Original

Comparison of salivary hemoglobin measurements for periodontitis screening

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Abstract: Salivary hemoglobin (Hb) for screening of periodontitis is approved under the pharmaceutical affairs law of Japan. Two reagents are commercially available for the modified fecal occult blood test: Saliva Hemo Plus and OC-AUTO S Latex Reagent. We simultaneously measured split specimens from 561 samples by using these two methods and compared the differences and agreement between both methods. Moreover, saliva samples were collected from 10 subjects at five time points during the day for analysis of circadian variations and fluctuation. The Pearson's correlation coefficient for these two reagents was 0.794. The Bland-Altman plot of differences in salivary Hb levels measured by the two reagents indicated that the difference included fixed errors (0.55 µg/mL). On analysis of circadian variations, no statistically significant differences were observed using the

Friedman test. However, fixed errors were observed between wake-up time and before dinner and before lunch and before dinner, and no random errors were observed by Bland-Altman analysis. In conclusion, the salivary Hb levels measured using OC-AUTO S Latex Reagent were lower than those measured using Saliva Hemo Plus, along with a tendency for higher levels in the morning. Thus, when performing salivary tests these observations must be considered.

Keywords: periodontal disease; salivary hemoglobin; saliva test.

Introduction

Periodontal disease is one of the most prevalent oral diseases among middle-aged and elderly people. Screening for periodontal disease plays an important role in its prevention and in improving oral health-related quality of life.

Conventionally, screening for periodontal disease is performed by pocket probing of representative teeth by dental professionals. However, pocket probing is associated with fundamental biological issues because patients

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with periodontitis are at a greater risk of bacteremia due to periodontal probing (1).

Several salivary biomarkers have been suggested to be correlated with periodontal conditions (2,3). These markers have been investigated for use in clinical diagnosis or screening. In our previous study, we reported that salivary lactate dehydrogenase had the highest sensitivity and specificity for periodontal disease (with at least one probing depth >4 mm) compared with other biochemical markers such as aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, creatinine, and blood urea nitrogen (4). However, salivary lactate dehydrogenase is not approved under the pharmaceutical affairs law in Japan for screening of periodontal disease as an *in vitro* diagnostic test; in addition, its sensitivity and specificity were <0.7. In contrast, salivary hemoglobin (Hb) is the only approved test. At an earlier stage, salivary Hb has been measured by colorimetric detection, and only free Hb (f-Hb) has been measured (4-6). Reportedly, the sensitivity and specificity of f-Hb were 0.81 and 0.27, respectively (4). This method revealed that contamination of blood from other species in food was detected. Therefore, the diagnostic precision of f-Hb in saliva was insufficient. Subsequently, methods for salivary Hb measurement have been improved. Two reagents based on the modified fecal occult blood tests are now commercially available, and these reagents use polyclonal antibodies, which are conjugated with colloidal gold (Saliva Hemo Plus, Alfresa Pharma Corporation, Osaka, Japan) or latex particles (OC-AUTO S Latex Reagent, Eiken Chemical Co., Ltd., Tokyo, Japan). The use of these methods increases the sensitivity of salivary Hb detection (7-9) and also improves sensitivity and specificity. Salivary Hb levels measured using Saliva Hemo Plus revealed a >0.7 sensitivity and specificity for bleeding (8). In addition, sensitivity and specificity of salivary Hb measured by OC-AUTO S Latex Reagent were also >0.7, according to the criteria of the Center for Disease Control and Prevention in partnership with the American Academy of Periodontology (10). In terms of likelihood ratio, the likelihood ratio for Saliva Hemo Plus, OC-AUTO S Latex Reagent, and f-Hb were 3.33, 3.22, and 1.42, respectively.

In order to promote saliva testing for screening of periodontal disease, basic data for validation are necessary. In this study, we simultaneously measured a single sample by using two methods and compared their differences and agreement. In addition, circadian variations and fluctuations during the day were assessed.

Materials and Methods

Study population

The study population was selected from subjects who registered for oral health promotional events sponsored by two dental associations (Ehime Dental Association and Minato-ku Shiba Dental Association). Inclusion criteria were adults aged >20 years with at least 20 teeth remaining and without dentures. In total, 561 subjects (410 male and 151 female) were selected (age: 54.8 ± 14.05 years).

The study population for analysis of circadian variations and fluctuations during the day was selected from the subjects who worked at Shikokuchuken Co. Ltd. Inclusion criteria were adults aged >20 years with at least 20 teeth remaining and without any dentures. In total, 10 subjects (six male and four female) were selected (age: 40.72 ± 9.06 years).

