MOLECULAR DETECTION OF ORAL *HELIcobacter pylori* WITH VACA, CAGA, AND DUPA VIRULENCE GENES IN RECURRENT APHTHOUS STOMATITIS PATIENTS IN Duhok, KURDISTAN REGION, IRAQ

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ABSTRACT

**Background:** Recurrent Aphthous Stomatitis (RAS) is an inflammatory condition of unknown etiology characterized by recurrent and painful lesions, with single or multiple ulcerations that are confined to the oral cavity mucosa. The current study aimed at the molecular detection of oral *H. Pylori* and its vcaA, cagA, and dupA virulence genes in patients with Recurrent Aphthous Stomatitis in Duhok, Kurdistan Region, Iraq.

**Method:** This is a cross-sectional study. It has been conducted in the laboratories of the college of medicine, Duhok city, Kurdistan Region, Iraq. A total of ninety-two individuals were included in the present study; forty-six patients with RAS consisted of 11 females and 35 males and forty six apparently healthy individuals as control group composed of 23 females and 23 males. A swab was taken from the RAS lesion of each in the patients' group and the control group's oral cavity (cheek) and submitted to a conventional PCR-based assay to detect the *H. Pylori* DNA using specific primers targeting 16SrRNA gene. The family history for RAS of both the patients and the control group was investigated. *H. pylori* virulence genes vcaA, dupA, and cagA(m1) were investigated in all extracted DNA samples using specific primers.

**Results:** *H. Pylori* DNA was detected only in 2 (4.34%) of the patients and one (2.17%) of the control group. The family history of RAS disease was positive in 24 (52.17%) of the cases, while only one individual (2.173%) of the control group had a positive family history of RAS disease. One of the *H. pylori* positive RAS patients showed a positive result for the three vcaA, dupA, and cagA(m1) virulence genes, whereas the other one was positive for only dupA and cagA(m1) virulence genes. In addition, the *H. pylori* positive healthy control showed a positive result for all the three vcaA, dupA, and cagA(m1) virulence genes.

**Conclusion:** There was no significant attribution *H. Pylori* in the etiology of RAS, while there was a highly significant relation of recurrent RAS with the family history of the patients (p < 0.01).

**Keywords:** cagA, dupA, *Helicobacter pylori*, Recurrent aphthous stomatitis, vacA.
MOLECULAR DETECTION OF ORAL HELICOBACTER PYLORI WITH VACA.

RAS study conducted on more than 10,000 young adults in more than 21 different countries; about 49.7% of females and 38.7% of males reported two or more previous episodes of RAS. Nearly about 25% of the participants reported at least one episode of RAS during the last year. RAS can be classified based on the size of the ulcer and the number of ulcerations into three clinical types; minor recurrent aphthous stomatitis (MiRAS), major recurrent aphthous stomatitis (MaRAS), and herpetiform ulceration (HU). The episodes of RAS are self-limiting and recover within one to two weeks without leaving any scars.

The underlying etiology is unknown, but there are several local factors and underlying systemic diseases and conditions that predispose to the appearance of RAS, including genetic factors, food allergens, local trauma, endocrine alterations (menstrual cycle), stress and anxiety, smoking cessation, certain chemical products, and microbial agents. Data introduced by some researchers suggest that the oral cavity may be a reservoir for *H. pylori* in some individuals, and the transmission of the disease may be via an oral-to-oral route. *H. pylori* is a Gram-negative, S-shaped bacterium that has long been associated with gastritis and chronically infected duodenal ulcers. Worldwide, the prevalence of *H. pylori* is about 50% (approximately 4.4 billion individuals infected). In developed countries, the prevalence rate is between 20 and 40%, and it reaches up to 90% in Africa and other non-developed countries. The variation of the prevalence of infection among populations is related to race, ethnicity, geographical location, or method of testing. *H. pylori*-associated diseases establishment and progression is attributed to a group of virulence factors. There is a number of genes encode these factors; they are the cytotoxic-associated gene A (*cagA*), which has been described as an oncoprotein, vacuolating cytotoxin gene A (*vacA*), which plays a significant role in immune modulation as well as in the induction of gastric cancer and duodenal ulcer promoting gene A (*dupA*). Detection of these virulence factors in *H. pylori* is vital in determining the risk of the disease. In some studies, the *cagA*, *dupA*, and *vacA* genes have been reported as potential virulence factors of gastroduodenal illnesses in children and adults.

