UNIVERSIDADE SAGRADO CORAÇÃO

LUCAS TREVIZANI RASMUSSEN

DIAGNÓSTICO E GENOTIPAGEM DO *HELICOBACTER PYLORI* EM AMOSTRAS DE BIÓPSIA GÁSTRICA, SALIVA E PLACA DENTAL

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Dissertação apresentada à Pró-reitoria de Pós-graduação como parte dos requisitos para obtenção do título de Mestre em Biologia Oral, Área de Concentração: Biologia Oral sob orientação do Prof. Dr. Spencer Luiz Marques Payão.

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Pró-reitoria de Pesquisa e Pós-graduação

ATA DA DEFESA DE DISSERTAÇÃO DE MESTRADO LUCAS TREVIZANI RASMUSSEN ALUNO (A) DO PROGRAMA DE MESTRADO EM BIOLOGIA ORAL - ÁREA DE CONCENTRAÇÃO: BIOLOGIA ORAL, DA UNIVERSIDADE DO SAGRADO CORAÇÃO, USC, BAURU.

No dia 28 de agosto de 2009, em sessão pública, na Universidade do Sagrado Coração, na presença da Banca Examinadora, composta pelos(as) docentes: ***Prof. Dr. Spencer Luiz Marques Payão***, Professor(a) Doutor(a) da Universidade do Sagrado Coração; ***Prof.ª Dr.ª Angela Mitie Otta Kinoshita***, Professor(a) Doutor(a) da Universidade do Sagrado Coração; e ***Prof. Dr. Valdeir Fagundes de Queiroz***, Professor(a) Doutor(a) do(a) Faculdade de Medicina de Marília; tiveram início os trabalhos de julgamento da Prova de DISSERTAÇÃO de MESTRADO para obtenção do Grau de Mestre em BIOLOGIA ORAL - ÁREA DE CONCENTRAÇÃO: BIOLOGIA ORAL, pelo(a) mestrando(a) **LUCAS TREVIZANI RASMUSSEN**. Os(as) examinadores(as), observando o tempo regulamentar, arguiram o(a) candidato(a) sobre a DISSERTAÇÃO que o(a) mesmo(a) havia apresentado, intitulada ***"DIAGNÓSTICO E GENOTIPAGEM DO HELICOBACTER PYLORI EM AMOSTRAS DE BIÓPSIA GÁTRICA, SALIVA E PLACA DENTAL"***, tendo o(a) candidato(a) procurado explicar e/ou rebater as críticas formuladas pelos(as) arguidores(as). Após a conclusão da prova de DISSERTAÇÃO de MESTRADO, foi suspensa a sessão pública e, em sessão secreta, os(as) argüidores(as) atribuíram seus conceitos. Reaberta a sessão pública, foram anunciados os resultados: ***Prof. Dr. Valdeir Fagundes de Queiroz,*** APROVADO ; ***Prof.ª Dr.ª Angela Mitie Otta Kinoshita,*** Aprovado ; ***Prof. Dr. Spencer Luiz Marques Payão*** , APROVADO . Conceito Final: APROVADO , fazendo jus, portanto, ao título de Mestre em BIOLOGIA ORAL - ÁREA DE CONCENTRAÇÃO: BIOLOGIA ORAL, de acordo com o artigo 43 do Regimento Geral da Pós-graduação da USC. Nada mais havendo a registrar, foi lavrada a presente ata, que vai por mim assinada, Angela Lemes de Moraes e pelos (as) Senhores (as) Membros da Comissão Examinadora.

Prof. Dr. Valdeir Fagundes de Queiroz

Prof.ª Dr.ª Angela Mitie Otta Kinoshita

Prof. Dr. Spencer Luiz Marques Payão
(Presidente da Banca e Orientador)

Angela Lemes de Moraes
(Secretária *Stricto Sensu*)

DEDICO ESTE TRABALHO

***A DEUS**, pelo destino traçado.*

*Aos meus pais, **Luis** e **Cristina**, por nunca duvidarem, pela incansável batalha e amor eterno.*

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"As mais lindas palavras de amor são ditas no silêncio de um olhar". (Leonardo da Vinci).

Lucas Trevizani Rasmussen

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"A mente que se abre a uma nova idéia jamais voltará ao seu tamanho original"

Albert Einstein

LISTA DE FIGURAS

LISTA DE ABREVIATURAS, SIGLAS E SÍMBOLOS

µL	Microlitro
mL	Mililitro
DNA	Ácido Desoxirribonucléico
kDa	Quilodaltons
Pb	Pares de Base
PCR	Reação em Cadeia da Polimerase
*bab*A	*Blood Group Antigen-Binding Adhesin*
*cag*A	*Cytotoxin association gene*
*vac*A	*Vacuolation cytotoxin gene*

SUMÁRIO

1.0. RESUMO

A bactéria *Helicobacter pylori* coloniza aproximadamente 50% da população mundial e está associada à gênese de gastrites, úlceras e câncer gástrico. Diferentes cepas de importância clínica vem sendo descritas por pesquisadores, que sugerem diversos marcadores de virulência entre os quais podemos destacar o gene *cag*A e o gene *vac*A que em vários estudos estão relacionados a gastrites e úlceras pépticas. Há controversas sobre a permanência do *H. pylori* na cavidade oral, e a literatura não é clara em afirmar se a cavidade oral é um reservatório da bactéria. O presente trabalho tem como objetivos diagnosticar o *Helicobacter pylori* e caracterizar a presença do gene *cagA* e os genótipos do gene *vacA* através da técnica de PCR e *Southern Blotting*, a partir de amostras de biópsia gástrica, saliva e placa dental. Foram analisadas amostras de biópsia gástrica, saliva e placa dental coletadas de pacientes adultos dispépticos. Houve uma diferença estatisticamente significante na detecção do *H. pylori* por *Southern blotting* e as demais técnicas, também verificamos um aumento significativo de positividade para o *H. pylori* associado ao gene *cag*A e alelos s1/m1 do gene *vac*A em amostras de biópsia gástrica, já as amostras de saliva e placa dental apresentaram uma menor prevalência da bactéria sem associação com os marcadores de virulência, entretanto a presença do *H. pylori,* em amostras de saliva e placa dental reforçam a premissa de que cavidade oral seja um reservatório da bactéria, e uma possível via de transmissão e reinfecção gástrica. Além disso, evidenciamos uma colonização mista em um único hospedeiro assim como a ausência de relação efetiva entre a colonização da saliva, placa dental e mucosa gástrica.

Palavras-chave:

Helicobacter pylori; Saliva; Placa dental, *cag*A; *vac*A; *Southern Blotting*

ABSTRACT

Purpose: The *Helicobacter pylori*, a gram negative bacterium to colonize human gastric mucosa and has been shown to be strongly associated with chronic gastritis, gastric and duodenal ulcerations and a risk factor for gastric carcinoma. Several studies suggest progressive gastric mucosa damage induced by the patogen. Extensive search for *Helicobacter pylori* virulence factors lead to the characterizations of a vacuolating cytotoxin A (*vac*A) and an associated cytotoxin A (*cag*A). The oral cavity has been implicated as a potential *H. pylori* reservoir, and may therefore be involved in the reinfection of the stomach, which sometimes follows treatment of *H. pylori* infection. The objectives of this study were (1) to determine the presence of *H. pylori* in the oral cavity and (2) to investigate the relationship between oral *H. pylori* and subsequent gastritis. **Methods:** Gastric biopsies, saliva and dental plaques were obtained from dyspeptic adults. DNA was extracted and evaluated for the *H. pylori* and *cag*A presence, *vac*A alleles, using polymerase chain reaction and Southern blotting methods. **Results:** Persons with gastritis had a high frequency of *Helicobacter pylori* positives in the stomach and there was a statistically significant correlation between gastric biopsies and oral cavity. **Conclusions:** We shown a high prevalence of the *H. pylori* and presence with *cag*A gene associated of *vac*A s1 alelles in gastric biopsies. Our results suggest a relation between gastric infection and the bacterium in the oral cavity, with a variation of cytotoxin genotype between saliva and dental plaque suggesting a reservoir of the species and that more than one *H. pylori* strains may exist in the saliva, dental plaque and stomach of the same patient.

INTRODUÇÃO

2.0. INTRODUÇÃO

A associação entre uma espiroqueta e a úlcera péptica foi descrita primeiramente por Doengues em 1939, entretanto Marshall & Warren em 1983 que descreveram uma bactéria gram-negativa de forma bacilo-espiral encontrada na mucosa gástrica, o qual foi denominada *Campylobacter pyloridis.* Tal descrição foi de início rejeitada pela comunidade cientifica, uma vez que, esses autores sugeriram a associação entre a bactéria e a gênese das doenças gástricas.

