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A Study on the Association between H. Pylori and Recurrent Aphthous Ulceration using Q-PCR

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Abstract: ***Background & Objective: Recurrent aphthous ulceration (RAU) is a common disease with unknown aetiology. We studied the possible involvement of* H. pylori *in the development of RAU considering that both (RAU and peptic ulcer) are immunologically mediated ulcers and have similar histologic features, and* H. pylori *is a causative factor in peptic/gastric ulcers. This study aims to measure the prevalence of* H. pylori *in patients with and without RAU and to determine the association between* H. pylori *and RAU. Methods: 30 subjects with Aphthous Ulcers and 30 healthy volunteers were included in the present study. The Real-Time Polymerase chain reaction (Q-PCR) technique was used to detect the presence of* H. pylori*. Independent t-tests were used for statistical analysis. Results: In the current study all patient with RAU lesions and without the lesion shows the presence of* H. pylori *DNA. The mean value for the urea A gene in the case group was 261.37 (SD=85.46) and for the control group, it was 210.15 (SD=113.98). There was a high level of urea A gene in the case group when compared to the control group and the difference between the two groups was statistically significant. Conclusion: H. pylori can be present in the normal oral cavity also, and maintains a commensal relationship with the human host because of its presence in both the case (patient with RAU) and control group (intact oral mucosa). Since its level was increased in patients with RAU as compared with normal oral mucosa it suggests that* H. pylori *may be associated with the pathogenesis of aphthous ulcers.***

Keywords: Recurrent Aphthous Ulceration, *H. Pylori*, gastric ulcer, Q-PCR.

1. Introduction

Recurrent Aphthous Ulceration or Stomatitis (RAU/RAS) is the most common oral ulcerative disease, affecting as many as 25% of the population worldwide [1], yet the least understood. [2]The origin of the word "aphthous" is from the Greek word "*aphthi*" which means burning or to set on fire, or to inflame. This was first used by Hippocrates (460-370 BC) to represent the clinical symptom of the disease. [3] The first valid clinical description of RAU was given by Von Mikulicz and Kummel in 1888. [4]

Oral mucosa and all gastrointestinal mucosa are derived from the ectoderm. They present similar development and structure. *H. pylori* is one of the etiological factors for gastric or peptic ulcers.[5] This has been detected in gastric secretions, faeces, saliva and the dental plaque of healthy individuals and also in patients with upper GI disorders. [6] It has also been proposed to be one of the etiological factors in the pathogenesis of RAU. In up to 70.8% of cases, patients with RAU appear to suffer from active *H. pylori* infection. [7]

Considering the similarities of histological features between gastric ulcers and RAU, and latter lesions often respond to treatment with broad-spectrum antibiotics such as tetracycline, it is reasonable to assume that *H. pylori* might also be involved in the development of RAU. Thus, studies should explore whether it is also associated with an oral ulcer as both are immunologically mediated ulcers. There are almost seven diagnostic assays for *H. pylori*, but none of these tests accurately quantifies the number of *H. pylori* that are present in samples. Amplification of DNA sequences specific to *H. pylori* genes by PCR appears to be the most sensitive method of detection. [8]

Some articles suggest that *H. pylori have* a probable role in RAU development, although there is limited documentation of the colonization and probable role of *H. pylori* in RAU. The aim of this study is to determine probable *H. pylori* infection in RAU by Real-time PCR.

There are mainly 3 types of RAU based on the clinical features: Minor aphthae (Mikulicz's aphthae; MiRAS),Major aphthae (Sutton's aphthae; MaRAS) and Herpetiform aphthae (HeRAS). A/c Stanley 1972 RAU has three different variants: Minor, Major, and Herpetiform Aphthous Ulcers. Based on the size of the ulcer, as suggested by De Meyer et al. (1977), RAU is classified as Minor (<1 cm) and Major (>1 cm). [9]

Table 1: Clinical characteristic of recurrent aphthous stomatitis according to their classification

Types	Size (mm)	No.	Depth	Scar	Duration (days)	Age	Frequency (%)	Localization
MiRAS	5-10	<10	Shallow	No	10-14	2nd decade	75-90	Non- keratinized oral mucosa
MaRAS	>10	1-3	Deep	Yes	>14	1st & 2nd decade	10-15	Keratinized and non-keratinized oral mucosa
HeRAS	<5	>10	Shallow	No	10-14	3rd decade	5-10	Non- keratinized oral mucosa.

