Relationship between Herpes simplex Virus Type-1 and Candida albicans in Pregnant Women with Aphthous Stomatitis in the oral cavity

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ABSTRACT
Background: This study was aimed to show the relationship between Herpes simplex virus type-1 (HSV-1) and Candida albicans, and to determine HSV-1 antigen in saliva of aphthous stomatitis patients by immunofluorescent (IF), as well as to determine HSV-1 antibodies immunoglobulin G (IgG) in saliva samples by enzyme linked immunosorbent assay (ELISA) test, in addition to study concentration of the total salivary protein.

Materials and methods: Sixty pregnant women with aphthae and thirty healthy control subjects were included in the study. Saliva samples were taken from all the subjects and examined by direct IF and ELISA, and using the saliva swab samples for isolation of Candida albicans. The isolated colonies were identified by germ tube formation, growth at 45°C, and measuring the total salivary protein.

Results: The results of the present study showed that aphthous were most prevalent at age group (26-30) years. The most commonly affected mucosa are the labial mucosa, buccal mucosa, tongue, and rarely the soft palate and floor of the mouth. Positive IF results were observed in 53.33% in patients and 6.67% in healthy control, while in ELISA, the positive results were found to be 63.33% in patients and 6.67% in control subjects. There was significant difference between anti HSV-1 IgG Abs and total salivary protein.

Conclusion: The present study showed there was no difference between Candida albicans and HSV-1. The present study indicated that HSV-1 was detected serologically in saliva of patients by ELISA and IF method. The results revealed positive association between HSV-1 and aphthous, and the virus may play a role in the occurrence of the aphthous. Saliva is regarded as a transudate of the serum and it contains the same antibodies as serum, and a similar range of IgG antibodies, but at a much lower concentration and it’s easily available and simply examined.


INTRODUCTION
Viruses are the smallest infectious microorganisms that can be observed by electron microscopy. They are composed of lipid envelope, protein coating and inner core of either RNA or DNA. They depend in their own reproduction on hosts’ cells (1). Herpes viruses comprise the largest family of viruses with oral manifestations. Eight types of herpes viruses are known to be pathogenic in human, with varying significance relative to oral diseases. HSV-1 is the most common virus cause oral and perioral viral infection (2). Herpes simplex virus is transmitted during close contact with an infected person who is shedding virus from the skin, saliva or secretions from the genitals (3). This horizontal transmission of the virus is more likely to occur when sores are present, although viral shedding, and therefore transmission, does occur in the absence of visible sores (4). In addition, vertical transmission of HSV may occurs between mother and child during childbirth, which can be fatal to the infant (5).

Herpes simplex virus -1 is a double stranded DNA (dsDNA) enveloped virus, the virion (virus particle) has 4 basic structures- the envelope, tegument, nucleocapsid, and a DNA-containing core (6). It is incurable and persisted during the lifetime of the host, often in latent form. Primarily infect mucosal surfaces following exposure to infected secretions, it causes a range of diseases from labialis and stomatitis to blinding keratitis and rarely encephalitis (7). Their clinical manifestations are variable and influenced by the portal of viral entry, degree of host immune competence as well as primary or secondary nature of the disease (8). Aphthous Stomatitis caused by HSV-1 is a common disease that causes the appearance of recurrent aphthous stomatitis in the oral mucosa, the incidence is of about 20% in the world population, and the disease is more frequent in females than males. Despite the high incidence many studies dedicated to unveiling its causes, it still is very controversial regarding etiology (9). This disease is currently defined as a disease characterized by aphtha lesions in the oral mucosa, in a recurrent fashion (every fortnight or monthly), for a minimum period of one year, and its onset is usually without evidences of associated systemic diseases (10). Incidence of aphthous stomatitis is greater in the second

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decade of life \(^{(11,12)}\). The aphthous stomatitis also caused by other pathogenic microbes including Candida, many species of Candida cause aphthous but *Candida albicans* is generally more virulent than any other species \(^{(13)}\). There are several putative virulence factors of *Candida albicans*, including the ability to form germ tubes\(^{(14)}\). Adherence to host cells, and secretion of proteinases \(^{(15-17)}\). The ability to make the transition from budding to hyphal growth is essential for virulence \(^{(18,19,20)}\). And the first stage in this transition is the formation of a germ tube \(^{(14)}\). The ulcerations are not infected with the virus. In these rare cases, HSV-1 may be responsible for the initiation of the autoimmune destruction; conversely, the immune dysregulation that produces aphthae may have allowed the release of the virion \(^{(21)}\). Saliva samples for antibodies detection is being considered to be able to substitute for serum in clinical diagnosis or screening purpose since salivaid regarded as a transudate of plasma, containing a similar range of antibodies \(^{(22)}\).