Características bioquímicas e genéticas desta nova espécie diferiam do gênero *Campylobacter.* Assim, surgiu o gênero *Helicobacter* e a bactéria foi definida como *Helicobacter pylori* (*H. pylori*). Somente em 1986 Rathbone e colaboradores, obtiveram o reconhecimento oficial da identificação do *Helicobacter pylori* na mucosa do estômago e duodeno de indivíduos adultos dispépticos.

Esta bactéria é classificada como gram-negativa, com 2 - 4 μm de comprimento e 0.5 - 1 μm de largura. Ocorrem sob duas formas morfológicas: bacilos e cocos, embora a forma bacilar espiral seja predominante. É considerado um microorganismo que cresce em condições de microaerofilia com temperatura ideal de 35ºC a 37ºC (Gatti *et al*., 2006).

O *H. pylori* apresenta características bioquímicas bem distintas, é catalase e oxidase positiva e uma grande produtora da enzima urease. As colônias são descritas morfologicamente como pequenas, cinzentas e delicadamente hemolítica. Este microorganismo é especializado na colonização do estômago humano podendo sobreviver a pH abaixo de 4.

Apresenta de 2 - 6 flagelos de aproximadamente 3 μm de comprimento arranjados na forma unipolar ou bipolar e uma alta mobilidade atingindo rapidamente a camada mucosa que reveste o epitélio gástrico, protegendo-o da acidez e dos

movimentos peristálticos (Marshall & Warren, 1983; Blaser, 1997; Kusters *et al*., 2006).

O *Helicobacter pylori* é também recoberto por estruturas fibrilares circulares as quais facilitam a sua adesão ás células epiteliais gástricas e dificultam sua eliminação. Esta aderência é mediada por diversas proteínas e glicopeptídeos de membrana (Gatti *et al*., 2006).

A urease é produzida em grande quantidade e converte a uréia endógena captada pelo *H. pylori* em amônia e gás carbônico tamponando o pH e criando uma camada neutra protetora em torno da superfície bacteriana, podendo também desestabilizar da camada de muco e causar conseqüentemente lesões sobre as células de revestimento com ativação do sistema imune (Megraud *et al*., 1992; Gatti *et al*., 2006).

O *H. pylori* apresenta um genoma de aproximadamente 1.7Mpb com 35 – 40% GC. Contém aproximadamente 1500 genes com duas cópias dos genes referentes às frações 16S, 23S e 5S rRNA (Kuster *et al*., 2006).

Algumas bactérias apresentam um ou mais plasmídeos, os quais parecem não carregar genes referentes a fatores de virulência ou que possam conferir resistência a antibióticos (Hofler *et al*., 2004).

Diferente de algumas bactérias o *H. pylori* é bastante heterogêneo sob o ponto de vista genético, apresentando regiões de plasticidade em seu genoma, o que se deve a possíveis mecanismos de adaptação as condições gástricas e resposta ao sistema imune (Kusters *et al*., 2006; Cover & Blaser, 2009).

Na cavidade oral essa heterogeneidade é ainda maior sendo explicada também pelo grande número de bactérias presentes e sua facilidade em adquirir material genético exógeno (Cover & Blaser, 2009).

Portanto, o presente trabalho visa diagnosticar a bactéria *Helicobacter pylori* em amostras de biópsia gástrica, saliva e placa dental de indivíduos adultos dispépticos, bem como verificar a presença do gene *cag*A e alelos do gene *vac*A, avaliando a cavidade oral como um reservatório da bactéria e a presença de diferentes cepas em um mesmo indivíduo.

REVISÃO DE LITERATURA

3.0. REVISÃO DE LITERATURA

3.1. Aspectos gerais do Helicobacter pylori

Segundo Marshall & Warren, (1983) e Peterson, (1991) o *H. pylori*, é uma bactéria Gram negativa conhecida por colonizar a mucosa gástrica humana, e tem sido fortemente associada com gastrite crônica, ulceração péptica e duodenal e é um risco para câncer gástrico, acrescentou Yuichi *et al*. (2007) e Souto *et al*. (2008).

Aproximadamente 50% da população mundial é infectada pelo *H. pylori* (Mitchell, 1999; Torres *et al*., 2009), o que a torna uma das infecções bacterianas mais comuns no mundo, (Parsonnet *et al.,* 1991; Hopkins, Girardi, Turney, 1996; De Vries *et al.,* 2007), sendo que, a prevalência da infecção em paises desenvolvidos varia entre 17 a 59% visto que as taxas são muito altas em paises em desenvolvimento (Souto *et al.,* 1998; Mitchell, 1999; Mitchell *et al*., 2003; O`Rourke *et al.,* 2003).

No Brasil a prevalência de *H. pylori* pode alcançar 80% em adultos (Souto *et al.,* 1998; Mitchell *et al*., 2003).

Os sintomas das doenças gástricas causadas pela *H. pylori* aparecem mais freqüentemente em adultos, embora a aquisição desta bactéria tenha ocorrido na infância (Bonamico *et al.,* 1997; Lehours, 2007).

Segundo Andersen & Rasmussen (2009) o *H. pylori* pode sobreviver por aproximadamente 14 dias em água destilada, entretanto em sua forma não cultivável pode persistir por até um ano em água.

De acordo com Rowland *et al.* (1999); Feldman (2001) e Kivi (2007), a infecção é adquirida por ingestão oral da bactéria e é principalmente transmitida entre os familiares na infância. Nahar *et al*. (2008) verificaram uma forte relação na

transmissão de mães para filhos sugerindo um possível fator de risco para o desenvolvimento de doenças gástricas.

Em países industrializados, a transmissão direta é de indivíduo para indivíduo por vômitos, saliva ou fezes; além disso, a transmissão pela água pode ser importante em países em desenvolvimento (Goodman *et al.,* 1996; Parsonnet, Shmuely, Haggerty, 1999; Delport *et al.,* 2007).

No entanto, Hulten *et al.* (1996) verificaram que o *Helicobacter pylori* pode ser transmitido através do consumo de vegetais não cozidos contaminados.

Atherton (1998) referiu que o desenvolvimento das doenças gástricas é influenciado pelo grau de virulência da cepa *H. pylori* infectante, e a susceptibilidade genética do hospedeiro e co-fatores ambientais.

Sheu *et al*. (2008) destacaram a heterogeneidade do *H. pylori* sugerindo uma grande diversidade genética otimizando a adaptação às condições da mucosa gástrica.

Para Mahdavi *et al.* (2002) a capacidade de adesão de muitas cepas de *H. pylori* durante a inflamação crônica, pode contribuir para virulência e a progressiva cronicidade da infecção.

A Agência Internacional para Pesquisa em Câncer IARC (1994), pertencente à Organização Mundial de Saúde, classificou *H. pylori* como grupo carcinogênico I.

Berroteran *et al.* (2002), Gebara *et al.* (2004) concluíram que a cavidade oral pode ser um reservatório para a infecção por *H. pylori* e as secreções orais podem ser um importante meio de transmissão.

A presença deste microorganismo na saliva e placa dental pode representar um fator de risco para reinfecção gastrintestinal e reincidência de úlcera após antibioticoterapia.

3.2. Cavidade Oral e Helicobacter pylori

A infecção pelo *H. pylori* apresenta um sucesso terapêutico em torno de 80 – 90% utilizando a tríplice terapia (dois antibióticos e inibidores da bomba de prótons; claritromicina, amoxicilina e omeprazol), porém reinfecções são freqüentes (Geraba *et al*., 2004).

Krajden (1989) foi o primeiro pesquisador a isolar o *H. pylori* na cavidade oral surgindo assim uma relação cavidade oral, *Helicobacter pylori* e estômago.

O primeiro estudo que destaca a influencia do *Helicobacter pylori* na cavidade oral relacionando-o com as condições da mucosa foi desenvolvido pelo grupo de Miyabayashi em 2000. Este estudo contou com 47 amostras que confirmou a relação entre a gastrite causada pela infecção e a colonização da cavidade oral. Os autores também propõem que na cavidade oral, o microorganismo fica inacessível aos antibióticos usados para eliminá-lo no estomago tornando o tratamento ineficiente.

Varios autores, dentre estes Krajden *et al.* (1989); Madmujar *et al.* (1990); Nguyen*,* El-Zaatari, Graham (1994); Loster *et al.* (2006); De Souza *et al.* (2006); Liu *et al*. (2008), relataram que a cavidade oral pode ser um reservatório da infecção, baseado em várias culturas e técnicas moleculares, como a Reação em Cadeia da Polimerase (PCR), com resultados diversos.

Segundo Oshowo *et al*. (1998), a reinfecção gástrica por bactérias que colonizavam a cavidade oral é possível, porém em menor impacto.

Wang *et al*. (2002) verificaram a existência de diferentes cepas do *H. pylori* em um mesmo hospedeiro, variando não só entre amostras, mas também entre locais de coleta de uma mesma amostra. Blaser *et al*. (1997) já ressaltava esta heterogeneidade do *H. pylori* afirmando que esta bactéria tem um grande potencial mutagênico.

