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Inhibition of *Helicobacter pylori* growth by oral Streptococci

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Abstract

Helicobacter pylori are an imperative gastrointestinal pathogen related with gastritis in addition to peptic ulcer disease. This study was designed to examine the *in vitro* influence of oral *Streptococci* on the growth of *H.pylori* isolated from different specimens. Sixty - seven specimens of peptic ulcer, saliva and dental plaque were collected from suspected patients and identified by culturing and PCR technique. Dental plaques swabs (67) from the same patients were collected, and cultured on suitable differential media to isolate and identify *Streptococcus spp*. .The effect of *S.oralis* and *S.mutans* was determined by growth inhibition of *H.pylori* isolates in solid and liquid medium as well as reduction the activity of urease as a marker to estimate viability of *H.pylori* . We concluded that important inhibition of growth, viability and urease activity has been achieved when *H.pylori* different isolates were dealt with *S.oralis* and *S.mutans* whole bacterial cell and supernatant. The study aimed to inspect the role of oral Streptococci in *H.pylori* activity besides pathogenicity.

Key words: *H.pylori*, peptic ulcer, dental plaque, oral Streptococci

INTRODUCTION

Helicobacter pylori (*H. pylori*) is one of the record public bacterial contagions in humans. It is a Gram negative, microaerophilic, rod-shaped that colonizes the gastric mucosa. As well as its existence in the human stomach was testified in every part of the world, the occurrence of *H. pylori* infection is sophisticated in developing countries than developed countries (1) . Spread of *H. pylori* could happen through fecal-oral, oral-oral routes and polluted food and water (2). *H. pylori* may be spread orally and has been noticed in dental plaque and saliva. Dental plaque can be one of the main causes of *H. pylori* re-infection and also be the cause of oral-oral spread that what confirmed by PCR and another new nucleic acid method(LAMP) ; Loop-mediated Isothermal Amplification (3). Oral cavity is a secondary location for *H. pylori* colonization and the existence of *H. pylori* in the oral cavity may augment the risk for gastric re-infection . The annual *H. pylori* recurrence rate is around 13% due to oral *H. pylori* infection (4,5). Disease expansion depends on some factors; the *H. pylori* strain, environmental and host features in addition to gastric microbiota (6).

Different oral bacteria were found to prevent the growth of *H. pylori* since oral cavity is colonized by different bacteria which interact competitively with each other in an attempt to survive and to prevent colonization by other bacterial forms like *H. pylori*. Numerous oral and intestinal bacteria; *Staphylococcus*, Enterococcus,Streptococcus ,*Lactobacillus* and *Bifidobacterium* besides opportunistic human pathogens can influence on the growth of *H.pylori* by producing inhibitory proteins have therapeutic use . Streptococci as *S. oralis* and *S.mutans* produce bactericidal compounds like hydrogen peroxide and substances similar to bacteriocins can inhibits *H.pylori* growth (7,8,9).

Recent data show that periodontal therapy possibly will play a role in reducing the levels and management of *H. pylori*-associated gastric disease in the oral cavity (3,5,10).

MATERIALS AND METHODS

Materials

Patients from the gastroenterology division of Al-Yarmouk hospital, were evaluated during 3 months. Clinical diagnosis was done under consultation of a specialist physician.

Sixty – seven samples from suspected patients were included in the current study who shoud not treated with antibiotics or other drugs; omeprazole, bismuth containing compounds like for three months ago . Samples from peptic ulcer, dental caries and saliva were collected to isolate *H.pylori* . Plaque and saliva samples were obtained before endoscopic examination.

Sixty - seven swabs from dental caries of the same patients were collected for *Streptococci spp*. isolation.

Specific primers ; 23S rRNA were used for amplification of *H.pylori* DNA by PCR ;

Table 1. Primers used in the study

Primer name	Primer sequence	Product size (bp)
Hp 23S 1942F	AGGATGCGTCAGTCGCAAGAT	367
Hp 23S 2308R	CCTGTGGATAACACAGGCCAGT	367

Methods

1- Samples of peptic ulcer ,dental caries and saliva were cultured on Brain Heart Infusion agar supplemented with trimethoprim (5µg /ml), vancomycin (8µg/ml) and polymyxin B (10 µg/ml) the plates were incubated at 37 °C in a microaerophilic humidified incubator (10% CO_2) for 3-6 days .