Therefore, the current study aims to detect *H. pylori* in oral samples of recurrent aphthous stomatitis patients and the presence of virulence genes *vacA*, *cagA*, and *dupA*.

MATERIALS AND METHODS

A cross-sectional conducted in the laboratories of Duhok medical college between the period June 2016 and January 2017, forty six RAS patients were included; they were presented with idiopathic recurrent aphthous stomatitis, the clinical diagnosis was achieved by a specialized dentist, the cases were recruited from high schools and the university of Duhok. Also, another, forty-six apparently healthy volunteer individuals were involved in the study as a control group. The ethical committee has approved the study of the health directorate of Duhok province. Verbal consent has been obtained from each subject. All the patients should have
experienced a minimum of three episodes of RAS within the last one year. Patients were subjected to an oral assessment protocol that included careful history review. All patients and healthy controls were questioned about gastroesophageal reflux’s classic symptoms (heartburn, acid taste, and regurgitation). In addition, they were asked whether they had been treated previously for gastroesophageal reflux disease or *H. pylori* infection in their stomachs. Well-known systemic disease patients (such as Behçet’s syndrome, Sweet’s syndrome, PFAPA syndrome, Reiter’s syndrome, Crohn’s disease, and gluten-sensitive enteropathy) were excluded. Patients under medication that could be associated with oral ulcers or *H. pylori* (such as antibiotics, proton pump inhibitors, H2 receptor blockers, Bismuth derivatives, non-steroidal anti-inflammatory drugs, chemotherapeutic agents, antibiotics, or vitamin supplements) for one month prior to the study were excluded. Women during pregnancy and menstruation were excluded as well. Swabs were collected from the oral cavity lesions of the 46 RAS patients and the cheek of the controls with the use of sterile cotton swabs. Genomic DNA was extracted from each of the oral swabs using DNA extraction kit (Qiagen, Germany) according to the manufacturer's instructions. PCR was conducted on extracted genomic DNA samples to detect the *H. pylori* DNA using a conventional PCR kit (Kapa Biosystem, USA). Primers used to target the *H. pylori* 16SrRNA gene were (F- 5’ GCG ACC TGC TGG AAC ATT AC 3’) and (R- 5’ CGT TAG CTG CAT TAC TGG AGA 3’) designed by Gramley et al. The primers are expected to yield a 138bp PCR product. The PCR reaction was conducted according to the method mentioned by Roesler et al. Briefly, the PCR reaction mix of each sample was made up to 25 μl. Each 25 μl PCR reaction mixture contained 12.5 μl PCR master mix (Promega, GoTag® Green Master Mix, USA), 0.5 μl each of primer (Metabion, Planegg, Germany), 5 μl of template DNA, and 6.5 μl of PCR grade water. For each PCR experiment, appropriate positive and negative controls were included. The *H. pylori* strain J99 and nuclease-free water were used as positive and negative controls, respectively. Forty thermal cycles were carried out, with each cycle consisting of a 30-second denaturation at 95°C, 1 min annealing at 60°C, and 1 min extension at 70°C and an additional 5 min extension at 70°C was needed to allow full product extension. To detect the PCR products, 5 μl of amplicons were electrophoresed in 2% agarose gel, ethidium bromide-stained, and visualized under UV light. *H. pylori* virulence genes vacA, dupA and cagA(m1) were investigated using PCR and the following specific primers: cagA(F: ACCGCTCGAGAACCCCTAGTCGGTGAA TGGG), (R: CAGGTACCGCGGCCGCTTAAGATTT TTGGAAACCAC),PCR product size 981 bp, vacAm1 (F: GGTCGATGTGCATGCTAGTGGG), (R:CCA TTGGTACCTGTAGAAAC), PCR product size 290 bp, dupA (F:GACGATTGAGCGATGGGAATAT), (R:CTGAGAAGCCTTATTATCTTGTTG), PCR product size 971 bp. The same PCR conditions are followed, as described previously. Chi-square test was used to describe the association between RAS and the oral *H. pylori* presence as well as
virulence factor genes as attributed risk factors, using the SPSS statistical software package version 18.0 (SPSS, Inc., Chicago, IL). P values < 0.05 were considered statistically significant.