Liu *et al.* (2008); Souto & Colombo (2008); Al Asgah *et al.* (2009); Rajendran *et al*. (2009) verificaram uma associação entre a presença do *H. pylori* em amostras de placa dental e doença periodontal, entretanto destacaram como principal responsável por essa ligação à higiene bucal deficiente nos pacientes estudados.

Não está claro se a cavidade oral é apenas um meio transitório ou permanente desta bactéria. Sabe-se que a detecção do *H. pylori* na cavidade oral não é necessariamente relacionada à presença do microorganismo na mucosa gástrica. Segundo Konturek *et al*. (2001) é necessário um grande número viável de bactérias para desencadear a infecção gástrica sendo o número de *H. pylori* extremamente baixo na cavidade oral impossibilitando uma reinfecção, porém possibilitando uma possível via de transmissão ou contaminação.

3.3. Métodos de diagnóstico

Os métodos de diagnóstico para infecção por *H. pylori* podem ser classificados em: métodos invasivos e não-invasivos (Culter *et al.,* 1995).

Van Leerdam & Tygtat, (2002) relataram que não há um único teste considerado ótimo para o diagnóstico da infecção por *H. pylori.*

Métodos não invasivos incluem: o teste respiratório da uréia, testes sorológicos e ensaio de banco de antígeno. O teste respiratório da uréia se confirma na abundância da bactéria, e a atividade da urease derivada da *H. pylori* no estômago; este teste detecta qualitativamente a infecção ativa com a sensibilidade e especificidade de mais de 90% (Drumm, Koletzko, Oderda*,* 2000).

Bazzoli (2001) destacou que se o teste da urease for negativo, amostras de biópsias adicionais estocadas em fixador podem ser enviadas para análise histológica.

Segundo Lu *et al.* (1999), a Reação em Cadeia da Polimerase (PCR) tem sido o método mais sensível e específico para detecção de *H. pylori*, dependendo da seleção dos oligonucleotídeos, representando um excelente teste alternativo.

Para Lage *et al.* (1995) e Kobayashi *et al.* (2002) a PCR é muito sensível e exata no diagnóstico da infecção por *H. pylori* comparado com outras técnicas invasivas em pacientes com úlcera péptica não hemorrágica.

Em trabalho realizado em nosso laboratório, Gatti *et al.* (2003) relataram que testes histológicos, urease e Reação da Polimerase em Cadeia foram empregados com sucesso, como métodos diagnósticos para *H. pylori.*

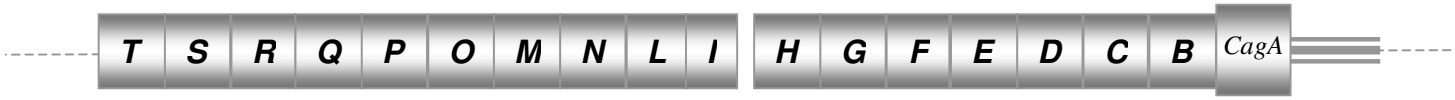
3.4. Marcadores de patogenicidade cagA e vacA

Cepas específicas com fatores de virulência podem influenciar no mecanismo de patogenicidade da bactéria. Estudos têm primeiramente observado dois principais fatores de virulência da bactéria; a ilha de patogenicidade Cag (Figura 1) (com o gene *cag*A como marcador) e a citotoxina vacuolizante *vac*A (Figura 2) (Rieder, Fischer, Haas, 2005).

O gene *cag*A está presente em aproximadamente 60-70% das cepas de *H. pylori* o qual codifica uma proteína imunodominante de alto peso molecular, 120-140 KDa (Covacci *et al*., 1993), localizado em uma ilha de patogenicidade cromossômica (Cag PAI) de 40 kb, que contém aproximadamente 30 genes com similaridade ao Sistema de Secreção Tipo IV, adquirido pela bactéria através de transferência horizontal (Yamaoka, 2008; Huang *et al*., 2009). A Ilha de patogenicidade Cag contém genes envolvidos na indução da síntese de quimiocinas pró-inflamatórias pelas células do hospedeiro (Cabtree *et al.,* 1995; Huang *et al.,* 1995; Hatakeyama, 2008).

Torres *et al*. (2009) verificaram a associação do gene *cag*A com mudanças nas vias de tradução, alterações estruturais no cito esqueleto celular e abundante síntese de Interleucina 8. Uma outra função do gene *cag*A mais recentemente descrita é a síntese de proteínas anti-oxidante as quais exercem uma função protetora para o *H. pylori*, reduzindo o efeito tóxico do oxigênio (Huang *et al*., 2009).

Figura 1 - Ilha de Patogenicidade Cag – IS605 (40kDa)
Sistema de secreção tipo IV.
Marais *et al*.(1999)



S – Proteína de membrana p60
R – Translocase (*secY*)
Q – Pré-pilina peptidase tipo 4
P – Proteína de formação fimbrial (*fmbI*)
O – Permease
M – Hook-associated proteína 3
L – Pré-proteina translocase *(secA*)
I – Subunidade D fimbrial

H – Protease extracelular *(proA*)
G – TCP biosíntese de pilus
E – *TraB, VirB4, PtlC*
D – *fliD*
C – *traH*

O gene *vacA* codifica uma proteína de 130-140 KDa, que contém uma seqüência sinal (s), responsável pelo transporte da proteína do citoplasma para o periplasma e uma região mediana (m) envolvida no reconhecimento de receptores de células epiteliais gástricas (Doorn *et al.,* 1998).

A região s do gene *vac*A , e a região m, são divergentes entre as cepas de *H. pylori*, podendo ser do tipo s1 (subdividida em s1a, s1b e s1c) ou s2 (Doorn *et al.,* 1998), e do tipo m1 os quais também podem ser subdivididos em m1a, m1*, m1-m2*, m2a e m2b (Chen-Wen *et al.,* 2000) e o mesmo encontra-se envolvido com o processo de vacuolização das células gástricas e úlcera péptica (Atherton *et al*., 1997). Os subtipos s1a estão envolvidos com uma maior atividade tóxica do

que o subtipo s1b segundo estudos realizados por Atherton *et al*. (1997) e Letley *et al.* (2000).

Rieder, Fischer, Haas (2005) e Harris *et al*. (2008) recentemente relataram que o gene *vacA* também está associado a alterações funcional da célula T inibindo a proliferação da mesma, sugerindo um possível mecanismo de como *H. pylori* pode evadir a resposta imune adaptativa para estabelecer a infecção crônica.

Notavelmente, a proteína *cag*A é freqüentemente expressa com a citotoxina vacuolizante (*vac*A), razão para a designação como proteína associada a uma citotoxina (Tummuru, Cover, Blaser, 1994; Xiang *et al.,*1995).

Para Tummuru, Cover, Blaser (1993) a expressão da proteína *cag*A em cepas *H. pylori* está altamente associada com ulceração péptica.

Rieder, Fischer, Haas (2005) concluíram que o gene *vac*A atua não só em células epiteliais, mas também em células do sistema imune, induzindo a imunosupressão, já o gene *cag*A parece interagir com várias moléculas dentro de células eucariontes induzindo a fosforilação.

Em outro trabalho do nosso grupo, Gatti *et al.* (2005) verificaram uma alta freqüência de cepas s1/m1 do gene *vac*A associados a gastrite crônica e úlcera péptica e uma significante correlação entre a presença do gene *cag*A com o alelo s1 e o gene *bab*A2 em pacientes com gastrite crônica. Dados semelhantes aos encontrados por Con *et al*. (2009) que verificaram uma alta incidência de atrofia gástrica e metaplasia intestinal associadas ao gene *cag*A e alelos s1/m1 do gene *vac*A em pacientes da Costa Rica.

Figura 2 - <u>Gene *vacA* - Diversidade Alélica</u>

Marais *et al.*(1999)

**** Toxicidade: s1>s2 e m1>m2***

OBJETIVOS

4.0. OBJETIVOS

O presente trabalho teve como objetivo:

4.1 Diagnosticar *Helicobacter pylori* através da técnica de PCR e *Southern blotting* a partir de material coletado da placa dental, saliva e mucosa gástrica;

4.2 Determinar os alelos “s” do gene que codifica a citotoxina vacuolizante (*vacA*) e a presença do gene que codifica a citotoxina associada (*cagA*), através da técnica de PCR, a partir de amostras positivas diagnosticadas em 4.1;

4.3 Avaliar a cavidade oral como um reservatório de *H. pylori* e a existência de diferentes cepas em um mesmo hospedeiro comparando as genotipagens dos três tipos de amostras.