This study was approved by the Ethics Committee of Tsurumi University, School of Dental Medicine (approval number: 430), and was conducted in accordance with the Helsinki 1964 Declaration.

Measuring salivary Hb

Stimulated whole saliva was collected by chewing on a gum base containing neither fragrant nor taste ingredients for five min. To examine salivary Hb levels, 100 μ L of the collected saliva was immediately transferred into a diluent solution with preserving agents and kept at 4°C. These samples were immediately transported to the medical examination laboratory. Salivary Hb levels were measured using Saliva Hemo Plus (Alfresa Pharma Corporation) and OC-AUTO S Latex Reagent (Eiken Chemical Co., Ltd.), according to the manufacturer's instructions (8-10).

For evaluation of circadian variations and fluctuations during the day, saliva samples were collected at five different time points: wake up, before lunch, 2 h after lunch, before dinner, and before bedtime. After confirming that the subjects had not consumed any food for at least 1 h, stimulated whole saliva was collected by chewing a gum base containing neither fragrant nor taste ingredients for 5 min. To examine salivary Hb levels, 100 μ L of the collected saliva was immediately transferred into a diluent solution with preserving agents and stored at 4°C. Salivary Hb levels were measured using Saliva Hemo Plus, according to the manufacturer's instructions.

Statistical analysis

In order to assess correlations between salivary Hb levels measured under different conditions, Pearson's correlation coefficient and linear regression formula were

calculated. *P* values of cross tabulations were calculated using chi-squared test and Fisher's exact test when counts were <5 per cell. *P* values for circadian variations were determined using the Friedman test. These statistical analyses were performed using IBM Statistical Package for the Social Sciences Statistics Ver 19.0 (IBM SPSS, Tokyo, Japan). To identify any systemic errors, Bland-Altman analysis was conducted (11-17). Differences in the two values were evaluated by means and 95% confidence intervals (CIs). The random errors were evaluated using the test of no correlation of mean and difference of the two values. This analysis was performed using S-Plus software Ver 6.0 (NTT DATA, Tokyo, Japan).

Results

Figure 1A presents a scatter plot of results for the 561 samples evaluated using the two reagents. Pearson's correlation coefficient was 0.794, and the formula for linear regression was $Y = 1.691X - 5.907$. Pearson's correlation coefficient and the regression formula were affected by certain samples with high values. Overall, 393 samples (70.0%, 393/561) presented values <10 µg/mL with both reagents, and the screening level of salivary Hb for periodontitis is 2.0 µg/mL. Figure 1B presents a magnified scatter plot for samples <10 µg/mL. Hence, the linear regression formula was changed to $Y = 0.7872X - 0.0408$.

To identify any systemic errors between salivary Hb levels calculated using these two reagents, Bland-Altman analysis was conducted for the aforementioned 393 samples. Figure 2 presents a Bland-Altman plot of differences in the salivary Hb levels measured using the two reagents against the mean values measured using the two reagents. The mean value of differences and 95% CI was 0.29 (95% CI%: 0.18-0.40); moreover, by performing the test of no correlation, a *P* value of 0.869 was obtained. Therefore, the difference included fixed errors and not random errors. This result indicated that salivary Hb levels measured using OC-AUTO S Latex Reagent were lower by 0.18-0.40 µg/mL than those measured using Saliva Hemo Plus. The optimal cutoff value of the OC-AUTO S Latex Reagent was calculated using ROC analysis with a cutoff point of 2.0 µg/mL of the OC-AUTO S Latex Reagent as a standard. The optimal cutoff point that showed the highest crude hit rate for the OC-AUTO S Latex Reagent was 1.45 µg/mL.

The cross tabulation for positive and negative results for the two reagents is presented in Table 1. Cross tabulation when the cutoff point for OC-AUTO S Latex Reagent was set as 1.45 is presented in Table 1B. The crude hit rate improved from 89.3 to 90.2%.

Fig. 1 Scatter plot of salivary hemoglobin (Hb) levels measured using the two reagents. A) All samples investigated in this study. B) Samples with salivary Hb levels < 10 µg/mL.