RESULTS

In the present study, ninety-two individuals were enrolled as two groups: the first group was the patients’ group, which composed of forty-six patients with RAS disease, consisting of 11 females (23.91%) and 35 males (76.08%), their ages range was (15-40 years). The family history of RAS disease was positive in 24 patients (52.17%). The second group was the control group of 46 healthy individuals, consisting of 23 females (50%) and 23 males (50%), their ages range was (15-30 years), only one individual (2.173%) had a positive family history of RAS disease. There was a significant difference between the two groups regarding gender (p < 0.05), and there was a highly significant difference between the two groups regarding family history (p < 0.001) in table 1.

Table 1: *H. pylori* DNA positive among RAS patients and the control group

<table>
<thead>
<tr>
<th>Studied groups</th>
<th>Gender</th>
<th><em>H. pylori</em> (+)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RAS patients</td>
<td>35 Male</td>
<td>2 (4.34%)</td>
</tr>
<tr>
<td></td>
<td>(76.08%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>11 Female</td>
<td>0 (0%)</td>
</tr>
<tr>
<td></td>
<td>(23.91%)</td>
<td></td>
</tr>
<tr>
<td>Healthy</td>
<td>23 Male</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>controls</td>
<td>(50%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>23 Female</td>
<td>1 (2.17%)</td>
</tr>
<tr>
<td></td>
<td>(50%)</td>
<td></td>
</tr>
</tbody>
</table>

The 138 bp PCR product of *16SrRNA* gene of *H. pylori* was detected in the extracted DNA from oral swabs of only two RAS patients (4.34%) both of them were males, and it was detected in only one female DNA sample (2.17%) of an apparently healthy individual (control group) (Figure 1). There was no statistically significant difference between the two groups concerning the *H. pylori* DNA.

![Figure 1](image.jpg)

*Figure 1: PCR products were obtained from extracted DNA of RAS lesions on 2% agarose gel electrophoresis, using specific primers targeting *16SrRNA* of the *Helicobacter pylori*. Lanes 7 and 8 are 138 bp products (*H. pylori* positive), lane L is a 50 bp DNA ladder, lane 13 is positive control, and the rest lanes are *H. pylori* negative in RAS patients.*

Table 2 shows the presence of *vacA*, *dupA*, and *cagA*(m1) virulence genes in *H. pylori* positive RAS cases and healthy controls. One of the *H. pylori* positive RAS patients showed positive results for the three *vacA*, *dupA*, and *cagA*(m1) virulence genes, whereas the other one was positive for only *dupA* and *cagA*(m1) virulence genes. Besides, the *H. pylori* positive healthy control showed positive results for all the three *vacA*, *dupA*, and *cagA*(m1) virulence genes table 2.
Table 2: The presence of vacA, dupA, and cagA(m1) virulence genes in H. pylori positive RAS cases and the healthy controls.

<table>
<thead>
<tr>
<th>Study groups</th>
<th>H. pylori positive cases</th>
<th>vacA</th>
<th>dupA</th>
<th>cagA (m1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RAS patients</td>
<td>1 (2.17%)</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Healthy control</td>
<td>1 (2.17%)</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