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5.0. REFERÊNCIAS BIBLIOGRÁFICAS

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ARTIGOS

6.0. ARTIGOS

6.1. Artigos enviados para Publicação em Periódicos Internacionais

Artigo 1. - *Helicobacter pylori, vacA and cagA gene in gastric mucosa, saliva and dental plaque.*
Submetido à revista **Journal of Medical Microbiology**, em 24 de julho de 2009.

Artigo 2. - *Helicobacter pylori detection in gastric biopsies, saliva and dental plaque of dyspeptic patients*.
A ser enviado: **APMIS: Acta Pathologica, Microbiologica et Immunologica Scandinavica**.

Artigo 1. - *Helicobacter pylori, vacA and cagA gene in gastric mucosa, saliva and dental plaque.*

Submetido à revista **Journal of Medical Microbiology**

Under Review - Check Status

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Helicobacter pylori, vacA and cagA gene in gastric mucosa, saliva and dental plaque

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*Helicobacter pylori, vac*A and *cag*A gene in gastric mucosa, saliva and dental plaque

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Running Title: *Helicobacter pylori and* Cytotoxin Genotypes

Subject Cateroty: Diagnostics, typing and identification

Summary

Helicobacter pylori (*H. pylori*) is a spiral-shaped Gram-negative flagellate bacterium and it related with gastritis and peptic ulcer disease. The vacuolating cytotoxin (*vac*A) and *cag*A gene, are important virulence factor involving gastric diseases. This study aims to: (1) to determine the presence of *H. pylori* in the oral cavity and gastric biopsies (2) verify *cag*A presence, *vac*A alleles in all samples. Gastric biopsies, saliva and dental plaques were obtained from 62 dyspeptic adults. DNA was extracted and evaluated for the *H. pylori* and *cag*A presence, *vac*A alleles, using polymerase chain reaction and Southern blotting methods. Persons with gastritis had a high frequency of *Helicobacter pylori* positives in the stomach ($p<0.0001$) and there was a statistically significant correlation between gastric biopsies and oral cavity ($p<0.0001$). We shown a high prevalence of the *H. pylori* and presence with *cag*A gene associated of *vac*A s1 alelles in gastric biopsies. Our results suggest a relation between gastric infection and the bacterium in the oral cavity, with a variation of cytotoxin genotype between saliva and dental plaque suggesting a reservoir of the species and that more than one *H. pylori* strains may exist in the saliva, dental plaque and stomach of the same patient.

Introduction

Helicobacter pylori (*H. pylori*) is a spiral-shaped Gram-negative flagellate bacterium that has been implicated as a major human gastric pathogen responsible for gastritis and peptic ulcer disease (Silva *et al*., 2009a). In Brazil, the prevalence of *H. pylori* infection may be as high as 80% in adults, whereas the prevalence is typically 30–70% in North America and Europe (Mitchell *et al.,* 1999; Brito *et al*., 2003*;* Mitchell *et al*., 2003; Tseng *et al*., 2006; Yamaoka *et al*., 2008).

The *vac*A gene is present in all *H. pylori* strains, and comprises two main regions that show significant sequence variability between strains: the signal region (s1 or s2) and the middle region (m1 or m2), these 2 parts of *vac*A gene determine cytotoxin production and are associated with pathogenicity of the bacterium. The vacA s1/m1 allelic combination exhibits the highest activity, while s2/m2 and the rare s2/m1 combinations are non-toxic (Erzin *et al*., 2006; Torres *et al*., 2009)

The *cag*A gene is present in approximately 60-70%, located within a 40-kilobases DNA fragment know as the *cag* pathogenicity island (*cag* PAI). It is of the major *H.pylori* virulence factor found more frequently in patients suffering from severe gastrointestinal diseases and your protein strong association of the gastric ulceration and gastric carcinoma. (Tummuru *et al.,* 1993; Tiwari *et al*., 2005, Cover & Blaser 2009). The *Helicobacter pylori* infection is associated with low socioeconomic status, crowded living condition, foods habits and poor personal higiene and is usually acquired in early childhood (Nahar *et al*., 2009). Despite these dramatic infection rates, the modes of acquisition and transmission of *H. pylori* remains unclear. Fecal-oral, oral-oral, and gastro-oral have all been implicated in the transmission of the bacteria. (Bonamico *et al*., 2004).

Several authors have reported the presence of *H. pylori* in the oral cavity and it was proposed that is would represent a source of *H. pylori* for gastric reinfection after

therapy (Kignel *et al*., 2005). Ishihara *et al.* (1997) suggest the *H. pylori* could be a part of the dental biofilm.

In this study we have analyzed *H. pylori* from gastric biopsies, saliva and dental plaque in symptomatic adults patients, genotyped *cag*A presence, *vac*A alleles and correlated them with histological findings. In addition, examine the relationship between oral *H. pylori* and subsequent gastritis.

Methods

Patients and Endoscopy

Sixty-two consecutive adults (27 male and 35 female with a mean age 52.56 years) presenting recurrent abdominal pain were participated in the study. All subjects were recruited from the Ambulatório de Endoscopia of the Faculdade de Medicina de Marília, São Paulo, Brazil. All subjects signed an informed consent form that was approved by the local ethics committee.

Three biopsies were obtained from gastric antrum of each patient. The first antrum specimen was used for the rapid urease test, the second specimen was used for histology and the third for molecular analysis. Dental plaque and saliva was also acquired from each patient. Subjects who were HIV-positive or had taken anti-inflammatory or antimicrobials drugs within the previous 2 months were excluded from the study. The endoscopic forceps were sterilized in 2% glutaraldehyde solution for a minimum of 20 minutes between each experiment.

Rapid urease test and histology

The presence of an infection was determined by a Rapid urease test, TUPF (Laborclin, BRAZIL). The manufacturer´s specifications and instructions were followed during the analysis. Samples were examined within 24h of collection. Urea hydrolysis and histopathologic examinations were conducted to determine the presence of *H. pylori*, in accordance with the updated Sydney System.

Collects and DNA extraction of Saliva and Dental Plaque

DNA extraction from the dental plaques and saliva was performed as previously described (Okada *et al.,* 2000; Kignel *et al*., 2005). Briefly, 10mL of dental plaque suspension and 1mL saliva were centrifuged for 5min at 10000rpm. The pellets were suspended in 480µl of digestion buffer (5mM EDTA, pH 8, 0.5 mol Tris-HCl, pH 7.5 and 5% tween 20) and 20µl proteinase K containing 100µg/mL and incubated at 55ºC overnight. DNA was extracted with an equal volume of phenol-chloroform twice and then precipitated with a double volume of ethanol 100%. Finally, the extracted DNA was resuspended in 80µl-100µl of TE buffer.

DNA extraction of Gastric biopsies, PCR for *H. pylori* diagnostic and Southern Blotting

DNA from the gastric biopsies was extracted using the QIAamp tissue (Qiagen, Hilden, Germany), according to the manufacturer´s instructions. PCR assays were performed with approximately 100 ng of total DNA using 1 set of oligonucleotides (Hpx1/Hpx2) that amplifies a 150-bp fragment corresponding to 16S-rRNA from *H. pylori* (Table1) (Scholte *et al.,* 1997). In each experiment, positive (strain 26695) and negative controls (water) were included. After separation in 2% agarose gels, PCR products were blotted to a Hybond N+ membrane and hybridized

with the specific PCR fragments labeled by chemiluminescent method (Amersham Pharmacia). The assay was considered positive when one of the PCR products was present.

Preparation of DNA Probe

The fragment of 150 base pairs was amplified from genomic DNA of culture *H. pylori* by PCR with the Hpx1 and Hpx2 primers and used as the probe in Southern hybridization. After amplification, the reaction mixture was electrophoresed in 2% agarose gels and the 150 base pair fragment of interest was recovered from an agarose gel and purified using GFX PCR DNA and Gel Band Purification Kit (Amersham Pharmacia, USA). The probe was synthetized using the Kit Gene Images AlkPhos Direct Labelling (Amersham Pharmacia, USA), according to the manufacturer´s instructions.

Cytotoxin Genotypes*: vac*A alleles and *cag*A gene

The *vac*A gene, “s” and “m” region genotyping and *cag*A gene detection were performed by PCR and Southern blotting, using one sets of oligonucleotides (Table 1) for each gene fragment. For *cag*A detection, primers Cag1/Cag2 amplify 232 fragments. The *vac*A “s” and “m” regions were genotyped with primer sets SA/SC, and MA/MB, respectively. Primers SA/SC amplify “s1” fragments of 176 base pair and “s2” fragments of 203 base pair. The “m1” fragments are 400 base pair and “m2” fragments of 475 base pair. (Atherthon *et al*., 1997; Doorn *et al*., 1998; Gatti *et al*., 2003).

Statistical Analysis

Statistical analysis was performed by χ^2, Fisher's exact test and Kappa test. The significance level was set at p-value of <0.05.

