6,11)	58 (33,2±7,48)	111 (33,94±6,96)

MI – Média de Idade; DP – Desvio Padrão

Tabela 2– Distribuição média dos valores de Profundidade de Sondagem (PS), Nível Clínico de Inserção (NCI), Índice de Placa (IP) e Índice de Sangramento Gengival (ISG), de acordo com a condição periodontal e o hábito de fumar

	Saúde periodontal			
	PS (mm)	NCI (mm)	IP (0/1)	ISG (0/1)
Fumante	3,45 ±1,65	2,94 ±1,64	0,61 ±0,33	0,47 ±0,32
Não-fumante	3,41 ±1,45	3,21 ±1,47	0,62 ±0,30	0,61 ±0,29
Total (M±DP)	3,43 ±1,54	3,08 ±1,55	0,61 ±0,31	0,54 ± 0,31
	Doença Periodontal			
	PS (mm)	NCI (mm)	IP (0/1)	ISG (0/1)
Fumante	4,65 ±1,54	4,70 ±1,62	0,62 ±0,32	0,55 ±0,29
Não-fumante	4,65 ±1,69	4,69 ±1,70	0,66 ± 0,30	0,54 ±0,30
Total (M±DP)	4,65 ±1,62	4,69±1,70 ±1,66	0,64 ±0,31	0,54 ± 0,30

M – Média; DP – Desvio Padrão; Teste *t* de Student e Mann-whitney

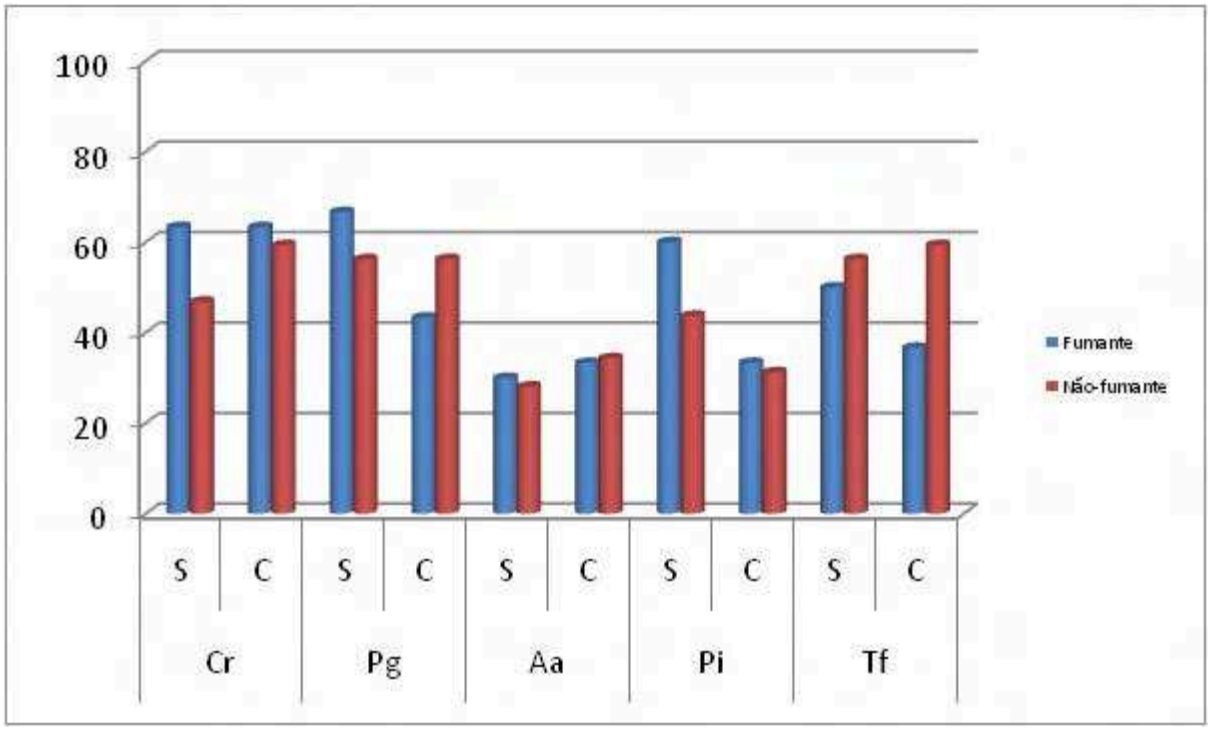

Figura 2 – Distribuição da freqüência dos patógenos periodontais nos indivíduos do grupo saúde periodontal de acordo com o hábito de fumar