MATERIAL AND METHODS

Ninety Iraqi pregnant women were included in this study in the consultant clinic of Gynecology in medical city , and the working in teaching laboratories during the period from beginning of November 2010 till the end of April 2011. The measurements included two groups:

1. **Patients group**: are sixty Pregnant women; age group range (15- 45 years) suffered from ulcers which were diagnosed clinically by dentist as aphthous stomatitis. The patients were subjected to a questionnaire on the disease manifestations, family history and clinical features, as shown in the . All the cases had received no treatment with no complain history of chronic or systemic diseases. The ulcers of all the sixty patients were of major aphthous stomatitis.

2. **Healthy group**: are thirty Pregnant women age group range from (15-45 years), without any history of oral mucosal lesions, and considered as control group.

**Saliva collection and preparation**

Five ml of whole unstimulated saliva were collected using disposable test tubes. Subjects were asked to refrain from eating, drinking, chewing and smoking one hour prior to collection of saliva. Saliva then centrifuged at 1500 rpm for 10 minutes; this was done within 1 hour after collection to eliminate debris and cellular matter, the supernatants were aspirated immediately into three pre labeled endproof tube and stored frozen at (-20 °C.) until assayed.

**Immunofluorescence for detection of HSV-1 Ag:**

ZEUS herpes simplex virus -1 IgG ( USA-Products Series: 9051) was used for detection of HSV-1 Ag by direct immunofluorescence assay according to manufacturer's protocol in the leaflet of the kit.

**ELISA method for the detection of HSV-1 Ab**

Enzyme immunoassay for the detection IgG antibodies to herpes simplex virus -1 by using HSV-1 IgG enzyme immunoassay, and its procedure as in the leaflet of the kit.

**Laboratory identification of Candida albicans**

**Specimen collection:** Specimen collection was done by using cotton tipped swab. The swab was taken from precipitation of saliva and inoculated directly on Sabouraud’s dextrose agar. The inoculated culture plates were incubated at 37°C for 72 hours and then kept at 4°C for further investigation.

**Microbiological identification:** Each swab was streaked onto the surface of Sabouraud’s dextrose agar, then incubated at 37°C for 48hrs. The isolated colonies were identified by:

**Colony morphology:** Colonies of *Candida albicans* appear smooth, creamy in color and typically medium sized 1.5 - 2 mm diameter which later develop into high convex, off - white colonies after two days \(^{(23)}\).

**Microscopic examination:** The slide was examined under light microscope, the rounded or oval yeast cells were stained Gram positive (stain violet).

**Germ tube formation:** Germ tube are filamentous out growth that arise from blastospores of *Candida albicans*, this was carried through lightly touching one representative colony with sterile platinum loop then it was suspended and incubated in one ml of human serum at 37°C for 2 hours after which it was examined under the light microscope to identify germ tube production of *Candida albicans* \(^{(24)}\).

**The growth at 37°C and 45°C:** This was done by using cotton tipped swab. The swab was taken precipitation of saliva and inoculated directly on Sabouraud’s dextrose agar (two plates). The inoculated culture plates were incubated at 37°C and 45°C for 24 hr after which it was examined to identify The growth in both degrees.

RESULTS

**Age**

Table 1 shows the distribution of study group by age for both patients group suffered from aphthous stomatitis and the control group without any history of oral mucosal lesions. As seen in the table the patients group and the healthy control
group were divided into six groups according to the age.

**Family history**

The distribution of study group according to the family history is seen in table 2. Concerning patients group, the positive family history recorded was higher than negative family history with statistically highly statistically significant difference (P<0.01). While the control group, showed the positive family history lower than negative family history with highly statistically significant difference (P<0.01).

**Type and Location of major ulcer**

The ulcers of all the sixty patients were of major aphthous stomatitis were diagnosis by dentist. The distribution of sample according to the location of ulcer showed that the labial mucosa was the most common sites (33.33%) followed by buccal mucosa (26.67%) then buccal vestibule (21.67%) then the tongue (8.33) then soft palate (5%), while the less common sites were the floor of the mouth (1.67%) as seen in table 3.

**The age of aphthous stomatitis onset and number of ulcers**

Regarding the age of aphthous stomatitis onset, all the patients group had the ulcer after the age of ten years, while for the number of ulcers, all the patients got one or two ulcers per episode.

**ELISA results**

Data of this study showing that the ELISA results (by using HSV-1 IgG enzyme immunoassay kit) As shown in table 4 the mean of anti HSV-1 IgG Abs in aphthous stomatitis patients was (0.329 Hu/ml) which is higher than mean in healthy control group (0.128 Hu/ml).The results showed statistically significant differences.