Up to now, the etiopathogenesis remains unclear. Evidence for a significant causative factor is still lacking and is considered multifactorial. The potential trigger factors include: genetic predisposition, food allergies, vitamin and microelement deficiencies, systemic diseases, increased oxidative stress, hormonal defects, mechanical injuries and anxiety [10]; however, evidence for a single causative factor is still unclear. Therefore, the treatment by current methods remains inadequate and often only palliative.

Among the potential factors some authors mention bacterial (*Streptococcus oralis, H. pylori)* and viral (HSV, VZV, CMV, and adenoviruses). The study's results; however, are ambiguous and conflicting. [11,12]

There is an important controversy on the significance of the presence of *H. pylori* in the oral cavity.*Krajden et al.* were able to isolate *H. pylori* from the dental plaque samples in 1 of 29 (3.4%) patients with *H. pylori* gastritis [13], and the isolate from the mouth was identical to that in the stomach [14].

Various diagnostic tests are available to detect *H. pylori* infection and are divided into invasive (histology, rapid urease test (RUT), and bacterial culture and non-invasive tests (serology, 13C-urea breath test (13C-UBT), and stool antigen test [15]. None of these techniques accurately quantifies the number of *H. pylori.*

The gold standard test in the stomach is culture; however, contrary to that, the oral cavity harbours many other faster-growing organisms that can grow in the culture [16]. Therefore, there are no "gold standard" tests to detect *H. pylori* in the oral cavity [17].

Some investigator believes that *H. pylori* belongs to the normal oral microbiota and maintains a commensal relationship with the human host. In contrast, other authors have suggested that *H. pylori* intermittently colonises oral cavities as a result of the ingestion of contaminated foods or as a secondary effect of gastro-oesophageal reflux. [18,19]

2. Methodology

This study was conducted on exfoliative cells collected from patients diagnosed clinically with RAU. Individuals with intact normal oral mucosa were taken as control. The study was undertaken on 60 subjects, divided into two groups (Group I and Group II, age 12-40 years) of 30 each.
Group I: Subjects with RAU

Group II: Healthy controls with Normal Oral mucosa (without the lesion) selected to act as a negative control group.
Endoscopic Gastric biopsy from gastric ulcer patient diagnosed positive for *H. pylori* by Rapid Urease Test (RUT) was taken as a positive control.

Ethical clearance was obtained from the study subjects. A written informed consent was obtained from the study subjects.

Exclusion criteria were cases with prior treatment for their current episode of RAU, patients with any mucosal lesion other than an aphthous ulcer, or who had any symptoms of chronic gastritis or peptic ulcers, or patients with a history of lip and cheek biting were excluded from the study.

At the time of the sample collection, the lesion had well-circumscribed margins and was surrounded by an erythematous halo. They varied in diameter from 2 to10 mm and the majority of the patients had 1 to 3 lesions during a single outbreak. The frequency of recurrence varied from 1-3 attacks per year to 1- 2 attacks per month. Samples were collected by swabbing the ulcer surfaces with a sterile *Microbrush* and were stored in a sterile Eppendorf tube containing 200µl of phosphate buffer saline (PBS) at PH- 7.4. The Eppendorf tubes containing the samples (swab tips and PBS) were then vortex mixed for 30 seconds and were stored at -20 oC until processing.

DNA extraction: The classical protocol for isolating DNA from a relatively large amount of tissue is the proteinase K phenol method but for isolating nucleic acid from a small amount of tissue, there are no established protocols.

To isolate H. *pylori* DNA from oral exfoliated cells four different DNA isolation techniques which included kits (Zymo kit, Gene all kit, Qiagen kit) and Phenol chloroform method were compared to determine which one produce a higher quality and yield of DNA from exfoliated cells. The extracted DNA was quantified by UV spectrophotometry at an Excitation wavelength -480 nm, Emission wavelength -530 nm using TECAN and MAGELLAN (Data Analysis Software).

A Difference in the amount of extracted DNA was found between the four different methods used. The best of four methods for obtaining a high yield and purity of H. *pylori* DNA has been through DNeasy Blood & Tissue Kit (QIAGEN). Thus, this method of isolation was used in our subsequent experiments to guarantee the accuracy and reliability of Q-PCR.

