Detection of Helicobacter pylori in dental plaque of Mexican children by real-time PCR

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Received 21 November 2013; revised 30 December 2013; accepted 7 January 2014

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ABSTRACT

Dental plaque in adult patients is well identified as a reservoir for Helicobacter pylori. This question still remains unclear in children. The aim of this study is to identify the presence of this bacterium in dental plaque of Mexican pediatric patients, using Real Time Polymerase Chain Reaction (qPCR). Forty patients from 2 to 11 years without dyspeptic symptoms were enrolled. Samples were collected from the subgingival space of the lingual side of the lower molars and cultured in selective medium. Therefore, qPCR analysis was conducted. According to the results obtained in this study, it was found that 35% of the pediatric population who participated tested qPCR positive for the presence of H. pylori in dental plaque samples. No significant associations were detected among isolation rate by gender or age. We found that dental plaque may be a reservoir for H. pylori. However, more research is needed to establish the way of the infection of pediatric population.

KEYWORDS
Helicobacter pylori; Oral Pathology; Children; Real Time Polymerase Chain Reaction; Anaerobic Bacteria

1. INTRODUCTION

Gastric cancer is one of the most common deadliest types [1] and it’s directly related to chronic infection of Helicobacter pylori [2-6]. Since 1984, Marsall and Warren associated H. pylori to chronic gastritis and peptic ulcers [7] and the International Agency for Research on Cancer classified it as a type I carcinogen in human beings [8].

There are many theories trying to explain the way H. pylori colonizes the stomach [9,10] and dental plaque is one of the recognized sites as a reservoir [11]. Positive findings on dental plaque in adults have been documented [12,13]. However, other studies show disagreeing results because they have failed in isolating the bacteria [14-16].

Prevalence studies of H. pylori infection have demonstrated an increase in the general population and an early exposure to the bacterium is the factor that explains why this organism is found in various age groups. Although the infection can be asymptomatic in children, sometimes it is reported to be accompanied by recurrent abdominal pain and dyspepsia symptoms [17-19]. In developed countries as Mexico, the prevalence of H. pylori seems to be lower in children and increases progressively with age [20].

The diagnosis of H. pylori is complex and is mostly done with the Urease test, which is poorly sensitive and leads to false negative results [21,22]. New studies have showed that the most feasible method for early detection
is real time polymerase chain reaction (qPCR). Using this technique, it has been possible to detect *H. pylori* in samples of dental plaque, canker sores, saliva, gastric acid and feces offering a better alternative for clinical diagnosis [15,23,24].

The aim of this work was to determine the frequency of *H. pylori* in dental plaque in children by a molecular diagnosis method called qPCR.

2. MATERIALS AND METHODS

This cross sectional study was conducted from July to August 2013 during follow-up visits from the Clinic of Pediatric Dentistry at the University of Nuevo Leon, Mexico. Forty subjects were enrolled and the inclusion criteria were: children from 2 to 11 years old, any gender, and apparently healthy. The exclusion criteria were: 1) actual history of antibiotic usage or during the previous 2 months, 2) systemic disease, and 3) abdominal pain. This protocol was registered in the institutional research committee. Prior the sampling the parents or guardian of the patient signed an informed consent form and declared their willingness to allow the use of the samples and their anonymous data for research purposes.

Sampling was by convenience according patients arrive to clinic until completed the sample size. A sterile wooden stick of 1 mm in diameter was used to get the dental plaque sample; it was modified on one end to create an active part as a shovel. The sample was taken from the lingual sulcus in the lower molars and placed in a microcentrifuge tubes containing: 150 μl bovine serum and 350 μl of trypticase soy.

Samples were grown on Campy Agar (10% sheep blood, Amphotericin, Cephalothin, Trimethoprim, Vancomycin and Polymyxin B). 200 μl of the sample were placed on the center of a Petri plate and incubated under microaerophilic conditions using a GasPak CO2 jar system (Becton Dickinson, USA). After 5 days, the bacteria were collected by scraping the colonies from the agar and cultured under the same conditions stated above for 3 days.

The DNA from the harvested bacteria was extracted by using Wizard ® Genomic DNA Purification Kit (cat A1120 Num. Promega, USA) following the manufacturer’s instructions. The samples were stored at −20°C until use for Real-Time Polymerase Chain Reaction (qPCR). The specific oligonucleotides probes were previously designed using the Primer Express 3.0 program (Applied Biosystems, USA) and selected for the 16S ribosomal subunit from *H. pylori*.

The qPCR reaction conditions was assessed 96-well plates, filled with 2.5 μl of each sample DNA, 5 μl de PCR reaction mixture (Universal TaqMan Master kit Probe I, Roche, Germany) and 0.150 μl of the designed primers. For the positive and negative controls, instead of sample DNA, were added pure DNA from the strain 43054 *H. pylori* and sterile water, respectively.

qPCR was carried out in a Light-Cycler 480II system (Roche, UK), programmed with the detection mode of HRM Dye, block of 96 and a reaction volume of 10 μl. The three stages were: pre-incubation at one cycle, fifty amplification cycles with quantification analysis mode and the cooling cycle.

The pre-incubation was conducted at a temperature of 95°C, at hold for 5 minutes and a ramp of 4°C/S, the amplification was carried out with three different temperatures: 95°C at a hold of 10 seconds and a ramp of 4°C/S, 55°C at a hold of 15 seconds and a ramp of 2°C/S, and finally at 72°C at hold of 10 sec, a ramp of 4°C/S and a single acquisition mode. The cooling program was at temperature of 40°C, a hold for 30 seconds and a 2 seconds ramp.