**Immunofluorescent results**

Results of direct immunofluorescent using the ZEUS herpes simplex virus-1 for detection of viral Antigen are summarized in table 5. Thirty two (53.33%) patients with aphthous stomatitis revealed positive reaction. While twenty eight (46.67%) patients revealed a negative reaction. The direct immunofluorescent method showed cells producing HSV-1 specific antigen, which was identified by the cytoplasmic apple-green fluorescence which is considered as a positive results figure 1. These were taken from patients having herpes labialis, while no specific fluorescence it will be considered as a negative results figure 2. Regarding control group two cases(6.67%) showed positive reaction as in figure 3, while twenty eight (93.33%) cases show a negative reaction figure 4. These results showed a statistically significant differences.

**Candida albicans isolation**

*Candida albicans* were isolated from the saliva samples of 60 patients on Sabouraud’s dextrose agar as following:

**Colony morphology:** Colonies of *Candida albicans* appear smooth, creamy in color and typically medium sized 1.5 - 2 mm diameter which later develop into high convex, off - white larger colonies after two days.

**Microscopic examination:** The slide was examined under light microscope the rounded or oval yeast cells were stained Gram positive (stain violet).

**Germ tube formation:** Germ tube are filamentous outgrowth that arise from blastospores of *Candida albicans*, this was carried through lightly touching one representative colony with a loop then was suspended and incubated in one ml of human serum at 37°C for 2 hours after which it was examined under the light microscope to identify germ tube production of *Candida albicans*.

**The growth at 37°C and 45°C**

All tested isolates showed growth at 37°C while only *Candida albicans* isolates grow at 45 °C (25) Thus, the test used to differentiate *Candida albicans* and *Candida dubliniensis* because *Candida dubliniensis* loss its ability to grow at 45 °C while *Candida albicans* grow due to its ability to form germ tube formation.

**Relation between HSV-1 Abs and Candida albicans**

In this study, there was no difference between positive and negative results of *Candida albicans* and anti HSV-1 IgG Abs in aphthous stomatitis patients (mean = 0.343 ± 0.022, mean = 0.329 ± 0.029).table 6.

![Figure 1: Positive Immunofluorescent picture in patient with aphthous stomatits showing large cells with ballooning degeneration.](image-url)
Relationship between Basic Sciences

**DISCUSSION**

**Age**

Sixty pregnant women with aphthous stomatitis were included in this study. The age of the patients was ranged between 15-45 years. The high incidence of recurrent aphthous stomatitis was noticed among age of 26-30 years which can be due to that they were under stress (the main etiological factor for recurrent aphthous stomatitis). The design of this study to deal with pregnancy women are due to the fast that female was more affected by recurrent aphthous stomatitis than male Ali (26), Al-Hijazi (28), Al-alttas (30).

**Family history**

It had been found in this study that 53.33% of RAS patients had family history of aphthae, in which familial aggregation and concordant disease support the involvement of genetic factors in the pathogenesis of the disease.

**Type and location of ulcers**

The major aphthous stomatitis was the most predominant type in this study. Regarding the location of the ulcer, the study showed that labial mucosa (33.33%) was the most common site for aphthous stomatitis. This could be explained on the basis that these sites were movable structures and mostly affected by trauma which was the most precipitating factor in developing the aphthous stomatitis. This result confirmed the observation of (Al-Hijazi (28), Ship and Arbor (29).

**The age of aphthous stomatitis onset and number of ulcers**

Most of the patients in the present study showed that the onset after ten year of aphthous stomatitis onset was after the age of ten year and were numbered from one or two ulcers and this finding confirmed the results observed by (Ali (26), Al-Hijazi (28), Al-alttas (30).

**ELISA**

The finding analyzed a positive antibody IgG against HSV-1 in the studied groups was detected in (63.33%) patients with RAS, while in control group it appears positive in (6.67%). ELISA results revealed that the mean values of anti HSV-1 IgG in patients with RAS was (0.329 Hu/ml) in comparison to among healthy control group (0.128 Hu/ml). This may refer to direct relationship between increased level of anti HSV-1 IgG in saliva of RAS patients and prevalence of the disease.

**Immunofluorescent**

The finding of Immunofluorescent results in this study showed a positive result in 32 out of 60 patients (63.33%) with aphthous stomatitis, in which the cells swell to a large size leading to ballooning degeneration and each field consist of many cells more than 10 cells as shown in figure (1), while in control subjects showed positive in 2 out of 30 healthy individuals (6.67%), in which the cells appear smaller in size and their number less than 10 per field as shown in figure (3). The positive results in control subjects could be attributed to the shedding of HSV-1 in the oral cavity which tends to be frequent and episodic.

**Relation between of HSV-1 and Candida albicans**

In this study, there was no difference between positive and negative results of Candida albicans and anti HSV-1 IgG Abs in aphthous stomatitis patients. The increasing of percentage of Candida albicans in patient with HSV-1 may be due to the ability of HSV-1 to suppress of the body immune system lead to HSV-1 infection. Which leads to growth and then changing of non pathogenic Candida albicans to become pathogenic (Rinallo and tropy (31).
REFERENCES


Table 1: Age Distribution of patients and control healthy group.

