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Comparison between HSV-1 Ag detection techniques by ELISA and real-time PCR in breast cancer patients suffering from periodontitis

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Saif S. Jasim* **MSc**
Ghada I. Taha* **BSc, PhD**

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Abstract

Background: Periodontitis is a long-standing infection that destroys the gums, periodontal ligaments, and the alveolar bone that supports the teeth. Inflammation of the gums and chronic periodontitis are both caused by the bacteria in the dental plaque and the herpes viruses, especially types 1 and 2 of the herpes simplex virus.

Objectives: To compare the ELISA and real-time PCR as ways to detect the herpes simplex virus in breast cancer patients with periodontitis who are receiving chemotherapy.

Cases and methods: In this case-control study, a total of 90 subjects (30 with periodontitis with breast cancer after receiving chemotherapy, 30 with periodontitis (without chemotherapy treatment), and 30 cases which do not have periodontitis (healthy gingiva) and without breast cancer as a healthy control group. The mean ±SD age was (51.06±8.521). Enzyme-linked immunosorbent assay (ELISA) kits were used for the analysis and Real-Time PCR to detection of Herpes simplex virus-1 in saliva samples.

Results: In the present study, the incidence of HSV-1 Ag in patients having periodontitis and receiving chemotherapy was high but not significantly so compared with those having periodontitis but without chemotherapy and the healthy control group. In contrast, the result of Real-time PCR was positive (13.3%) and (10.0%), which proves the presence of HSV in most patients with periodontitis without chemotherapy and with periodontitis receiving chemotherapy but was not significant ($p>0.05$).

Conclusion: Real-time PCR was able to back up ELISA's findings in this study. Since ELISA is more cost-effective than real-time PCR, it is advised that the first screening may be performed using ELISA, and then positive samples might be investigated with real-time PCR to confirm the presence of HSV-DNA

Keywords: Periodontitis, HSV-1, real-time PCR, ELISA, breast cancer, chemotherapy.

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Introduction:

Periodontal disease is a chronic dental illness characterized by inflammatory conditions affecting the dentition's supporting tissues. Predominantly aerobic gram-negative bacteria on the tooth surface as microbial biofilms and other microbial substances gain access to the gingival tissue and initiate an inflammatory response, which destroys the periodontal ligament and alveolar bone leading to tooth loss (1).

Periodontitis is caused by a multifactorial immune response to periodontopathic bacteria. Oral microbiota plays a major part in the etiology of numerous inflammatory periodontal diseases. However, bacterial etiologies and disease types vary. Chronic periodontitis is the most common type (2). Periodontitis is caused by periodontal infections, vulnerable hosts, and a lack of helpful bacterial species (3). When prone to periodontitis, the major host defense system is dominated by the microbial assault, biofilms expand laterally and apically, the junctional epithelium becomes an ulcerated, highly porous pocket epithelium, and inflammation intensifies. Periodontitis has high levels of pro-inflammatory cytokines, prostaglandins, and matrix metalloproteinases and low levels of anti-inflammatory and tissue inhibitors, while periodontal health has the opposite (4). Herpes viruses cause the second most common diseases after flu and cold viruses. They may cause sickness immediately or wait and induce shingles (5). Herpes simplex viruses type 1 are in supra- and subgingival plaque. HSV-1 causes most oral herpes. HSV-1 may be vertically passed from mother-to-baby before or after delivery, but most children catch it via saliva contact with an infected person. HSV-1, EBV, and CMV are associated with extremely frequent periodontal lesions, directly infect

***Corresponding Author:** *Dept. of Basic Science in Oral Microbiology, College of Dentistry, University of Baghdad.**saifsalahbarca2016@gmail.com
ghada_ibraheem@codental.uobaghdad.edu.iq

gingival epithelial cells, and are strongly connected with disease severity (6).

Cancer and its therapy disrupt immunological function. Leukocyte depletion, dysregulated inflammation, decreased pathogen identification, and graft-versus-host responses make cancer patients very susceptible to lower respiratory tract infections. Cancer patients have functional and anatomical abnormalities. Recurrent healthcare visits in cancer patients increase nosocomial and drug-resistant pathogen exposure (7).