To confirm the stability of salivary Hb levels, samples provided by the Ehime Dental Association were measured at one and three days after sampling. Scatter plots for salivary Hb levels by using Saliva Hemo Plus and OC-AUTO S Latex Reagent are shown in Fig. 3A and B, respectively. Pearson's correlation coefficients were 0.993 for Saliva Hemo Plus and 0.992 for OC-AUTO S Latex Reagent. By conducting Bland-Altman analysis of the 50 samples with salivary Hb levels <10 µg/mL, the differences did not include fixed or random errors. The means and *P* values of the differences were 0.163 (95% CI: 0.001-0.319; *P* = 0.269) for Saliva Hemo Plus and 0.198 (95% CI: 0.001-0.392; *P* = 0.559) for OC-AUTO

Table 1 Cross tabulation of positive and negative test results for the two reagents

(A)		OC-AUTO S Latex Reagent		<i>P</i>
		<2.0 µg/mL	≥2.0 µg/mL	
Saliva Hemo Plus	<2.0 µg/mL	282	12	<0.001
	≥2.0 µg/mL	48	219	

(B)		OC-AUTO S Latex Reagent		<i>P</i>
		<1.45 µg/mL	≥1.45 µg/mL	
Saliva Hemo Plus	<2.0 µg/mL	272	22	<0.001
	≥2.0 µg/mL	33	234	

Same samples were measured by two reagents. According to manufacturer's instructions, the cutoff point for periodontitis screening is 2.0 µg/mL (A). On conducting Bland-Altman analysis, fixed error was found. When the cutoff point of the OC-AUTO S Latex Reagent was changed to 1.45 (B), the crude hit rate improved from 89.3 to 90.2%. *P* values were calculated using chi-squared test.

Fig. 2 Bland-Altman plot for samples with salivary Hb levels <10 µg/mL. A total of 393 sample (70.0%) with salivary Hb levels < 10 µg/mL were analyzed using Bland-Altman analysis. Mean value and 95% confidence interval (CI) of the difference was 0.29 (95% CI%: 0.18-0.40) and *P* value was 0.869 when calculated using the test of no correlation. The differences by both reagents included fixed errors and no random errors. The dotted line indicates 95% CIs of the differences. Hb: hemoglobin.

S Latex Reagent, respectively. The cross tabulations for samples with <2.0 and ≥2.0 µg/mL and <1.45 and ≥1.45 µg/mL are summarized in Table 2. The crude hit rates were >95%.

Circadian variations and fluctuations during the day were measured in samples from 10 subjects. The results are presented in Fig. 4. Friedman test revealed no statistically significant differences. However, on conducting Bland-Altman analysis, fixed errors were observed between wake-up time and before dinner, and before lunch and before dinner, but no random errors

Table 2 Cross tabulation of positive and negative test results obtained at 1 and 3 days after sampling

(A)		Saliva Hemo Plus		<i>P</i>
		3 days		
		<2.0 µg/mL	≥2.0 µg/mL	
1 day	<2.0 µg/mL	38	0	<0.001
	≥2.0 µg/mL	1	23	

(B)		OC-AUTO S Latex Reagent		<i>P</i>
		3 days		
		<2.0 µg/mL	≥2.0 µg/mL	
1 day	<2.0 µg/mL	37	0	<0.001
	≥2.0 µg/mL	1	24	

(C)		OC-AUTO S Latex Reagent		<i>P</i>
		3 days		
		<1.45 µg/mL	≥1.45 µg/mL	
1 day	<1.45 µg/mL	35	0	<0.001
	≥1.45 µg/mL	2	25	

Within 3 days, limited effects of the degradation of the saliva samples were observed for periodontitis screening if the samples were kept in the specific diluent containing preservatives. *P* values were calculated using Fisher's exact test.

were observed (Table 3). The higher values tended to be obtained in the morning.

Discussion

Screening for periodontal diseases should be simple, easy, and cost-effective; in addition, samples must be collected using noninvasive techniques. The Community Periodontal Index (CPI), which is a traditional screening method used for periodontal diseases and involves the use of periodontal probing, may be uncomfortable or even painful for certain subjects. Although CPI was designed

Fig. 3 Stability of salivary Hb levels: salivary Hb within 3 days. A) Saliva Hemo Plus. B) OC-AUTO S Latex Reagent. Figures present the scatter plot of salivary Hb levels measured at 1 and 3 days after sampling. Pearson's correlation coefficients were 0.993 for Saliva Hemo Plus and 0.992 for OC-AUTO S Latex Reagent. Moreover, the regression formulas were (3 days) = $1.084 \times (1 \text{ day}) - 1.451$ for Saliva Hemo Plus and (3 days) = $0.913 \times (1 \text{ day}) + 0.449$ for OC-AUTO S Latex Reagent. Hb: hemoglobin.

for use in large-scale populations, the personnel cost of a trained dentist is a major issue. On the other hand, training and calibration of the dentists or examiners for salivary Hb tests is minimal. Moreover, salivary Hb tests can be simultaneously used in testing several subjects.