2- Swabs from dental caries were cultured on blood, chocolate, McConkey and mannitol salt agar aerobically 37°C for 24hrs. .

3- Total RNA was extracted from a precipitated sample of *H.pylori* according to (11) . Five µl of each amplified sample was analyzed by 1.5% agarose gel electrophoresis and stained with 0.5 µg/ml of ethidium bromide.

4- Supernatants of *S.oralis* and *S.mutans* were obtained from overnight cultures, centrifuged at 10000 × *g* for 20 min. at 4 °C ,filtered through 0.22 µm pore size filter.

5- *H. pylori* liquid culture was prepared in BHIB without antibiotics supplemented with 0.25 % yeast extract in shaking incubator.

6- For growth inhibition experiment ; 3 *H.pylori* isolates were chosen ;the first one from peptic ulcer source, second from dental plaque . last from saliva . Two isolates of oral Streptococci ; *S.mutans* and *S.oralis* highly biofilm producers were chosen . The experiment was done as following :

(a) Solid culture method

H.pylori isolates were cultured on BHI agar plates (10^7 CFU/ml) ,wells were done in the agar by sterile cork borer ,100 µl of both *S.oralis* and *S.mutans* suspensions (10^7 CFU/ml) were added in wells. Plates were incubated for 48-72 hrs. under microaerophilic conditions at 37 °C . Diameters of inhibition zones were measured (mm) .

(b) Liquid culture assay.

H. pylori (10^8 CFU/ml) liquid culture prepared in (5) was incubated under microaerophilic conditions at 37°C in the presence of *S.oralis* and *S.mutans* supernatant separately . The viability of *H. pylori* during 24 - 48 hrs. was estimated

by determination of viable CFU on BHI agar plates following incubation at 37°C under microaerophilic conditions.

7- Urease activity was determined by phenol red method (12). 10 µl of *H. pylori* cell suspension was added to 300 µl of urease reaction buffer (20% wt/vol) urea and 0.012% phenol red in phosphate buffer saline (pH = 6.5) on a microtiter plate, the plate was incubated for 2 hrs. at 37°C. The absorbance at 550 nm was measure.

Statistical analysis. All statistical analyses were performed using SPSS, version 18.0 (SPSS Inc., NY, USA). Chi-square tests were used to compare categorical measures between groups (Fisher's exact test where appropriate).

Results And Discussion

Identification

We studied the potential inhibitory effect of *Streptococci spp. : S. oralis* and *S.mutans* on *H. pylori* by *in vitro* assay.

Table 2 . Samples for isolation *H.pylori*

No.samples	*positive for H.pylori (%)*	*negative for H.pylori (%)*
67	31(46.3)	36(53.8)
Pu:44	19(43.2)	25(56.8)
Dc:18	11(61.1)	7(38.9)
Sa:5	1(20)	4(80)

Pu ; peptic ulcer, Dc; dental carries ,Sa; saliva

Most *H. pylori*-positive specimens were from peptic ulcer and the samples of oral cavity of dental plaque were more than saliva (18:5) *H. pylori* isolates from patients with peptic ulcers, dental caries and saliva were diagnosed by Gram staining and biochemical examination, the urease test the most important test , the results were established by culturing the isolates , there was difficulty of cultivating *H.pylori* from the oral cavity and sometimes *H. pylori* may be present in the oral cavity in an unculturable state therefore PCR technique was performed.

The PCR amplification product was then gel electrophoresed and analyzed under UV transilluminator . PCR amplification products were compared with standard molecular markers and analyzed. Gel electrophoresis results of PCR showing that 27 isolates were positive for *H. pylori* while 11 isolates were positive by culture.

Table 3. Identification results by culture and PCR

Test	**positive(%)**	**negative(%)**
Culture	11(35.5)	20(64.5)
PCR	27(87)	4(13)
P value	0.001	0.001

The culture medium used in the present study was selective for *H. pylori* , in addition to specific primer in (PCR) thus possibility of false-positive results because of other urease-producing baceria in the oral cavity is eradicated.