Breast cancer (BC) is a complex disease with a multifactorial etiology. Both the innate and acquired arms of the immune system are thought to be important in the anti-tumor response, and the interaction between host immune system and tumor cells has been studied for decades (8, 9)

Chronic periodontal disease is common among postmenopausal breast cancer survivors. Periodontal disease may increase breast cancer risk (10). Immune system cells and inflammatory mediators affect the tumor micro-environment (11). Chemotherapy remains the principal cancer treatment (12). Chemotherapeutics target cancer cells and treats metastatic cancer, unlike surgery and radiation. Modern chemotherapeutic medications are cytostatic or cytotoxic to slow or destroy cancer cells, but they also affect fast-growing normal cells. HSV-1 is more common in periodontal pocket lesions than HSV-2 (13). Since asymptomatic HSV seropositive people may isolate HSV from their saliva, saliva's involvement in intra-oral herpes simplex virus (HSV) management is contentious. It worsens lesions in many trials. HSV-1 infection lowers immunological regulators and causes inflammation, which may worsen periodontal disease (14). Surgery, radiation, and chemotherapy treat cancer. Chemotherapy is used to treat hematological malignancies, lymphoproliferative diseases, and solid tumors as adjuvant or neoadjuvant. Unlike surgery, chemotherapy is severe and cyclic, causing nausea, vomiting, and diarrhea, with extended treatment, hospitalizations, and chemotherapeutic adverse effects (15). Chemotherapy greatly boosts HSV-1 detection (16). Chemotherapy requires robust innate and adaptive immune systems, activates effector T cells, and inhibits immunosuppressing cells. Antibodies may indicate a past or latent HSV infection, making antibody detection unreliable. Recently, researchers utilized PCR. Restriction fragment length polymorphism (RFLP) analysis of HSV PCR amplicons can type the virus and distinguish HSV-1 from HSV-2 (17). Molecular strain-typing polymorphisms are determined using public database sequence information and an appropriate restriction endonuclease site. This can detect change at one nucleotide (16,17). This research also compared ELISA and real-time PCR methods for measuring herpes simplex virus type I in breast cancer patients with periodontitis after chemotherapy.

Cases and methods:

A total of 90 females were included in the study and divided into three groups. The first group includes 30 cases diagnosed as periodontitis with breast cancer after receiving chemotherapy (all of these 30 cases received the same treatment and were at the same stage of chemotherapy). The second group includes 30 cases of periodontitis (without chemotherapeutic treatment). The third group includes 30 healthy controls who are without periodontitis (healthy gingiva)

Ethical approval: The research was carried out in accordance with the principles outlined in the Declaration of Helsinki, and was approved by the college's in-house ethics committee of Dentistry, University of Baghdad. This clinical study was a prospective study, which collected only the participants' clinical data, and did not interfere with the participants' treatment plans. Therefore, this study did not pose physical risks to the participants. Participants' information was protected from leakage. The application for the exemption of informed consent was submitted, and approved.

Inclusion Criteria: Ages of patients between (40-75) years, patients with breast cancer, only women, patients undergoing chemotherapy, and at least 20 teeth in the oral cavity.

Exclusion Criteria: Previous periodontal therapy for the last 6 months and pregnant women.

Sample collection: Unstimulated whole-saliva samples were collected from all participants before the clinical periodontal examination between 9 AM and 11 AM, at least one hour after the last meal. The participants had not brushed their teeth for 45 minutes prior to the sample collection. Before collecting the sample, all participants rinsed their mouths, and the sample was collected using the standard spitting method after 1-3 minutes, The sample was stored in a cooling box, then HSV-1 was measured by ELISA Technique and Real-Time PCR.

Enzyme-Linked Immunosorbent Assay (ELISA): Detection of herpes virus (HSV-1) by using Human HSV-1 IgG enzyme immunoassay kit in saliva samples.

Principle of the Procedure: Diluted samples are incubated with HSV-1 antigens bound to the solid surface of a microtiter well. If IgG antibodies against HSV are present in the samples, they will bind to the antigen forming antigen-antibody complexes. The residual sample is eliminated by aspirating and washing. Conjugate (horseradish peroxidase-labeled anti-human IgG) is added and will bind to these complexes. Unbound conjugate is removed by aspiration and washing. The substrate is then added

and incubated. In the presence of the bound enzyme, the substrate is converted to an end product. The absorbance of this end product can be read spectrophotometrically at 450 nm (reference 600-630 nm) and is directly proportional to the concentration of IgG antibodies to HSV-1 antigen present in the saliva sample.