Fig. 4 Circadian variations and fluctuations during the day for salivary Hb levels. P value was calculated using the Friedman test. Hb: hemoglobin.

The salivary Hb tests meet the criteria of simple, easy, and cost-effective, and are expected to become the alternative screening method to CPI.

Three salivary Hb tests are currently used as *in vitro* diagnostic methods for screening of periodontal disease and are approved under the pharmaceutical affairs law in Japan. The two methods that are the subject of this study, Saliva Hemo Plus and OC-AUTO S Latex Reagent, provide quantitative and comparative results. The third method is a qualitative colormetric method that immunologically detects human salivary Hb by means of a colloidal gold-labeled antihuman Hb polyclonal antibody (18). Therefore, in this study a comparison with the Perioscreen test (Sunstar, Osaka, Japan) was not performed.

From the scatter plots in Fig. 1, results obtained by Saliva Hemo Plus showed a strong correlation with those obtained by OC-AUTO S Latex Reagent; this suggests that both Saliva Hemo Plus and OC-AUTO S Latex Reagent can be reliably used for screening of periodontal disease. However, the salivary Hb levels measured by OC-AUTO S Latex Reagent tended to be lower than those measured by Saliva Hemo Plus. The Bland-Altman plot indicated that the difference included fixed errors and not random errors (Fig. 2). The fixed errors may originate from several factors. First, the two reagents used different antibody reactions; Saliva Hemo Plus uses colloidal gold agglutination reaction and OC-AUTO S Latex Reagent uses latex agglutination reaction. Second, certain components in saliva may affect the agglutination reaction. The standard curve for evaluation of a standard material is dependent on the measuring device as well as on the reagents.

Table 3 Results of Bland-Altman analysis for circadian variations of salivary hemoglobin (Hb) levels

		Before lunch	2 h after lunch	Before dinner	Before bedtime
Wake up	Difference	-0.373	0.922	1.274*	0.648
	(95% CI)	(-1.451-0.908)	(-0.216-2.220)	(0.101-2.447)	(-0.571-1.864)
	<i>P</i> value	0.254	0.151	0.168	0.056
Before lunch	Difference		1.256	1.574*	0.921
	(95% CI)		(-0.045-2.575)	(0.225-2.868)	(-0.186-2.028)
	<i>P</i> value		0.070	0.072	0.100
2 h after lunch	Difference			0.282	-0.344
	(95% CI)			(-0.343-0.908)	(-1.171-0.484)
	<i>P</i> value			0.111	0.099
Before dinner	Difference				-0.626
	(95% CI)				(-1.476-0.224)
	<i>P</i> value				0.180

P values for correlation between mean values in and differences of, salivary Hb levels (μg/mL). *P* values were calculated using the test of no correlation. * indicates statistical significance. Fixed errors were observed between wake-up time and before dinner and before lunch and before dinner. There were no *P* values < 0.05. Therefore, there were no fixed errors.

The results of these experiments provided information that circadian variations may influence salivary Hb levels, while the stability of salivary Hb may not affect salivary Hb levels. Figure 4 and Table 3 present circadian variations and fluctuations during the day in salivary Hb levels of 10 subjects. Saliva samples from nine periodontally healthy subjects and one with periodontitis who was treated in a dental office, were evaluated. In the periodontally healthy subjects, except for one subject, higher values tended to be obtained in the morning; however, the salivary Hb levels were in the reference range. One periodontally healthy subject and one subject with periodontitis revealed levels that were over the reference range in the morning. The differences were not statistically significant during the day by Friedman test; this may be because the small sample size and the high standard deviation affected the results. However, higher values tended to be obtained in the morning compared with before dinner by the Bland-Altman analysis (Table 3). Hence, sampling time for each subject is critical as circadian variations may affect salivary Hb levels.

In this study, salivary Hb levels measured by OC-AUTO S Latex Reagent tended to be lower than those measured by Saliva Hemo Plus. Higher values tended to be observed in the morning. These two issues must be taken into consideration to promote widespread use of salivary Hb testing.

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Conflict of interest

The authors have no conflict of interest to declare.

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