PCR is a sensitive and reliable test for detecting oral *H. pylori* since PCR detects low numbers of bacteria . Also non-viable or non-infectious *H. pylori* can be detected.

The source and transmission route of *H. pylori* infection are still unclear, and more reliable methods for the recognition of the bacterium in the oral cavity are needed . (14,15).

A total of 67 dental plaque samples were analyzed. Forty –two *Streptococcus spp.* isolates were identified by colony characters, Gram stain, catalase and oxidase tests, optochin and bacitracin susceptibility. *S. mutans* and *S.oralis* were identified confirmatory by using VITEK-2 compact system (Biomerierx).Results of growth inhibition test were expressed as +++,++,+ ,table 4.

Table 4. Effect of *S.oralis* and *S.mutans* on *H.pylori* growth

Streptococcus spp.	***H.pylori* Pu1**	***H.pylori* Dc5**	***H.pylori* Sa**	**P value**
S. oralis SBP 22	+	++	+++	0.001
S.mutans SBP 39	++	+++	+++	0.01

SBF; strong biofilm producer , Pu;peptic ulcer, Dc;dental carries, Sa;saliva
+ : inhibition zone ≤ 5mm
++: inhibition zone ≥ 5mm
+++ :inhibition zone > 10 mm

As noticed from table -4 that *S.mutans* isolate SBF 39 was more potent than *S.oralis* SBF 22 as it strongly inhibits the growth with large inhibition zone(> **10 mm)** particularly with the oral *H.pylori* .

Also growth inhibition was studied in liquid medium to confirm the results of inhibition in solid medium . Influence of *S.oralis* and *S.mutans* supernatants on bacterial cell viability of *H.pylori* was determined by CFU on BHI agar after (5 -10-15-20-24) hrs. Results were drawn as in the curve below ; fig.1:

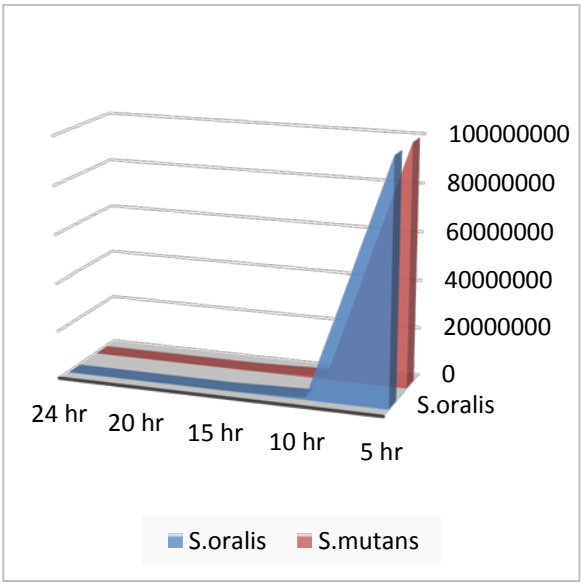

Figure 1. Effect of *S .oralis* and *S.mutant* supernatants on viability of *H.pylori* after 24 hrs. incubation period at 37

Noticebale decrease in viable number of *H.pylori* were recorded with incubation time , after 10 hrs. of incubation with *S.oralis* supernatant , the bacterial number declined to 10^6 cell/ml ,10^3 cell/ml after 10,20 hrs. respectively and identical results regarding *S.mutans* effect were observed .

Inhibitory action may attributed to bacteriocin-like substances produced by *S.oralis* and *S.mutans* as well as H_2O_2 . *H.pylori* possibly susceptible to inhibitory effect of these compounds. Watanabe *et al*.,2009 (16) have recorded the bactericidal effect of *Streptococcus sanguinis* supernatant to inhibit the growth of *P.aeruginosa* using the minimum bactericidal concentration method. The viable number of *P. aeruginosa* was decreased with time after exposing to the filtrated supernatant of *S. sanguinis*, and collapsed bacteria were detected with electron microscopy. Another study was documented that *Streptococcus pneumoniae* secretes hydrogen peroxide leading to DNA damage and apoptosis in lung cells (17).