<table>
<thead>
<tr>
<th>Age group (year)</th>
<th>Patients group</th>
<th>Control group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No.</td>
<td>%</td>
</tr>
<tr>
<td>15-20</td>
<td>5</td>
<td>8.33</td>
</tr>
<tr>
<td>21-25</td>
<td>16</td>
<td>26.67</td>
</tr>
<tr>
<td>26-30</td>
<td>18</td>
<td>30.0</td>
</tr>
<tr>
<td>31-35</td>
<td>11</td>
<td>18.33</td>
</tr>
<tr>
<td>36-40</td>
<td>9</td>
<td>15.0</td>
</tr>
<tr>
<td>&gt;40</td>
<td>1</td>
<td>1.67</td>
</tr>
<tr>
<td>Total</td>
<td>60</td>
<td>100%</td>
</tr>
<tr>
<td>χ² value</td>
<td>--</td>
<td>6.03 **</td>
</tr>
</tbody>
</table>

** (P<0.01).
Table 2: Distribution of study groups according to family history

<table>
<thead>
<tr>
<th>Subject</th>
<th>Positive family</th>
<th>Negative family</th>
<th>Total</th>
<th>( \chi^2 ) value</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No.</td>
<td>%</td>
<td>No.</td>
<td>%</td>
<td></td>
</tr>
<tr>
<td>Patients</td>
<td>32</td>
<td>53.33</td>
<td>28</td>
<td>46.67</td>
<td>60</td>
</tr>
<tr>
<td>Control</td>
<td>2</td>
<td>6.67</td>
<td>28</td>
<td>93.33</td>
<td>30</td>
</tr>
<tr>
<td>( \chi^2 ) value</td>
<td>--</td>
<td>9.48**</td>
<td>--</td>
<td>11.63**</td>
<td>--</td>
</tr>
</tbody>
</table>

* (P<0.05), ** (P<0.01).

Table 3: Distribution of ulcer according to their location among patients group.

<table>
<thead>
<tr>
<th>Location</th>
<th>Patients</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No.</td>
</tr>
<tr>
<td>Tongue</td>
<td>5</td>
</tr>
<tr>
<td>Buccal mucosa</td>
<td>16</td>
</tr>
<tr>
<td>Labial mucosa</td>
<td>20</td>
</tr>
<tr>
<td>Buccal vestibule</td>
<td>13</td>
</tr>
<tr>
<td>Labial vestibule</td>
<td>2</td>
</tr>
<tr>
<td>Soft palate</td>
<td>3</td>
</tr>
<tr>
<td>Floor of the mouth</td>
<td>1</td>
</tr>
<tr>
<td>Total</td>
<td>60</td>
</tr>
</tbody>
</table>

\( \chi^2 \) value: -- 6.49 **

** (P<0.01).

Table 4: Difference in mean of anti HSV-1 IgG Ab among study groups

<table>
<thead>
<tr>
<th>Subject</th>
<th>No.</th>
<th>Mean ± SD of total protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patients</td>
<td>38</td>
<td>0.329 ± 0.18</td>
</tr>
<tr>
<td>Control</td>
<td>30</td>
<td>0.128 ± 0.13</td>
</tr>
<tr>
<td>LSD value</td>
<td>-</td>
<td>0.051 *</td>
</tr>
</tbody>
</table>

* (P<0.05).

Table 5: Distribution of study groups in relation to result of IF.

<table>
<thead>
<tr>
<th>Subject</th>
<th>Positive IF</th>
<th>Negative IF</th>
<th>Total</th>
<th>( \chi^2 ) value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No.</td>
<td>%</td>
<td>No.</td>
<td>%</td>
</tr>
<tr>
<td>Patients</td>
<td>32</td>
<td>53.33</td>
<td>28</td>
<td>46.67</td>
</tr>
<tr>
<td>Control</td>
<td>2</td>
<td>6.67</td>
<td>28</td>
<td>93.33</td>
</tr>
<tr>
<td>( \chi^2 ) value</td>
<td>--</td>
<td>9.48**</td>
<td>--</td>
<td>11.63**</td>
</tr>
</tbody>
</table>

* (P<0.05), ** (P<0.01).

Table 6: Comparison between positive and negative of Candida albicans of patients according to ELISA results (mean ± SD).

<table>
<thead>
<tr>
<th>Subject</th>
<th>No.</th>
<th>Mean ± SD of ELISA results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
<td>28</td>
<td>0.343 ± 1.164</td>
</tr>
<tr>
<td>Negative</td>
<td>10</td>
<td>0.329 ± 9.169</td>
</tr>
</tbody>
</table>
| LSD value | -  | NS: The difference is non-significant.