Results and Discussion

Detection of *Helicobacter pylori* from gastric biopsies by PCR, PCR/Southern blotting, histology and urease test

All 62 (100%) adult patients were analised by PCR and hybridization by Southern blotting, *H. pylori* DNA was detected in 50 (80.6%) (Table 2; Figure 1A and 1B). Forty five (72.6%) patients were diagnosed with chronic gastritis through histologic analysis and 17 (27.4%) were diagnosed with normal mucosa without gastric alterations. Of the 45 patients who were diagnosed with chornic gastritis 41 were *H. pylori* positive in gastric biopsies.

The histological analysis showed the presence of *H. pylori* in only 19 (30.6%) subjects and urease test detected *H. pylori* infection in 27 (43.5%) patients, both techniques showed decreased sensitivites and specifitcty. The results are showed in table 2 (p>0.0001).

A previous study (Tiwari *et al.,* 2005; Loster *et al*., 2006; Harris *et al*., 2008; Souto *&* Colombo 2008) used the technique by PCR to disgnostic of *H. pylori,* they have shown results like ours, which 95% of success in the isolation of *H. pylori* in the stomach. Li *et al*. (1995) and Song *et al*. (2000) have evidenced a significant increase of sensitivity after using Southern blotting, also verifying a high prevalence.

The high sensitivity and specificity of the PCR test with Southern blotting and hybridization did not reveal false positives, false negatives or contamination, due to the fact that the probe was synthesized from genomic DNA of a culture *H. pylori* by PCR, which excludes false results. (Clayton *et al*., 1992; Song *et al*., 1999)

Detection of *Helicobacter pylori* in saliva and dental plaque by Southern blotting

In oral cavity the *H. pylori* has been demonstrated earlier, although the numbers are somewhat discrepant. It is not clear whether the oral cavity is a permanent or a temporary reservoir for this microorganism.

In this study the *H. pylori* was found in the saliva from 26 (42%) and in the dental plaque from 29 (47%) of the studied individuals. (Table 3, Figure 1A and 1B)

Of the 50 patients who were *H. pylori* positive by gastric biopsies, 16 (32%) have presented the *H. pylori* only stomach, and from all 12 patients who were negative in stomach, six were found *H. pylori* in oral cavity. The *Helicobacter pylori* DNA was detected in gastric mucosa, saliva and dental plaque in 10 (16%) patients simultaneously. However, 24 (35%) subjects harbored *H. pylori* in gastric biopsies and oral cavity (saliva or dental plaque).

No statistically significant difference was observed between strains in the saliva and dental plaque. However, a statistically significant correlation was observed between gastric biopsies and the oral cavity ($p<0.0001$).

Li *et al*. (1995) verified 75% (*n= 56*) of positives samples of saliva, similar data of the ones described by Wang *et al*. (2002) who found the bacterium in 71% (*n=31*) samples of saliva and Al Asgah *et al.* (2009) who reported a significant association between the presence of *H. pylori* in stomach and dental plaque.

Silva et al. (2009b) detected the *H. pylori* in 16 of 30 saliva samples and 11 dental plaque samples, however Tiwari *et al. (2005)* have studied 120 dyspeptics patients and they have detected the *H.pylori* in all of the samples of gastric and

saliva. Both authors suggest saliva as a way of transmission and a possible way for gastric re-infections.

Detection of *cag*A and *vac*A alelles of *Helicobacter pylori* isolates from gastric biopsies, saliva and dental plaque

In our *H. pylori*, we isolated 25 strains of gastric biopsies (50%) of the common *vac*A genotype s1/m1 and only 12 strains (24%) were found to be *vac*A s2/m2. Ten strains *H. pylori* was found to have *vac*A genotype s1/m2, and 3 strains showed *vac*A genotype s1/m1/m2 suggesting a coinfection with two different strains of *H. pylori.* The *cag*A gene was detected in 72% (36 strains) of our *H. pylori*. Of the 36 *H. pylori* that were positive for *cag*A, 32 strains (88%) were associated with the toxin-producing *vac*A s1 and only 4 (22%) *cag*A-positive strains were vacA s2.(Table 3; Figure 2A, 2B, 3A and 3B)

Other studies from Brazilian patients found 67% (*n=137*), 79% (*n=208*) and 48% (*n=89*) of cagA-positive strains (Ribeiro et al., 2003; Oliveira *et al*., 2003; Gatti *et al*., 2005). Several studies have described an association between s2/m2 and cagA-negative strains and between s1/m1 and cagA-positive strains. The results of the present study have confirmed this association.

Previous study reported that infection with triple-positive strains has been associated with a higher degree of inflammation and gastroduodenal lesion. In Brazilian dyspeptic patients seen to have a lower rate (32.6%) of tripe-positive strains and ours results showed similar percentages (42%) of both types strains (Torres *et al.,* 2009).

*Cag*A gene was detected in 13 (50%) samples of saliva. Of the 13 strains who were *cag*A-positives, 7 were associated with *vac*A s1, only two strains were found to

be *vac*A s2 and four strains were s1/s2. The middle region (m1 or m2) of *vac*A gene were genotyped only *H. pylori* gastric isolates, the middle region of *vac*A was not detected in *H. pylori* isolates from saliva and dental plaque, probably due to heterogeneity in the *vac*A gene described previously (Torres *et al.,* 2009) and the large amount contamination in saliva and dental plaque can be inhibited the reaction of PCR. In dental plaque only 7 of 29 strains (24%) have *cag*A gene and 3 strains were associated with the toxin-producing *vac*A s1. However 11 strains *cag*A-negative showed associated with *vac*A genotypes s1.

No statistic differences was observed when we compared the association between *cag*A presence and *vac*A alelles in saliva or dental plaque but in gastric biopsies the association between strains *cag*A-positives and *vac*A genotypes s1/m1 showed a significant correlation. The association between *cag*A presence and vacA alelles in all strains is described in Table 3. (Figure 2A, 2B, 3A and 3B)

The *vac*A s1 alelle was found in 53.8% (*n=50) H. pylori* isolates from saliva and 48.2% (*n=50*) isolates from dental plaque, the *cag*A gene was detected in 13 (50%) strains of saliva and only 7 (24%) strains of dental plaque of positive *H. pylori* samples. No association was found whith *vac*A s1 alelles and the presence of *cag*A gene in oral cavity. We was detected 11 samples of oral cavity (4 saliva and 7 dental plaque) with *vac*A alelles s1 and s2 simultaneously suggesting the presence of multiple *H. pylori* strains in oral cavity the same subject.

Wang *et al*. (2002) verified 23% (*n=31*) presence of *cag*A gene and 77% (*n=31*) *vac*A s1 in samples of saliva, ours results showed a great presence of *cag*A gene in saliva but in dental plaque we found only 24% (*n=29*) of *cag*A gene.

We found also a significante cytotoxin gennotypic diversity among *H. pylori* from stomach, saliva and dental plaque. This is in agreement with Wang *et al.* (2002)

who evidencied of tremendous genetic diversity. The heterogeneity of *H. pylori* may be due to genotypic variation among strains and or variations in *H. pylori* populations within as individual host, as proposed by Blaser (1997).

Differences of the frequency of *H. pylori* oral cavity could be observed in the literature, it may be a consequence of the results from differences study in populations, oral health status, *H pylori* infection, type and number of clinical samples, complexity of the oral flora and methods of detection. (Souto & Colombo, 2008*).* Kignel *et al.* (2005) reported that the levels of bacterium in oral cavity may be too low to be detected by one round PCR and they emphazised that the local for samples collected in the oral cavity can influence the prevalence of the microorganism.

Song *et al*. (2000) presented results that showed the varied settling of the *H. pylori*, showing a prevalence of 82% in the molar region, 64% in pre-molar region and 59% (*n=39*) in the region of the incisors. Loster *et al.* (2006) suggest that this fact because of the dental plaque as well as the posterior region of the oral cavity are less oxygenated contributing for the settling of the *H.pylori* .

Considering saliva and dental plaque as samples of the oral cavity our results approximately present 70% (*n=62*) of positivity in the oral cavity, however with a variation of positivity between saliva and dental plaque already described above.

We conclude from our analyses that a high prevalence of the *H. pylori* and yours main virulence factor genes in Brazilian *H. pylori* isolates. Also observed high presence with *cag*A gene associated of *vac*A s1 alelles in gastric biopsies. Our results suggest a relation between gastric infection and the bacterium in the oral cavity. However *H. pylori* was present in the oral cavity with variable of cytotoxin genotype and variable distribution between saliva and dental plaque suggesting that

a reservoir of the species and a possible association to a gastric re-infection. The date sugest also that more than one *H. pylori* strains may exist in the saliva, dental plaque and stomach of the same patient.