Detection of HSV by real-time PCR: (REALQUALITY RS-HSV 1): The REALQUALITY RS-HSV-1 kit is an IVD device for detection and quantification of the DNA of Herpes simplex virus type 1 (HSV 1). For quantification of the viral DNA present in the sample, this device has to be used in combination with **REALQUALITY RQ-HSV 1 STANDARD, code RQ-06-SM.**

The test is based on Real-Time PCR on DNA extracted from human clinical samples. This in vitro diagnostic test for the detection and quantification of HSV 1 is an auxiliary device for diagnosis and monitoring of HSV 1 infections.

The kit includes a ready-to-use PCR master mix that contains all reagents needed for the reaction as well as the component(s) listed below:

1. ROX™: An inert colorant that exhibits stable fluorescent properties throughout all amplification cycles. On some Real-Time PCR instruments (Applied Biosystems, Stratagene, etc.) it is used for normalization, in order to compensate for differences between wells due to pipetting errors or limitations of the instrument;

2. dUTP/UNG system: A system that prevents contamination from previous amplification runs. The dUTPs are used to incorporate uracil residues into the amplification product during the amplification session. At the beginning of each new run, the UNG enzyme degrades any single or double-stranded DNA containing uracil. This way any amplification products from former sessions are eliminated.

3. Internal control consisting of a recombinant DNA fragment of the β-globin gene (BG) was used to analyze acellular samples. It allows verification of the extraction procedure and detection of PCR inhibition. This internal control was standardized for 10 µL of control to be added to the sample before the extraction and eluting of the DNA extract in a volume of 60 µL.

Statistical analysis: The data was analyzed using (SPSS) version 25. Mean±SD was calculated for quantitative variables, while frequencies and percentages were calculated for qualitative data. The Chi-square test was used to test for association between variables and ANOVA was used to compare group means.

Results:

Table 1 shows that the age distribution of the two periodontitis groups was comparable with those 50 years old or less were 40% or less of the group, while this age group constituted more than 80% of the control group. This was also clear when the mean±SD was calculated for each group. The chi-square indicates a highly significant association between age and periodontitis (P<0.05).

The table also reveals a significant difference (p<0.05) concerning the number of teeth, between the three study groups, with comparable means for the two periodontitis groups (24.60±0.411) and (24.73±0.521) respectively and the control group (26.97±0.309).

Table 1: Age distribution and number of teeth of the three study groups

Variable	Study Groups			P value
	Periodontitis without chemotherapy No. (%)	Periodontitis with chemotherapy No. (%)	No Periodontitis, No chemotherapy (Controls) No. (%)	
Age group (year)				
<=50	11 (36.7)	12 (40.0)	25 (83.3)	<0.0001
> 50	19 (63.3)	18 (60.0)	5 (16.7)	
Total	30	30	30	
Mean ±SD	54.03±8.075	53.83±9.281	45.30±4.481	
Number of teeth				
Mean±SE	24.60±0.411	24.73±0.521	26.97±0.309	0.0001

Table 2 shows that the mean±SE of HSV-1 Ag in the saliva of patients with periodontitis receiving chemotherapy (0.23±0.004) was higher compared with those with periodontitis not receiving chemotherapy (0.222±0.004) and healthy controls (0.220±0.003), but not significantly so (p>0.05).

Table 2: Levels of HSV-1_Ag in the study groups

ANOVA Test	Study Groups			P value
	Periodontitis without chemotherapy Mean±SE	Periodontitis with chemotherapy Mean±SE	No Periodontitis, No chemotherapy (Controls) Mean±SE	-
HSV-1 Ag (ng/L)	0.22±0.004	0.23±0.004	0.22±0.003	0.071

Table 3 shows that out of 90 saliva samples collected in this study 4 (13.3%) and 3 (10.0%) were found to have HSV-1 Ag in patients with periodontitis without chemotherapy and patients with periodontitis receiving chemotherapy using real-time PCR technique. The result was not significant (p>0.05).