DISCUSSION

The RAS is a chronic inflammatory disorder characterized by the appearance of one or more ulcers on the oral mucosa with nonspecific histological features that persist for several days to several weeks, causing pain, and recurs after different periods of remission. The etiology of RAS is unknown. But there are several theories and studies that have been suggested and conducted to look for the etiology and predisposing factors of the RAS. The present study aimed to evaluate the attribution of H. pylori as an etiologic factor in RAS in Duhok city, Iraq. The molecular technique (PCR) was used to detect the H. pylori in the RAS lesion. In the current study, the age range of the RAS patients was 16-28 years, and the mean was 22±2.2 years, and of the control group was 15-30 years with a mean of 18.41±3.2 years. Infection with H. pylori is, basically, asymptomatic, and the individual will be a carrier through life till the time when eradication treatment is done. The exact mechanism by which H. pylori induces tissue injury is not clear. Some immune-mediated mechanisms are suggested. Due to the similarity between the histological characteristics of gastric ulcers and oral aphthous ulcers, which respond to the treatment by broad-spectrum antibiotics, it looks logical to suppose that H. pylori could play a role in the etiopathogenesis of RAS disease, but still, the data regarding the potential relation between RAS and H. Pylori infection are limited and controversial. In the current study, we found that there was no significant attribution of the H. pylori infection with the RAS, since it was detected in only 4.3% and 2.1% of RAS and controls, respectively. Other authors came out with results consistent with our results, while in other studies, the results were inconsistent with ours when Yi-Jian et al. (2015) found a significant association of H. pylori infections with oral diseases including periodontal diseases and caries. In a review study, a search in PubMed (MEDLINE) databases was made of articles published up until July 2015, Gomes, et al. stated that the H. pylori could be occasionally detected in RAS lesions and the eradication of the infection may affect the clinical course of RAS lesions by undetermined mechanisms. However, most of the studies do not support the association of RAS ulcers with the presence of the bacteria in the oral cavity, and the presence of the bacteria in the ulcer may reflect a passenger infection and not the trigger event. There is no convincing evidence of a direct cause-consequence effect of H. pylori infection and RAS ulcer development. This association requires further investigation by well-design prospective studies. So, the relationship between H. pylori and RAS remains controversial, and these discrepancies in the findings of different studies remain unexplained, but some factors could explain these discrepancies; such as the small sample size of RAS patients as in our study.
study, variations in techniques and tests used in studies, the variations of the ways used in the collection of specimens, the density of the bacterial samples taken with swabs, the differences of ethnicities of the studied patient populations, and various primers and target DNA used in the PCR assays. In the present study, RAS's family history was positive in about (52.2%) of the patients' group, while (2.2%) of the control group had a family history of RAS. The current study results are consistent with those of Zand et al., 2012 who reported that the rates of positive family history of RAS in patients and control group were (54.2%) and (9%) respectively, which is a statistically significant risk factor for having RAS, and in the same time, they found that the family history (among the predisposing factors of RAS disease) have the strongest correlation with the RAS. Also, Compilato et al. (2010) reported that family history was significantly associated with RAS. Some of the detected gene polymorphisms, such as pro-inflammatory cytokine encoding genes, explain the increased susceptibility to developing stimulated immune response to some oral antigens leads to aphthous formation erosions and oral ulcers. Based on the previous data, family history should be highly considered during the management of RAS patients.

CONCLUSIONS
There was no significant association between the prevalence of H. pylori and the RAS. The family history of the included subjects had a highly significant impact on the etiology of RAS of the patients involved in the study.

CONFLICTS OF INTEREST
The authors have nothing to declare.

ACKNOWLEDGMENTS
Many thanks go to the staff of Duhok Medical Research Center for their help.

REFERENCES


Molecular Detection of Oral *Helicobacter pylori* with VACA.


پوخته

ناساندنا مولیکول‌یا به کتریا زری دارگه‌ی و فاکته‌یین گگکر
نام‌سرا دهفی یا دووبرار ل دموک‌یی کوریستانت‌ی عراق

پیکشگی

نه‌سه‌ی را دهفی یا دووبرار رووش‌یا گنگ‌یی ماکرو زری دهفی‌یی. فاکته‌یین سه‌دهما هنده‌ی ری‌ه نی دیارن

نیکولینه شارمانه نکه‌ت بو دیارکرکا مولیکولی‌یا به کتریا زری دارگه‌ی و فاکته‌یین گگکر

ل دهفی نه‌خوش‌ین نام‌سرا دهفی یا دووبرار و دیارکرکا په‌پومن‌یا وان دگه‌ی ثیک.