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Table 1 - Primers and PCR condition for *H.pylori* detection and genotyping

Primers	Sequence (5' – 3')	PCR condition
Hpx1	5'-CTGGAGARACTAAGYCCTCC-3'	94°C, 5 min, 40 cycles: 94°C, 1 min/59°C, 1 min/72°C, 1 min, and 72°C, 7 min
Hpx2	5'-GAGGAATACTCATTGCGAAGGCGA-3'	
SA	5'-ATGGAAATACAACAAACACAC-3'	94 °C, 5 min, 40 cycles: 94°C, 1 min/53°C, 1 min/72°C, 1 min, and 72°C, 7 min
SC	5'-CCTGARACCGTTCCTACAGC-3'	
MA	5'-CACAGCCACTTTYAATAACGA-3'	94°C, 5 min, 40 cycles: 94°C, 1 min/53°C, 1 min/72°C, 1 min, and 72°C, 7 min
MB	5'-CGTCAAAATAATTCCAAGGG-3'	
Cag1	5'-ATGACTAACGAAACTATTGATC-3'	94°C, 5 min, 40 cycles: 94°C, 1 min/53°C, 1 min/72°C, 1 min, and 72°C, 7 min
Cag2	5'-CAGGATTTTTGATCGCTTTATT-3'	

[a] R (A or G), W (A or T), and Y (C or T).

Table 2 – Diagnostic of *H. pylori* from gastric biopsies of 62 dispeptic patients

Helicobacter pylori	Southern Blotting	Urease test	Histologic
H. pylori +	50 (80.6%)	27 (43.5%)	19 (30.6%)
H. pylori -	12 (19.4%)	35 (56.5%)	43 (69.4%)
Total		62 (100%)	

*p < 0,0001

Table 3 – Southern Blotting detection of *H. pylori* DNA and *cag*A and *vac*A genes in *H. pylori* from gastric biopsies, saliva and dental plaque.

			*vac*A				
Samples	*H. pylori +*	*cag*A +	m1	*m2*	*s1*	*s2*	*s1/s2*
Gastric B.	50 (80.6%)	36 (72%)	28 (56%)	22 (44%)	38 (76%)	12 (24%)	—
Saliva	26 (42%)	13 (50%)	—	—	14 (54%)	8 (31%)	4 (15%)
Dental Plaque	29 (47%)	07 (24%)	—	—	14 (48%)	8 (27%)	7 (25%)

*p < 0,0001

1A

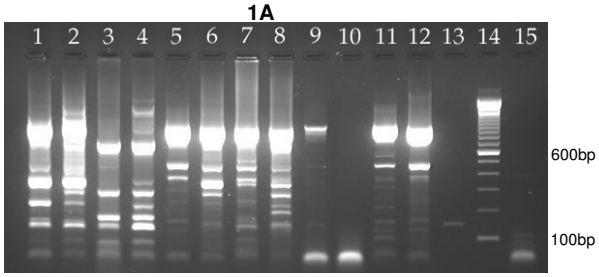

Figure 1 – A) Agarose gel 2% showing detection of *H. pylori* in strains of gastric biopsies, saliva and dental plaque. "Slots" 1, 3, 5 and 7– strains of saliva; "Slots" 2, 4, 6 and 8 strains of dental plaque; "Slots" 9, 10, 11 and 12, strains of gastric biopsies; "Slots" 13 and 15 controls positive and negative, respectively; "Slot" 14 Ladder Marker 100 bp *(*Invitrogen).

1B

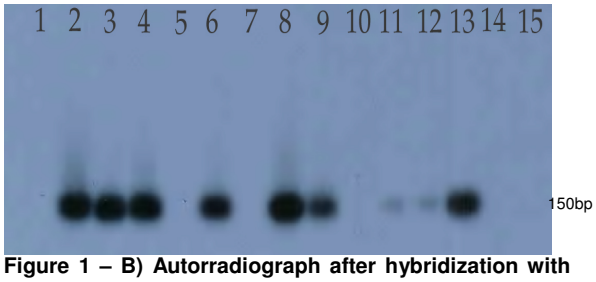

Figure 1 – B) Autorradiograph after hybridization with specific chemiluminescent probe referent figure 1 A "Slots" 1, 5 and 7, Strains of negative saliva. "Slot" 3, strain of positive saliva."Slots" 2, 4 ,6 and 8 strains of positives dental plaque. "Slot" 10, strain negative gastric biopsy."Slot" 9, 11 and 12, strain positives gastric biopsies. "Slot" 13 and 15 positive and negative control respectively**.**

2A

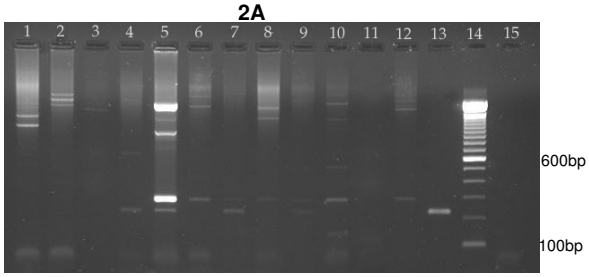

Figura 2 – A) Agarose gel 2% showing detection of *cag*A gene of *H pylori* isolates of saliva and dental plaque. "Slots" 1, 2, 3, 4, 5 and 6 strains of saliva, 7, 8, 9, 10, 11 and 12, strains of dental plaque; "Slot" 13 positive control; "Slot" 15 negative control; "Slot" 14 Ladder Marker 100 bp *(*Invitrogen)

2B

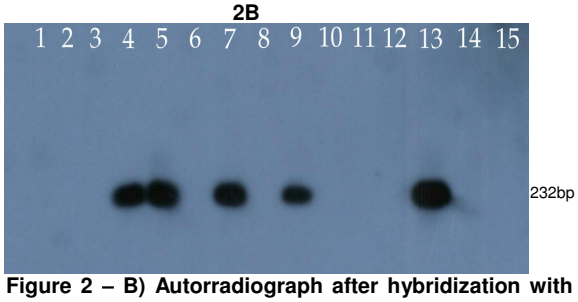

Figure 2 – B) Autorradiograph after hybridization with specific chemiluminescent probe referent figure 2 A. "Slots" 1, 2, 3, 6, 8, 10,11 and 12 strans *cag*A-negative; "Slots" 4, 5, 7 e 9, strains *cag*A-positives "Slot" 13 positive control; "Slot" 15 negative control.

3A

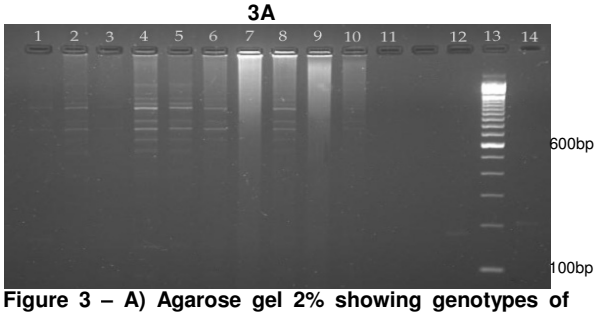

Figure 3 – A) Agarose gel 2% showing genotypes of *vac*A gene, alelles s1/s2 from *H. pylori* of gastric biopsies, saliva and dental plaque "Slots" 1, 2, 3 and 4 strains of gastric mucosa; "Slots" 5, 7 and 9 strains of saliva; "Slots" 6, 8, 10, strains of dental plaque; "Slots" 11 negative control; "Slot" 12 and 14 positives control s1/s2 respectively; "Slot" 13 Ladder Marker 100 bp (Invitrogen)

3B

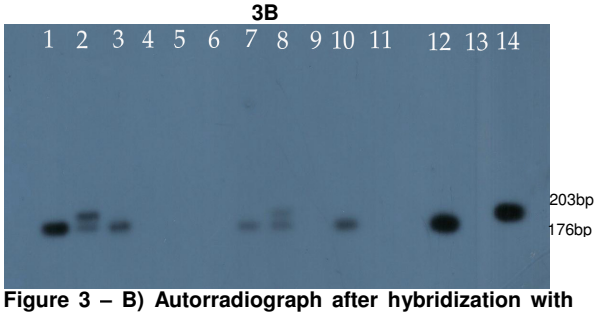

Figure 3 – B) Autorradiograph after hybridization with specific chemiluminescent probe referent figure 3 A "Slots" 1, 3, 7 and 10 *vac*A alelle s1. "Slots" 2 and 8, *vac*A alelles s1/s2. "Slots" 11 negative control "Slot" "Slot" 12 and 14 positives control s1 and s2 respectively

Artigo 2. - *Helicobacter pylori detection in gastric biopsies, saliva and dental plaque of dyspeptic patients*.

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"*Helicobacter pylori* detection in gastric biopsies, saliva and dental plaque of dyspeptic patients"

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Running head: Molecular Diagnostic and *H. pylori*

Rasmussen LT, de Labio RW, Gatti LL, Silva LC, Queiroz VF, Smith MAC, *Payão SLM. *Helicobacter pylori* detection in gastric biopsies, saliva and dental plaque of dyspeptic patients.