Table 3: Distribution of HSV-1 Ag by study group using Real-time PCR

HSV-1_RT	Study Groups			Total No. (%)	P value
	Periodontitis without chemotherapy No. (%)	Periodontitis with chemotherapy No. (%)	No Periodontitis, No chemotherapy (Controls) No. (%)		
Negative	26 (86.7)	27 (90.0)	30 (100.0)	83 (92.2)	0.133
Positive	4 (13.3)	3 (10.0)	0 (0.0)	7 (7.8)	
Total No.	30 (100.0)	30 (100.0)	30 (100.0)	90 (100.0)	

Table 4 shows that the mean value of HSV-1 Ag level as detected by ELISA test was significantly higher in patients having periodontitis with chemotherapy and those having periodontitis without chemotherapy with positive PCR test results compared to those with negative PCR result (0.280±0.017), (0229±0.004), (0.258±0.002), (0.216±0.004) respectively, ($p<0.05$). In the control group, no positive HSV-1 Ag was detected, while for those who showed a negative PCR test, the mean value was (0.223±0.003).

Table 4: HSV-1 Ag level by Real-time PCR test and ELISA test in the study groups

HSV-1 by Real time PCR	Study Groups					
	Periodontitis without chemotherapy		Periodontitis with chemotherapy		No Periodontitis, No chemotherapy (Control)	
	HSV-1_Ag		HSV-1_Ag		HSV-1_Ag	
	Mean	±SE	Mean	±SE	Mean	±SE
Negative	0.22	0.004	0.23	0.004	0.22	0.003
Positive	0.26	0.002	0.28	0.017	--	--
P value	0.0001		0.0001		--	

Discussion:

The results of the present study revealed that the mean age of the patients in the groups of periodontitis with and without chemotherapy was significantly higher than that of the control group, which is consistent with the results of Beck et al and Siaili et al which indicated that chronic periodontal disease prevalence increases with age and stays steady beyond 50-59 (18, 19). This may be due to molecular alterations in periodontal cells that increase bone loss in older periodontitis patients.

The result of the current study showed a lower mean number of teeth in patients with periodontitis with and without chemotherapy than in the control group. This is in agreement with the results of Al-Ghurabi, who reported a higher number of teeth lost with a greater extent of severe periodontal disease (20). Periodontal disease is one of the main causes of tooth loss worldwide (21).

In the present study, the HSV-1 Ag detection rate was higher in patients having periodontitis and receiving chemotherapy, compared with those having periodontitis without chemotherapy and with the control group. Patients receiving chemotherapy have several risk factors for viral reactivation. Physical trauma, emotional stress, hunger, and immunological compromise. Thus, such patients are more likely to reactivate viruses than healthy people (21), which explains the results obtained in the current study. This is in agreement with the results of Whitley and Roizman and Bruce et al indicating that quick replication and reactivation occurs when chemotherapy or immunosuppression disrupts the virus-host equilibrium (17, 22). Knaup et al suggested that HSV reactivation may harm oral mucosa, aggravate somatotopic cancer treatment lesions, and propagate viruses. HSV reactivation in cancer patients is rare and may go misdiagnosed, although research varies by malignancy and diagnostic laboratory techniques. Several causes may trigger this imbalance and viral reactivation. Lip vesicles or saliva viral release may result from latent HSV reactivation (22, 23).

ELISA and PCR findings differ. According to Kumar et al, antibody testing cannot distinguish between present and prior herpes infections. Since antibodies take time to generate, there may not have been a positive antibody test in the past (24). According to Bezold et al and Al-Hasnawi, PCR utilized for the diagnosis of herpes simplex virus type 1 and type 2 infections from saliva samples is concordant with most positive ELISA results (25, 26). According to Lee et al when herpes lesions are present, As HSV IgG antibodies last forever, PCR testing are advised (27). In addition to Al-Hasnawi's report, researchers reported that the kit for extracting the DNA of a virus from saliva revealed a good yield of DNA purity and concentration (26). Herpesvirus-based oncolytic vectors have been tested against various malignancies using various chemotherapeutic drugs (28). Chemotherapy-induced oral mucositis (CIOM) in hematological malignancies patients receiving high-dose chemotherapy was strongly related to oral HSV-1 reactivation (29). According to Saito et al, systemic chemotherapy suppresses systemic immunity and decreases peripheral lymphocyte count, which may lead to latent HSV-1 reactivation (30). Due to the high seropositive percentage for HSV-1 and the lack of clinical signs of initial HSV infection, all HSV PCR-positive cases are considered as "reactivations" (31).

Conclusion:

The study concluded that HSV-1 viral prevalence in periodontitis indicates a strong relationship between

these viruses and periodontal diseases, especially in breast cancer patients because of a low immune response after receiving chemotherapy. The results of the real-time PCR confirmed those of ELISA. As ELISA is cheaper than real-time PCR, initial screening may be done using ELISA, and then positive samples can be explored by real-time PCR to confirm HSV DNA.