S – Amostras de sulco gengival, C – Amostras de mucosa jugal; Teste Qui-quadrado

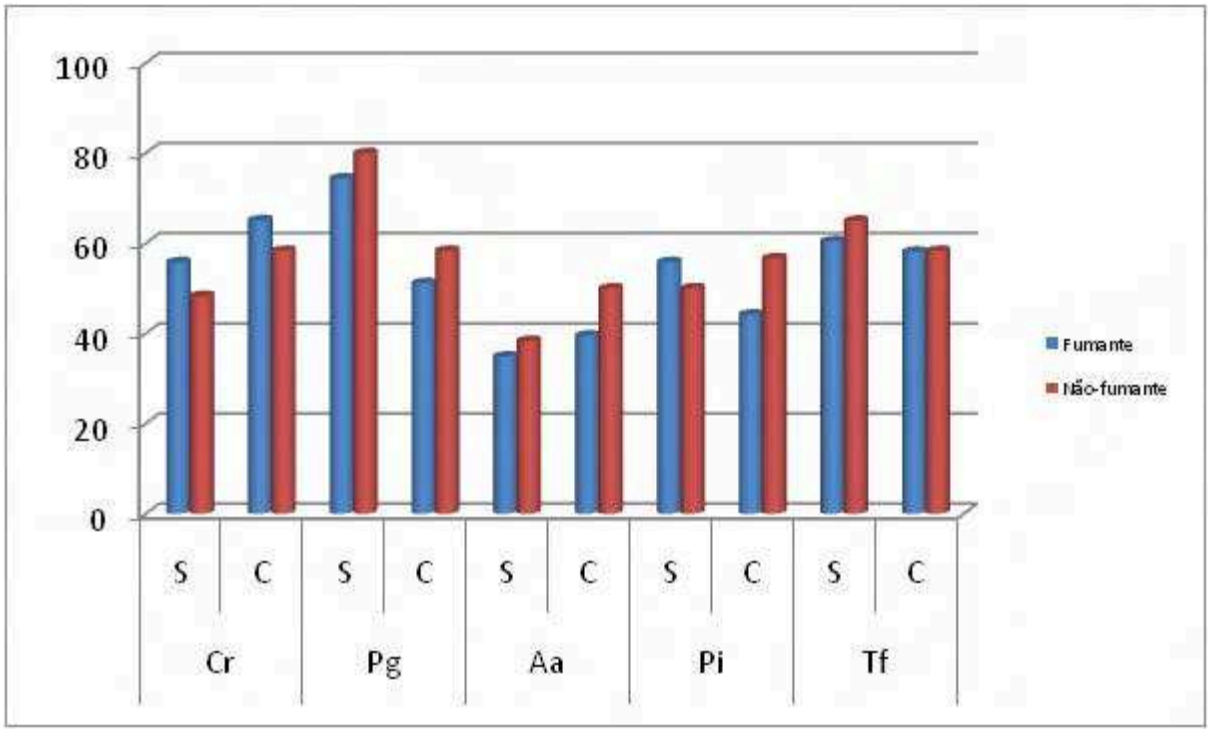

Figura 3 – Distribuição da freqüência dos patógenos periodontais nos indivíduos do grupo doença periodontal de acordo com o hábito de fumar

S – Amostras de sulco gengival, C – Amostras de mucosa jugal; Teste Qui-quadrado

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3 CONCLUSÕES

Diante dos resultados obtidos no presente estudo podemos concluir que:

A) A colonização por patógenos periodontais ocorre precocemente e se altera ao longo da vida, sendo muitas vezes direcionada pela presença dos elementos dentários que no presente estudo influenciou sobretudo a ocorrência de *P. gingivalis*, *P. intermedia* e *T. forsythia*.

B) A condição periodontal materna influenciou a ocorrência de patógenos periodontais em recém-nascidos. Similarmente, o status periodontal infantil acarretou aumento nas freqüências bacterianas.

C) O tabagismo não alterou os perfis microbiológicos observados.

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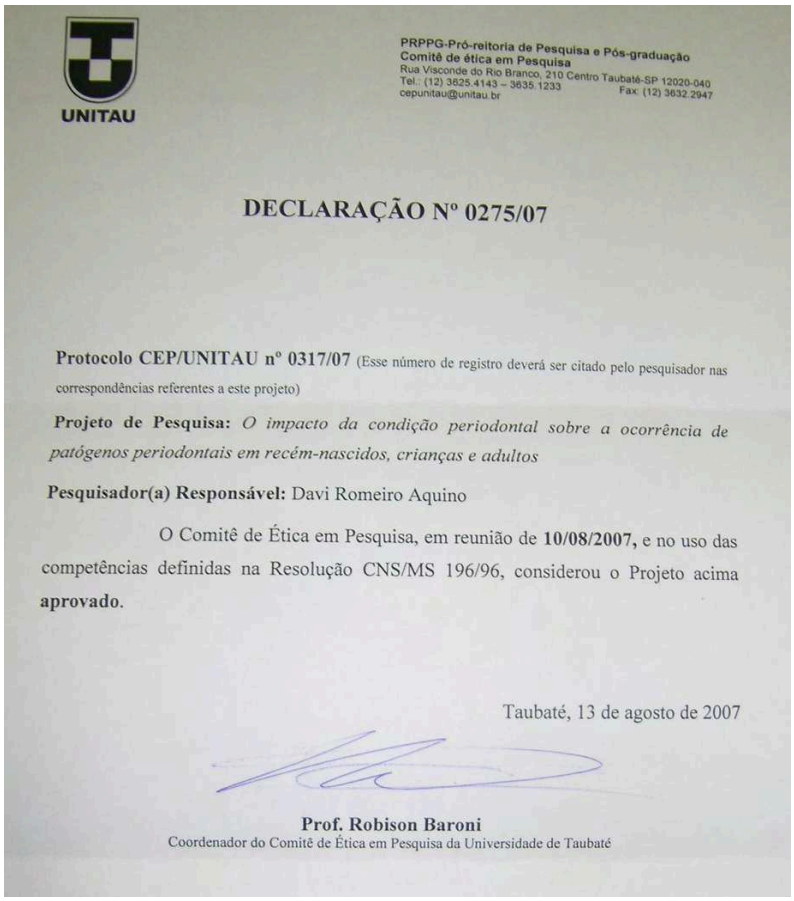
ANEXOS