Primer designing: The genes or nucleic acid sequences targeted for amplification in previous studies were: cagE, UreA, Urease C, 23srRNA, and 26 kDa Helicobacter species-specific antigen (SSA), and Urease gene. We targeted for amplification a DNA sequence internal to the urea A gene, specific for H. *pylori.* Primers complimentary to this sequence have been demonstrated to have high specificity for detection of the *H. pylori.*

To design oligonucleotides specific for *H. pylori*, multiple sequences of the output regions were searched against GenBank sequences with the BLAST (Basic Local Alignment Search Tool) to find any sequence similarities and ensure the specificity of primers on National Centre for Biotechnology Information Website (NCBI) **(** https://www.ncbi.nlm.nih.gov/BLAST/) and analysed using Primer3 Input (version 0.4.0). Primers were synthesized commercially from Eurofins.

Primers Urea A F 5'-GGC TGA ATT GAT GCA AGA AG-3' and Urea A R 5'-GGT ATG CAC GGT TAC GAG TT-

3' targeting the Urea A gene of *H pylori* with Accession no. CP024072.1 and LC090364.1 were used.

Primers were received in a lyophilized i.e. freeze-dry state hence it was resuspended in molecular grade H2O according to the protocol and stored at -20oC.

The specificity of Q- PCR amplification of urea A gene was tested on gastric biopsy which was diagnosed for H. *pylori* Gastritis by endoscopy and Rapid Urease Test (RUT) by a gastroenterologist and further tested with conventional PCR and agarose gel electrophoresis which has shown positive result representing a 100- bp DNA ladder.

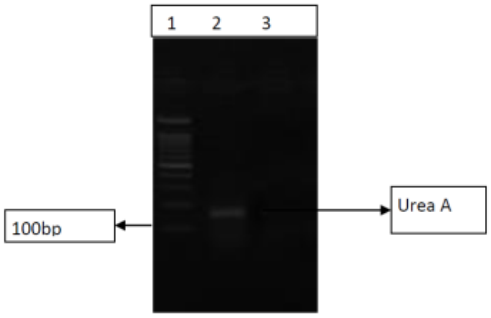

Figure 1: 100 - bp DNA ladder on gel electrophoresis. Lane 1: represents a 100- bp DNA ladder. Lane 2: H. *pylori* genomic DNA from gastric ulcer patient. Lane 3: negative control

Real time PCR for samples: Q-PCR was carried out in a final volume of 20 µl reaction mixture, containing 1 µl of DNA, 10 µL of Red Taq Master Mix 2x, 1µM of each complementary primer specific for urea A. Each amplification reaction cycle consisted of 95 OC for 15min for denaturation when using genomic DNA, annealing at 95 OC for 10 seconds and extension at 49 OC for 20 sec. Samples were amplified for 35 consecutive cycles. The relative quantity of Urea A gene copies in test samples was estimated based on the Ct value of positive control samples. Quantitative results were expressed by the determination of the threshold of detection or the crossing point (Cp) which marked the cycle when the value of the fluorescence of a given sample significantly exceeded the baseline signal. Each sample was tested in duplicate (cycle run twice) and the final Cp was the mean of the two results. Then the Cp was plotted against the known concentration of bacterium to obtain the standard curve.

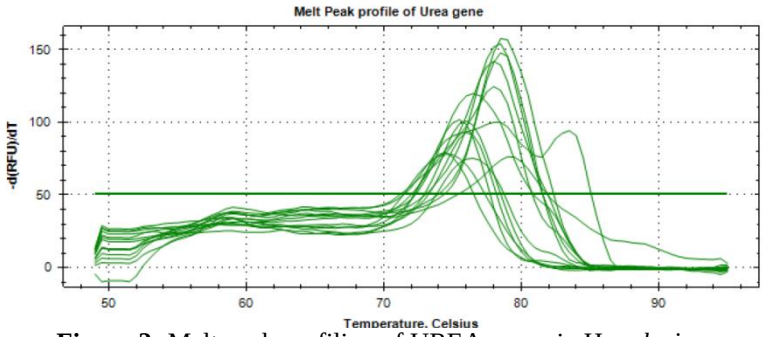

Figure 2: Melt peak profiling of UREA genes in H. *pylori*

Statistical Analysis:
Data collected during survey was entered in excel sheet and was subjected to statistical analyses**.** The data were analysed using the statistical package SPSS version 25.0. Descriptive statistics: Mean, standard deviation and independent t-test were used to analyse the data. P < 0.05 was considered as level of significance.