Summary

Helicobacter pylori is an important human pathogen that causes chronic gastritis and is associated with the development of peptic ulcer disease and gastric malignancies. The oral cavity has been implicated as a potential *H. pylori* reservoir, and may therefore be involved in the reinfection of the stomach, which sometimes follows treatment of *H. pylori* infection. The objectives of this study were (1) to determine the presence of *H. pylori* in the oral cavity and (2) to examine the relationship between oral *H. pylori* and subsequent gastritis. Gastric biopsies, saliva samples and dental plaques were obtained from all 78 dyspeptic adults. DNA was extracted and evaluated for the presence of *H. pylori* using polymerase chain reaction and Southern blotting methods. Persons with gastritis had a high frequency of *Helicobacter pylori* positives in the stomach ($p<0.0001$) and there was a statistically significant correlation between gastric biopsies and oral cavity ($p<0.0001$). Our results suggest a relation between gastric infection and the bacterium in the oral cavity. Despite this, *H. pylori* was present in the oral cavity with variable distribution between saliva and dental plaques, suggesting the existence of a reservoir of the species and a potential association with gastric reinfection.

Keywords: *Helicobacter pylori*, Southern Blotting, Saliva, Dental Plaque

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1. Introduction

Helicobacter pylori (H. pylori) is a spiral-shaped, Gram-negative bacterium, which persistently colonizes gastric mucosa of humans. This bacterium plays an important role in the initiation of gastrointestinal diseases, particularly peptic and duodenal ulcers, as well as gastric cancer and lymphoid tissue (MALT) lymphoma. *H. pylori* is estimated to inhabit a least half the world's human population (1-3).

Numerous retrospective and prospective studies have shown a significant correlation between *H. pylori* infection and distal gastric cancer risk (4, 5). Additionally, *Helicobacter pylori* infection is associated with low socioeconomic status, crowded living condition and poor personal hygiene. The infection is usually acquired in early childhood (6).

The prevalence of *H. pylori* infection in gastric biopsies appears to be higher in developing countries compared to developed countries. In Brazil, the prevalence of *H. pylori* infection may be as high as 80% in adults, whereas the prevalence is typically 30–70% in North America and Europe (7-12). Despite these dramatic infection rates, the modes of acquisition and transmission of *H. pylori* remains unclear. Fecal-oral, oral-oral, and gastro-oral have all been implicated in the transmission of the bacteria. Previous work has found that subjects infected with *H. pylori* excrete the bacteria in feces (13).

It is well-established that the principal ecological niche for *H. pylori* is the gastric mucosa. Recent studies utilizing polymerase chain reaction (PCR) technique, have demonstrated that *H. pylori* can be found in either transient or permanent forms in the human oral cavity. This body region provides an excellent niche for microaerophilic environments and is therefore a potential reservoir for *H. pylori* (14, 15). Some investigators believe that *H. pylori* belongs to the normal microbiota of the human oral cavity and maintains a commensal relationship with human host. Contrarily, other authors have suggested that *H. pylori* intermittently colonizes oral cavities as result of the ingestion of contaminated foods or secondary to gastro-esophageal reflux (16-18)

One of the first investigations on the influence of oral *H. pylori* on the stomach condition was carried out by Miyabayashi et al. (19). This study confirmed the relationship between gastritis induced by *H. pylori* infection and oral colonization of the bacterium. Moreover, these authors also attempted to elucidate the resistance of

oral *H. pylori* to typical triple *anti-H. pylori* therapy used to eradicate the germ from the stomach. They determined that patients with oral *H. pylori* were at a significantly greater risk of gastric reinfection following successful therapy. This study emphasized a clear link between the presence *of H. pylori* in the oral cavity and the infection of gastric mucosa (14).

Previous research has layed the groundwork for the present study. While *H. pylori* has been suggested to colonize in the mouth of persons with gastritis, however this hypothesis is unclear. Therefore, the objectives of this study were (1) to determine the presence of *H. pylori* in the oral cavity and (2) to examine the relationship between oral *H. pylori* and subsequent gastritis

2. Materials and Methods

2.1. Patients and Endoscopy

Seventy-eight consecutive adults (36 male and 42 female with a mean age 51.2 years) presenting recurrent abdominal pain were participated in the study. All subjects were recruited from the Ambulatório de Endoscopia of the Faculdade de Medicina de Marília, São Paulo, Brazil. All subjects signed an informed consent form that was approved by the local ethics committee.

Three biopsies were obtained from gastric antrum of each patient. The first antrum specimen was used for the rapid urease test, the second specimen was used for histology and the third for molecular analysis. Dental plaque and saliva was also acquired from each subject. Subjects who were HIV-positive or had taken anti-inflammatory or antimicrobials drugs within the previous 2 months were excluded from the study. The endoscopic forceps were sterilized in 2% glutaraldehyde solution for a minimum of 20 minutes between each experiment.

2.2. Rapid urease test and histology

The presence of an infection was determined by a Rapid urease test, TUPF (Laborclin, BRAZIL). The manufacturer´s specifications and instructions were followed during the analysis. Samples were examined within 24h of collection. Urea hydrolysis and histopathologic examinations were conducted to determine the presence of *H. pylori*, in accordance with the updated Sydney System.

2.3. Collection and DNA extraction from Saliva and Dental Plaque

Saliva and plaque samples were collected prior to the endoscopic examination from each subject. Salivary flow was stimulated for each patient and 3 mL of saliva was collected in test tubes. Dental plaque of all oral cavity (incisors, canines, premolars and molars) was removed with a sterile curette and transferred to 15 mL of phosphate buffered saline.

DNA extraction from the dental plaques and saliva was performed as previously described (4-20). Briefly, 10mL of dental plaque suspension and 1mL saliva were centrifuged for 5min at 10000rpm. The pellets were suspended in 480µl of digestion buffer (5mM EDTA, pH 8, 0.5 mol Tris-HCl, pH 7.5 and 5% tween 20) and 20µl proteinase K containing 100µg/mL and incubated at 55ºC overnight. DNA was extracted with an equal volume of phenol-chloroform twice and then precipitated with a double volume of ethanol 100%. Finally, the extracted DNA was resuspended in 80µl-100µl of TE buffer.

2.4. DNA extraction from Gastric biopsies, diagnostic PCR and Southern blotting

DNA from the gastric biopsies was extracted using the QIAamp tissue (Qiagen, Hilden, Germany), according to the manufacturer´s instructions. PCR assays were performed with approximately 100 ng of total DNA using Hpx1 - 5'-CTGGAGARACTAAGYCCTCC-3' and Hpx2 - 5'-GAGGAATACTCATTGCGAAGGCGA-3' oligonucleotides, that amplifies a 150-bp fragment corresponding to 16S-rRNA from *H. pylori* (21). In each experiment, positive (strain 26695) and negative controls (water) were included. After separation in 2% agarose gels, PCR products were blotted to a Hybond N+ membrane and hybridized with the specific PCR fragments labeled by chemiluminescent method (Amersham Pharmacia). The assay was considered positive when one of the PCR products was present. (Primers: R: A or G and Y: C or T)

2.5. Preparation of DNA Probe

The fragment of 150 base pairs was amplified from genomic DNA of culture *H. pylori* by PCR with the Hpx1 and Hpx2 primers and used as the probe in Southern hybridization. After amplification, the reaction mixture was electrophoresed in 2% agarose gels and the 150 base pair fragment of interest was recovered from an

agarose gel and purified using GFX PCR DNA and Gel Band Purification Kit (Amersham Pharmacia, USA). The probe was synthetized using the Kit Gene Images AlkPhos Direct Labelling (Amersham Pharmacia, USA), according to the manufacturer´s instructions.

2.6. Statistical analysis

Statistical analysis was performed by χ^2 and Kappa test. The significance level was set at p – value of < 0.05.

3. Results

3.1. Detection of *Helicobacter pylori* from gastric biopsies by PCR, Southern blotting, histology and urease test

All 78 adult patients were analyzed by PCR and hybridization via Southern blotting and 66 (84.6%) were positive by *H. pylori* infection ($p<0.0001$) (Fig. 1A and 1B). Histological analysis revealed that 54 (69.2%) patients had chronic gastritis, while 24 (30.8%) patients demonstrated normal mucosa without gastric alterations.

The histological analysis showed the presence of *H. pylori* in 21 (27%) subjects and urease test detected *H. pylori* infection in 30 (38.5%) patients, both techniques showed decreased sensitivities and specificity. These results are presented in Table 1.