ANEXO A – Cópia da carta de aprovação pelo Comitê de Ética Institucional



UNITAU

PRPPG-Pró-reitoria de Pesquisa e Pós-graduação
Comitê de ética em Pesquisa
Rua Visconde do Rio Branco, 210 Centro Taubaté-SP 12020-040
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DECLARAÇÃO Nº 0275/07

Protocolo CEP/UNITAU nº 0317/07 (Esse número de registro deverá ser citado pelo pesquisador nas correspondências referentes a este projeto)

Projeto de Pesquisa: *O impacto da condição periodontal sobre a ocorrência de patógenos periodontais em recém-nascidos, crianças e adultos*

Pesquisador(a) Responsável: Davi Romeiro Aquino

O Comitê de Ética em Pesquisa, em reunião de **10/08/2007,** e no uso das competências definidas na Resolução CNS/MS 196/96, considerou o Projeto acima **aprovado**.

Taubaté, 13 de agosto de 2007

Prof. Robison Baroni
Coordenador do Comitê de Ética em Pesquisa da Universidade de Taubaté

ANEXO B – Comprovante de submissão, revisão da língua inglesa e ficha de avaliação de contato mãe-filho

11-Feb-2009

Dear Prof. Cortelli,

Your manuscript entitled "The impact of the maternal's periodontal status on the detection of periodontal pathogens in newborn children" has been successfully submitted online and is presently being given full consideration for publication in the International Journal of Paediatric Dentistry.

Your manuscript ID is IJPD-02-09-1113.

Please mention the above manuscript ID in all future correspondence or when calling the editorial office with questions. If there are any changes in your mailing or e-mail addresses, please log into Manuscript Central at http://mc.manuscriptcentral.com/ijpd and edit your user information accordingly.

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Yours sincerely,

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ANEXO B – Comprovante de submissão, revisão da língua inglesa e ficha de avaliação de contato mãe-filho – continuação...

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ANEXO B – Comprovante de submissão, revisão da língua inglesa e ficha de avaliação de contato mãe-filho – continuação ...

Mãe (nome e telefone)	
Recém-nascido (nome)	
Médico (nome)	
Prontuário médico (número)	
1 Tipo de parto	Natural ( ) cesariana ( )
2 Sentiu dor após o parto?	
3 Teve que tomar remédio?	Sim ( ) Não ( ) Qual?
4 Sentiu sono após o parto?	
5 Ficou acamada após o parto?	Sim ( ) Não ( )
Por quanto tempo?	____ dias, ____ semanas
Quem cuidou do bebê nesse tempo?	
6 Número e idade dos outros filhos	____ filhos / idades ___, ___, ___, ___, ___, ___, ____, ____
7 Tipo de amamentação	Materna ( ) Mamadeira ( )
De quanto em quanto tempo ele mama?	
Quem prepara e dá as mamadeiras?	
O que é colocado na mamadeira?	
Você limpa o peito antes de dar mama?	Sim ( ) Não ( ) O que usa para limpar?
Você limpa a boca do bebê depois da mamada?	Sim ( ) Não ( ) O que usa para limpar?
8 O bebê usa chupeta?	Sim ( ) Não ( ) O que usa para limpar?
9 Quem troca as fraldas do bebê?	
De quanto em quanto tempo é feita essa troca?	
10 Quem dá banho no bebê?	
Em qual horário?	
11 Quem leva o bebê para tomar sol ou passear?	
Em qual horário?	
12 Quem leva o bebê para tomar vacinas?	
13 Você leva o bebê quando sai de casa?	
14 Quais horários seu bebê dorme?	Manhã
	Tarde
	Noite
15 Quem faz almoço na sua casa?	
Nessa hora o bebê fica com você?	
16 Quem lava e passa roupa na sua casa?	
Nessa hora o bebê fica com você?	
17 Quem limpa sua casa?	
Nessa hora o bebê fica com você?	
18 Em que períodos do dia seu bebê fica com outras pessoas?	Manhã ( ) ____ horas
	Tarde ( ) ____ horas
	Noite ( ) ____ horas
Quem são essas pessoas?	
19 Quem te ajuda com o bebê?	
Quando?	
20 Você beija seu bebê na boca?	

ANEXO C – Declaração de não infração de direitos autorais

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