3. Results and Discussions

The mean age in the present study was 24.7±6.914 for the case group and 22.9±7.563 for the control group. The difference between the groups was not statistically significant. (p=0.34).

Out of 60 study participants, 26(43.3%) were males and 34(56.6%) were females. In the case group, 12(40.0%) were males and 18(60.0%) were females. In the control group, 14(46.6%) were males and 16(53.3%) were females. The difference between the groups was not statistically significant. (p=0.60)

Out of 30 study participants, 22 (73.3%) had minor and 8 (26.6%) had a major aphthous ulcer.

The mean value for the urea A gene was 261.37 (SD=85.46) in the case and 210.15 (SD=113.98) for the control group. All patients with and without the lesion showed the presence of H. *pylori* DNA but there was a high level of urea A gene in the case group when compared to the control group and the difference between the two groups was statistically significant (P< 0.05).

Such a result demonstrates that because of its presence in both the case group (patient with RAU) and control group (intact oral mucosa) H. *pylori* can be present in the normal oral cavity also and maintains a commensal relationship with a human host. Since its level was increased in patients with RAU as compared with the normal oral mucosa, it suggests that H. *pylori* may be associated with the pathogenesis of an aphthous ulcer.

Table 1: Comparison of relative mean quantification value of (urea A) gene in samples

Group	Cycle -1 Mean (SD)	Cycle -2 Mean (SD)
Case (RAU)	52.16(17.26)	261.37(85.46)
Control (Intact mucosa)	40.49(19.14)	210.15(113.98)

Table 2: Comparison of mean quantitative value of (Urea A) gene copy number in H. *pylori* between case and control group for cycle -1

Relative quantity of (urea A) gene (cycle-1)	Mean	t-value	P-value
Case (RAU)	52.16±17.26	-2.486	0.01*
Control (Intact mucosa)	40.49±19.14		

t denotes t test value *statistically significant p<0.05

Table 3: Comparison of mean quantitative value of (Urea A) gene copy number in H. pylori between case and control group for cycle-2

Relative quantity of (urea A) gene (cycle-2)	Mean	t-value	P-value
Case (RAU)	261.37±85.46	-1.969	0.05*
Control (Intact mucosa)	210.15±113.98		

t denotes t test value *statistically significant p<0.05

Inconsistent differences in the frequency of H. *pylori* in the oral cavity are found in the literature. These differences may be a consequence of variations in the demographics of subjects, oral health status, *H. pylori* infection status, type and number of clinical samples, complexity of the oral microbiota and methods of detection.[20]

Methodological differences such as variation in collection and in bacterial density of samples, method employed for DNA extraction, primers and PCR reaction may partly explain such disparity. One could speculate about the possibility of amplification of another species of Helicobacter- like organism found in oral cavity such as *Campylobacter rectus* and *C. curvus.[21]*

The other problem is that because H. *pylori* gene can be detected using PCR, it is not clear whether the gene found belongs to live bacteria or not. It detects the DNA of bacteria that are also not viable and also detects small numbers of bacteria that may not have a significant impact on oral cavity infections. PCR assays for *H. pylori* have a wide cross-reactivity and are positive when other microorganisms contain those sequences. [22]

The PCR assay described in the present work has been shown to be very sensitive and specific because of the use the primer used which is 100 % specific for *H. pylori*. A Gene Bank search (BLAST) indicated that this sequence of DNA had no homology or only negligible homology with other genes which decreases the possibility of unspecific amplification. Q-PCR technique was fast and PCR for detection could be performed in 2-3 hours, including DNA extraction.[23]

4. Conclusion and Future Scope

In the current study, all patients with RAU and without the lesion showed the presence of H. *pylori* DNA but there was a high level of urea A gene in the case group when compared to the control group and the difference between the two groups was statistically significant.

Since its level was increased in patients with RAU as compared with normal oral mucosa it suggests that *H. pylori* may be associated with the pathogenesis of aphthous ulcers.

Two mechanisms can be involved in the formation of aphthous ulcers by H. *Pylori: -*

1) It can act as an opportunistic organism and may lead to the formation of an aphthous ulcer.
2) The aphthous ulcer can be formed due to a decreased immune response such as in case of stress or malnutrition which can be secondarily infected by H. *Pylori*.

In conclusion, the present study gives support to the assumption that *H. pylori* could be involved in RAU development.

The issue of whether *H. pylori* is a permanent or transient member of oral microflora will need to be further investigated. Further studies with a larger sample population would be warranted.

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