3.2. Detection of *Helicobacter pylori* in saliva and dental plaque by Southern blotting

H. pylori was found in the saliva of 33 (42.3%) patients. The bacteria was also present in the dental plaque in 37 (47.4%) of individuals (Table 2, Fig. 1A and 1B). Of the 66 patients who were *H. pylori* positive as determined by gastric biopsies, 19 (28.8%) presented with *H. pylori* only in the stomach. Twelve patients were negative for *H. pylori* in the stomach, and of these, six were found to have *H. pylori* in the oral cavity. *Helicobacter pylori* DNA was detected in the gastric mucosa, saliva and dental plaque of 14 (21%) patients. However, 29 (44%) subjects harbored *H. pylori* in gastric biopsies and in the oral cavity in either the saliva or the dental plaque.

No statistically significant difference was observed between strains in the saliva and dental plaque. However, a statistically significant correlation was observed between gastric biopsies and the oral cavity ($p<0.0001$).

4. Discussion

H. pylori is the causative agent of chronic superficial gastritis and plays an important role in the etiology of peptic ulcer disease. Evidence suggests that *H. pylori* infection preexists in gastric carcinoma and is a risk factor for the development of other gastric diseases (22). Because of the importance of this bacterium in the development of chronic ailments, we evaluated the association among the presence of *H. pylori* in gastric biopsies and in the saliva and dental plaques of the same adult dyspeptic individuals. In our study, 46 (59%) of the patients presented *H. pylori* infection as determined by PCR. Furthermore, after detection of the PCR fragments by Southern blotting and hybridization, the number of *H. pylori* infected patients increased from 46 to 66, with 25.6% percent of positive samples showing a significant increase (Table 1).

Previous studies (14, 18, 23, 24) used PCR to diagnose an infection of *H. pylori.* These previous results are comparable to the results of the present study, in which there was a 95% success in the isolation of *H. pylori* in the stomach. Li et al. (25) and Song et al.(26) showed a significant increase in the sensitivity after using Southern blotting and also verified a high prevalence of infection.

Two different methods were used for DNA extraction from oral samples and stomach samples, because the oral samples show many contaminants and the technical using phenol-chloroform reveal a great sensitivity eliminating this contamination.

The high sensitivity and specificity of the PCR test with Southern blotting and hybridization did not reveal false positives, false negatives or contamination, due to the fact that the probe was synthesized from genomic DNA of a culture *H. pylori* by PCR, which excludes false results (27, 28). In addition, we used a pair of primers, designated Hpx1/Hpx2 that were specific for a 150 bp fragment of 16S rRNA of *H. pylori.* This is the most conserved region of and introduces a higher sensitivity and specificity when compared to other primers specific to *H. pylori*.

Our results parallel previous studies have revealed that the prevalence of the organism in adults can exceed 80%. However, it is important to remember that the infection rates can vary dramatically by geographic area, age, race and socioeconomic status (10, 18).

Several authors (14, 16, 18, 29, 30, 31, 32) reported that the oral cavity can be a reservoir of *H. pylori,* making treatment difficult and exposing the individual to a higher risk of gastric re-infection. However, Okada et al.(20); Dye et al.(33) and Olivier et al.(34) have characterized the bacterium in the oral cavity as being mainly transient. Despite this, these authors do not discard the hypothesis that an association between the presence of the bacteria in the stomach and mouth exists.

In our study, 33 (42.3%) and 37 (47.4%) of the patients had *H. pylori* in sample of saliva and dental plaque, respectively. Li et al.(25) found 75% of positive samples in saliva, which is similar to the results of Wang et al.(35) who found the bacterium in 71% of saliva samples. Santosh et al.(24) studied 120 dyspeptic patients and detected *H. pylori* in all of the gastric and saliva samples. Both authors suggested saliva is a method of transmission and may potentially induce gastric re-infections, which supports the results of the present study.

Song et al. analyzed 117 samples of dental plaque from 42 patients and verified the presence of the bacterium in 68% of the samples. This is similar to the results of Liu et al.(36) that found positive samples in 59% of the subjects and from our results that we found 47.4% of the positives samples. It is interesting to note that these studies differ from the results of Kignel et al.(4) who found a low prevalence in samples of gastric biopsies and only one positive sample in dental plaque. Similarly, Souto et al.(18) found 20% and 33% of subjects had positive samples in saliva and dental plaque, respectively.

Silva et al.(32) used a control group (individuals with no gastric disease who were *H. pylori* positive) and a case group (individuals with gastric disease who were *H. pylori* positive). The results from that study showed the presence of *H. pylori* in saliva and dental plaques from only the case group, suggesting an association between oral cavity and gastric disease. These findings further support the results of our present study.

Inconsistencies differences in the frequency of *H. pylori* in the oral cavity are found in the literature. This may be a consequence of differences in the demographics of subjects, oral health status, *H pylori* infection status, type and number of clinical samples, complexity of the oral flora and methods of detection (18).

Kignel et al. reported that the levels of bacterium in the oral cavity may be too low to be detected by one round of PCR. They further emphasized that the location in

the mouth used for the collection of the samples can influence the prevalence of the microorganism. This was further supported by the results of Song et al. found a prevalence of 82% in the molar region, 64% in pre-molar region and 59% in the region of the incisors. Loster et al.(14) suggested that this variation is the result of dental plaques could be due the differents levels of oxygenation, that can alter the settling of the *H. pylori*. The prevalence of infection from saliva and dental plaques, collectively, was 70% in this study. Then, our results verified a possible correlation between the prevalence of *H. pylori* in the oral cavity and infection in the stomach in Brazilian adult patients.

5. Conclusion

Our results suggest a relation between gastric infection and the bacterium in the oral cavity. Despite this, *H. pylori* was present in the oral cavity with variable distribution between saliva and dental plaques, suggesting the existence of a reservoir of the species and a potential association with gastric reinfection.

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Table 1 Comparison of the diagnostic methods for *Helicobacter pylori* (urease test, histology, PCR and Southern blotting) in 78 dyspeptic adults.

H. pylori	Urease test	Histology	PCR	Southern blotting
H. pylori-positive	30 (38.5%)	21 (27%)	46 (59%)	66 (84.6%)
H. pylori-negative	48 (61.5%)	57 (73%)	32 (41%)	12 (15.6%)
Total		78 (100%)		

*p < 0.0001

Table 2 Detection of *H. pylori* DNA in gastric biopsies saliva and dental plaque by Southern blotting

H. pylori	Gastric mucosa	Saliva	Dental plaque
H. pylori-positive	66 (84.6%)	33 (42.3%)	37 (47.4%)
H. pylori-negative	12 (15.6%)	45 (57.7%)	41 (52.6%)
Total		78 (100%)	

*p < 0.0001

1A

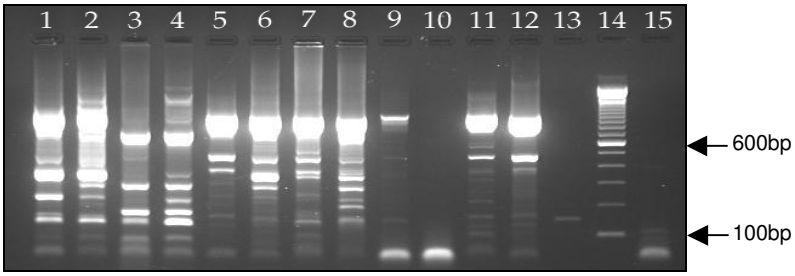

Figure 1 – A) Agarose gel 2% showing *H. pylori* PCR products of strains of gastric biopsies, saliva and dental plaques. "Slots" 1,3,5 and 7– saliva samples; "Slots" 2, 4 ,6 and 8 dental plaques samples; "Slots" 9, 10, 11 and 12, gastric biopsies samples; "Slots" 13 and 15 controls positive and negative, respectively; "Slot" 14 Ladder Marker 100 bp *(*Invitrogen).

1B

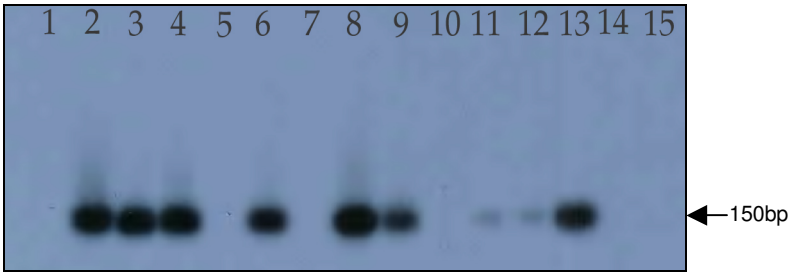

Figure 1 – B) Autorradiograph after hybridization with specific chemiluminescent probe referent to figure 1A "Slots" 1, 5 and 7, *H. pylori*-negative saliva samples. "Slot" 3, *H. pylori*-positive saliva sample."Slots" 2, 4 ,6 and 8 *H. pylori*-positive dental plaques samples. "Slot" 10, *H. pylori*-negative gastric biopsy sample."Slot" 9, 11 and 12, *H. pylori*-positive gastric biopsies samples. "Slot" 13 and 15 control positive and negative, respectively**.**

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