ریک‌ین کاری

46 نه‌خوش‌ین بو نام‌سرا دهفی یا دووبرار، 11 می و 35 نیز هاننامه دستنی‌شنانکنکنی، مه‌روسا 46 کسین ساخن‌ی زی و دگکی

کوما کونترول کو زود 23 می و 23 نیز هاننامه دیارکرکن زکوما نه‌خوش‌ان نام‌سرا دهفی و زکوما کونترول چنلیکا دهفی سواب ماننّه

بینه دیارکرکن.

 dup A, cag A, racA

و دیرگن‌ن داکو جینا 16 Sr RNA یو به‌کتریا زری دارگه‌ی و فاکته‌یین گگکر

نمه‌جِم

ترش‌نه‌خوش‌ی بین 16 سر RNA دیارکرکن دهم دوم (43.4%) نه‌خوش‌ان کو تینک توان فاکته‌یین گگکر

Sr RNA 16 مه‌پورون واین دن فاکته‌یین گگکر cag, dup A, cag A, racA

هاننامه دیارکرکن لجم تینک (20.2%) زکوما کونترول کو فاکته‌یین گگکر مه‌پورون د دیروکا کوما نه‌خوشان‌دا

dup A, cag A, racA

هامه دیارکرکن لجم دیروکا 24% (51.2%) هاننامه دیارکرکن دهم دوم ده‌ه مان دمدا بینن تینک کس (20.2%) یا

کوما کونترولا خدونا پاشناهکنی مالپانون بو به‌خوش‌یا نام‌سرا دهفی یا دووبرار.

دهرنه‌چاپ

مهم دیارکرک کو کتریا زری دارگه‌ی نایننامه نگاری یپ‌پایبویا نام‌سرا دهفی یا دووبراره، د همان دمدا بینن تینک‌لیا گونگ و یوز هدیه

ناماها نام‌سرا دهفی یا دووبرار و دیروکا مالپانون دا
الخلاصة

الكشف الجزيئي عن البكتيريا الملتوية البوابية وعوامل الضراوة vacA, cagA, dupA لدى مرضى التقرحات الفموية المتكررة في دهوك – أقليم كردستان - العراق

خلفية البحث

تعتبر التقرحات الفموية المتكررة من الحالات المرضية التي تصيب الأغشية المخاطية الفم. لاتزال العوامل المسببة للمرض غير واضحة. الدراسة الحالية تهدف إلى الكشف الجزيئي عن البكتيريا الملتوية البوابية وعوامل الضراوة vacA, cagA, dupA لدى مرضى التقرحات الفموية المتكررة والتحقق من مدى العلاقة بينهما.

المرضى وطريق البحث

تم تشخيص 46 فرداً من ذوي التقرحات الفموية المتكرر، 11 من الإناث و35 من الذكور وكذلك تم اختيار 46 من الأفراد الأصحاء كمجموعة سيطرة وتتكون من 23 إناث و23 من الذكور. أخذت مسحة من الفم لكل فرد من مجموعة الدراسة ومن تجويف الفم (الخد) من مجموعة السيطرة للكشف عن الحمض النووي للبكتيريا 16SrRNA الملوية المعدية وعوامل الضراوة vacA, cagA, dupA وللكشف عن عوامل الضراوة vacA, cagA, dupA.

النتائج

تم الكشف عن الحمض النووي للبكتيريا الملتوية المعدية في حالتين (4.34٪) من مجموعة cagA, dupA, واحدها احتوت على عوامل الضراوة vacA, cagA, dupA، وتم الكشف عن الحمض النووي للبكتيريا 16SrRNA في حالة واحدة فقط (2.17٪) من مجموعة السيطرة. تم العثور على خلفية عائلية في مرض التقرحات الفموية المتكررة في 24 (52.17٪) من مجموعة الدراسة، في حين أن شخص واحد فقط (2.17٪) من مجموعة السيطرة كان لديه خلفية عائلية لمرض التقرحات الفموية المتكرر.

الاستنتاجات

توصلنا إلى أنه لم يكن هناك تأثير للكبتيريا الملتوية المعدية في التقرحات الفموية المتكررة، في حين كانت هناك علاقة معنوية عالية بين التقرحات الفموية المتكررة والتاريخ العائلي للمرضى.