Authors' contributions
Saif S. Jasim: MSc student
Ghada I. Taha: Supervisor

Authors' Declaration
Conflicts of Interest: None.
We hereby confirm that all the Figures and Tables in the manuscript are ours. Besides, the Figures and images, which are not ours, have been given permission for re-publication and attached to the manuscript. Authors sign on ethical consideration's approval-Ethical Clearance: The project was approved by the local ethical committee of Dentistry, University of Baghdad according to the code number (No, 402821).

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مقارنة بين تقنيات الكشف عن مستضدات فيروس الهربس البسيط نمط 1 بواسطة فحص الإليزا وتفاعل البلمرة المتسلسل بالزمن الحقيقي في مريضات سرطان الثدي اللواتي يعانين من إلتهاب اللثة

د.سيف صلاح الدين جاسم / قسم العلوم الأساسية في ميكروبيولوجيا الفم، كلية طب الأسنان، جامعة بغداد.
غادة ابراهيم طه

الخلاصة

الخلفية: إلتهاب دواعم السن هو عدوى طويلة الأمد تدمر اللثة والأربطة اللثوية والعظام السنخية التي تدعم الأسنان. يحدث إلتهاب اللثة وإلتهاب دواعم السن بسبب البكتيريا الموجودة في لوحة الأسنان وفيروسات الهربس، وخاصة النوعين 1 و 2 من فيروس الهربس البسيط.

الهدف: مقارنة تقنية الأليزا وتفاعل البلمرة المتسلسل بالزمن الحقيقي كطرق للعثور على فيروس الهربس البسيط من النوع الأول في مرضى سرطان الثدي الذين يعانون من إلتهاب دواعم السن والذين يتلقون العلاج الكيميائي.

المواد والطرق: شملت الدراسة 90 شخصا كالتالي: 30 مريضة لديهن إلتهاب دواعم السن مع سرطان الثدي بعد تلقي العلاج الكيميائي، و 30 مريضة لديهن إلتهاب دواعم السن مع سرطان الثدي بدون علاج كيميائي و 30 سيدة لا يعانين من إلتهاب دواعم السن (اللثة صحية) كمجموعة تحكم. كان متوسط العمر (51.06) سنة. تم استخدام الأليزا للتحليل وتفاعل البلمرة المتسلسل بالزمن الحقيقي للكشف عن فيروس الهربس البسيط من النوع الأول في عينات اللعاب.

النتائج: في الدراسة الحالية، زاد معدل الإصابة بمستضد فيروس الهربس البسيط من النوع الأول في مجموعة المرضى الذين يعانون من التهاب دواعم السن ويتلقون العلاج الكيميائي ولكن بشكل غير ملحوظ ($p>0.05$) مقارنة بالمرضى الذين لديهم التهاب دواعم السن ولايتلقون العلاج الكيميائي ومجموعة المرضى الذين لديهم لثة صحية. في المقابل، كانت نتيجة تفاعل البوليميراز المتسلسل بالزمن الحقيقي إيجابية (13.3٪) و (10.0٪) مما يثبت وجود فيروس الهربس البسيط في المرضى الذين يعانون من التهاب دواعم السن بدون علاج كيميائي والتهاب دواعم السن الذين يتلقون العلاج الكيميائي ولكنه لم يكن معنويا" ($p>0.05$).

الخاتمة: تمكن تفاعل البلمرة المتسلسل بالزمن الحقيقي من دعم نتائج اختبار الأليزا في هذه الدراسة. ومع ذلك، نظرا لأن الأليزا أكثر فعالية من حيث التكلفة من تفاعل البلمرة المتسلسل بالزمن الحقيقي، فمن الممكن إجراء الفحص الأول باستخدام الأليزا، ومن ثم قد يتم فحص العينات الإيجابية باستخدام تفاعل البوليميراز المتسلسل في الوقت الحقيقي لتأكيد وجود الحمض النووي الرايبوزي منقوص الاوكسجين (الدنا) الخاص بفيروس الهربس البسيط من النوع الأول.

الكلمات المفتاحية: التهاب اللثة، فيروس الهربس البسيط نمط 1، تفاعل البلمرة المتسلسل بالزمن الحقيقي، فحص الإليزا، سرطان الثدي، العلاج الكيميائي.