The sample size of 40 subjects was calculated using Stata IC12 with a power of 80% and a confidence level of 95%. All data were stored in an Excel Microsoft Database. The data were analyzed by SPSS IBM Statistics v19. Proportion of isolations of *H. pylori* was determined and next, the association of isolation according genre were determined by Chi square. Differences in the mean age according isolation were evaluated by t student test. Values were considered statistically significant if p value < 0.05.

3. RESULTS

The study population consisted of 40 healthy pediatric patients, 13 females (32.5%) and 27 males (67.5). The mean age was 5.35 ± 2.23, mode and median were 5. The distribution of patients according to age is shown in Table 1 where the range is 9, while the minimum and maximum were 2 and 11 years respectively.

The prevalence of *H. pylori* isolation was 35% in the total sample, while the isolation frequencies by age and gender are presented in Tables 1 and 2 respectively.

There is no significant association within the occurrences of isolation and age (p = 0.32) (Table 1), the mean age of patients with and without isolation (p = 0.14) (Table 3) and between gender and isolation of *H. pylori* p = 0.09 (Table 2).

4. DISCUSSION

The infection with *H. pylori* is considered carcinogen and is recognized as the primary cause of gastric cancer [8]. Several studies have concluded that *H. pylori* is existent in the oral cavity and proposed that the mouth could be the source of infection and reinfection after eradication treatments for stomach infections [11-13]. More specialized studies postulate that *H. pylori* exists in peri-
odontal pockets, because it re-creates an excellent atmosphere for microaerophilic and anaerobic microorganisms. However, many authors have concluded that the oral cavity is not a reservoir for this bacterium [14-16] and these contradictory results can be explained by differences in the studied population, the possible contamination of the sample, collection methods and poorly sensitive diagnostic techniques.

In this study, 35% of the children tested positive for *H. pylori*. All the subjects appeared otherwise healthy, and none of them reported any gastrointestinal disease. Studies in non-dyspeptic pediatric population are consistent with our results obtaining similar prevalence rates [25,26], unlike when conducted in adult populations where it was only possible to isolate *H. pylori* in the dyspeptic patients [27]. Our results do not show association between age and *H. pylori* isolation, and most of the patients under 5 years have higher isolation rates 11/17 (64.7%) than those over 5 years 3/9(33.3%) (Table 1), opposing with Oliveira et al. [28] and the prevalence was 16% in patients less than 2 years of age, 37% in children from 3 to 5 and 64% in patients 15 to 18 years old.

The plaque sample was collected from the lingual sulcus in the lower molars because it was not constantly swept by the salivary flow and was a half anaerobic zone. We consider this a niche with the appropriate pH, redox potential, and nutrient availability to sustain the bacteria [29]. In this study, we cultured the bacteria in specific medium, in order to eliminate other pathogenic bacteria. Detection of *H. pylori* has been achieved by several methods, some effective and others with low sensitivity, but the qPCR is the most suitable for detecting low numbers of *H. pylori* in the oral cavity [15]. The primer used for qPCR is specific for *H. pylori* and eliminates the possibility of false-positive results. The highly sensitive 16S rRNA gene was used for the DNA amplification. This cellular component has been shown to be suitable for demonstrating phylogenetic diversity of bacteria [30]. Although sensitivity is tenfold more than other PCR methods, this nonspecifically amplifies human DNA [23, 31].

In conclusion, this study strongly supports the hypothesis that the mouth of children may be a reservoir for *H. pylori*. Further studies will be required to confirm the importance of any relationship between oral and gastric strain and possible routes of transmission. It is necessary to pay attention to dental plaque as a possible second reservoir for *H. pylori* colonization.

**ACKNOWLEDGEMENTS**

The authors would like to thank to the Consejo Nacional de Ciencia

| Table 1. Shows distribution of patients by age and isolation of *H. pylori*. |
| --- |
| AGE (Years) | NEGATIVE | POSITIVE | Sum | p value |
| n | % | n | % |
| 2 | 1 | 100 | 0 | 0 | 1 |
| 3 | 7 | 87 | 1 | 13 | 8 |
| 4 | 2 | 40 | 3 | 60 | 5 |
| 5 | 7 | 50 | 7 | 50 | 14 |
| 6 | 3 | 100 | 0 | 0 | 3 |
| 7 | 1 | 100 | 0 | 0 | 1 |
| 8 | 3 | 75 | 1 | 25 | 4 |
| 10 | 1 | 33 | 2 | 67 | 3 |
| 11 | 1 | 100 | 0 | 0 | 1 |
| Sum | 26 | 65 | 14 | 35 | 40 |

*Chi square p value.

| Table 2. Shows distribution of patients by genre and isolation of *H. pylori*. |
| --- |
| GENRE | NEGATIVE | POSITIVE | Sum | p value |
| n | % | n | % |
| FEMALE | 11 | 84 | 2 | 16 | 13 |
| MALE | 15 | 55 | 12 | 45 | 27 |
| Sum | 26 | 65 | 14 | 35 | 40 |

*Fisher exact test p value.

| Table 3. Shows means age of patients according isolation of *H. pylori*. |
| --- |
| n | Mean | Median | Range | IC: 95% | p value |
| Negative | 26 | 5.08 | 5 | 9 | 4.2 | 5.95 |
| Positive | 14 | 5.86 | 5 | 7 | 4.5 | 7.21 |
| Total | 40 | 5.35 | 5 | 9 | 4.3 | 6.14 |

* t student p value.
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