The effect of *S.oralis* and *S.mutans* on urease activity of *H.pylori* reflects the viability of bacterial cells in liquid *H.pylori* cultures. Results were shown in fig.2:

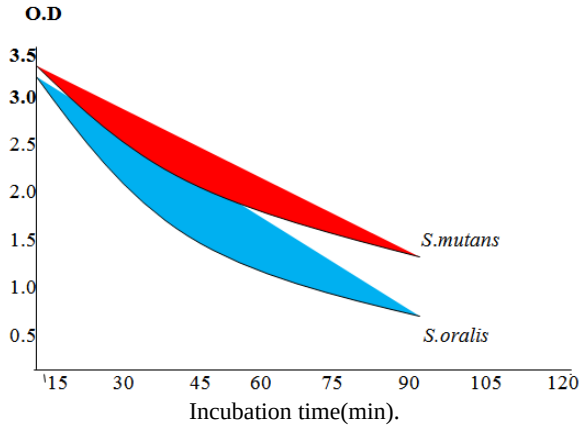

Figure 2. Reduction in the urease activity of *H. pylori* incubated with *S. oralis* supernatant (a) and *S.mutans* supernatant (b) for 2 hrs. at 37°C by measuring optical density at 550 nm

Urease is an imperative virulence factor for *H.pylori* and is critical in gastric colonization ,the specific inhibition of this enzyme has been suggested to fight this bacteria which infects billions of persons throughout the world(18) . *H. pylori* is the only urease-positive bacterium inhabits the stomach so the urease test is a appropriate detection test for routine on gastric specimens while oral cavity is habitation to a number of urease positive species, like *Streptococcus spp*., *Haemophilus spp*., and *Actinomyces spp*. , it is unsuitable to conclude that high urease activity in dental plaque is indicative of the presence of *H. pylori* (19).

The oral cavity may be a lasting reservoir for *H. pylori* and multiple *H. pylori* strains are present at the same time (14). Sangita and Jithendra 2012(10) were indicated to the inhibitory activity of *Lactobacillus casei in vitro* on growth of *H.pylori* and ability to reduce colonization and inflammation *in vivo* .They reported the ability to detect *H. pylori* in dental plaque samples that offers the potential for a non-invasive test for infection and would provide support for the oral spread as the principal mode of transmission, therefore the detection of *H. pylori* in dental plaque of dyspeptic patients cannot be ignored and might correspond to a risk factor for reinfection after systemic therapy .

Ishihara *et al.*, 1997 (7) have indicated that the oral bacteria produce bacteriocin-like inhibitory proteins against *H.pylori* because the growth inhibitory activities were negatively affected by heating at 80 C for 60 min. or by protease treatment. It is possible that *H. pylori* converts to the coccoid form when exposed to the inhibitory effects of oral microorganisms (20).

Gao *et al*., 2011(2) concluded that *H.pylori* is present at various parts of oral cavity, it might be an important source of gastric *H.pylori* , the detection rates of oral specimens were dental plaques (82.3%), gargles (51.1%) and scrapings of dorsal mucosa of tongue (37.5%) respectively.

The *H. pylori* comes from the stomach reflux can live in the oral cavity for a few hours only because of the high oxygen concentration in the oral cavity. Oxygen is essential determinant factor, because it influences the ability of oral bacteria to grow and multiply. Also oral bacteria prevents the growth of other bacteria when produce organic acids, H_2O_2, fatty acids, and bacteriocins(21).

The influence of a large number of oral and intestinal commensals as well as opportunistic bacteria on the growth of *H.pylori* was examined *in vitro*. Some of the bacteria that were tested could serve as probiotics. Others are opportunistic pathogens, but their inhibitory products might be of therapeutic use (8).

The frequency of the presence of oral *H. pylori* in the current study is low compared with that of the stomach, that because of the antagonistic effect of oral microorganisms against *H. pylori* in the oral cavity.

It was founded that PCR was highly sensitive with adequate specificity for detection of oral *H. pylori*, when compared to culture.

Results present special interest to isolation and characterization of the promising inhibitory substances useful in treatment of *H.pylori* infection.

Further investigations should be carried out to prove oral cavity as the prospective reservoir of *H. pylori* , and additional successful culturing techniques for oral *H. pylori* are required to confirm its viability and ability to cause infection. More investigations, for characterization of the inhibitory substances shoud be done .

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