Performance Evaluation of a Salivary Amylase Biosensor for Stress Assessment in Military Field Research

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Background: A convenient biosensor for real-time measurement of biomarkers for in-field psychophysiological stress research and military operations is desirable. We evaluated a hand-held device for measuring salivary amylase as a stress marker in medical technicians undergoing combat casualty care training using two different modalities in operating room and field settings. **Methods:** Salivary amylase activity was measured by two biosensor methods: directly sampling saliva with a test strip placed under the tongue or pipetting a fixed volume of precollected saliva onto the test strip, followed by analyzing the sample on the strip using a biosensor. The two methods were compared for their accuracy and sensitivity to detect the stress response using an enzyme assay method as a standard. **Results:** The measurements from the under-the-tongue method were not as consistent with those from the standard assay method as the values obtained from the pipetting

method. The under-the-tongue method did not detect any significant increase in the amylase activity due to stress in the operating room ($P > 0.1$), in contrast to the significant increases observed using the pipetting method and assay method with a significance level less than 0.05 and 0.1, respectively. Furthermore, the under-the-tongue method showed no increased amylase activity in the field testing, while both the pipetting method and assay method showed increased amylase activity in the same group ($P < 0.1$). **Conclusion:** The accuracy and consistency of the biosensors need to be improved when used to directly measure salivary amylase activity under the tongue for stress assessment in military medical training. J. Clin. Lab. Anal. 30:223–230, 2016. © 2015 Her Majesty the Queen in Right of Canada. Journal of Clinical Laboratory Analysis published by Wiley Periodicals, Inc. Reproduced with the permission DRDC Editorial Board.

Key words: biosensor; military medical training; salivary amylase; stress

INTRODUCTION

Military operations and training involve a broad spectrum of stressors (1–4). Although the resultant stress may not always lead to negative consequences, it can impair soldier's well-being and operational performance (5, 6). Psychosocial measures of stress based on self-reports have been used, but are limited by reliability and validity issues (7). Alternatively, physiological means of quantifying stress using biomarkers are useful to reduce self-report bias (8). However, it can be difficult to measure some biomarkers in a field environment.

Salivary amylase has been widely used as a noninvasive biomarker for the sympathetic nervous system in response to stress (9–11). It is normally measured by saliva collection, processing, storage, and analysis using commercial

kits (12). Recently, a salivary amylase biosensor (Nipro Corporation Cocoro Meter, Toyosaki-Kita-ku, Osaka, Japan) has been developed for point-of-care testing. It is considered simple to operate, produces fast results, is durable, and requires minimal maintenance and resources (13, 14). Though it has been used for assessing stress (15–18), and driver fatigue (19), it has not been rigorously

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examined in the combat training environment. This study was conducted to further evaluate the performance of the biosensor by comparing it to a standard assay method in a military field training environment. This experiment was part of a larger study comparing two medical training modalities (live tissue and high-fidelity patient simulator) for Canadian Forces Medical Technicians.

MATERIALS AND METHODS

Participants

Twenty healthy Canadian Armed Forces Medical Technicians (Med Techs) undergoing combat casualty life-saving training were used for this study (mean age 27 ± 6 , 70% male). The participants were recruited by the Principal Investigator (PI) of the main study as part of their trauma training requirements. The PI was not part of the Med Techs chain of command so had no influence over the participants. Participants were screened to ensure that they were free from psychotropic medication, steroids or drug abuse, any transitory or chronic conditions (i.e., Addison's syndrome, Cushing's syndrome, etc.). Written informed consent was obtained from all the participants. The study was approved by Human Research Ethics Committee at Defence Research and Development Canada. In addition, in conducting this research, the authors adhered to the "Guide to the care and use of experimental animals" and "The Ethics of Animal Experimentation" published by the Canadian Council of Animal Care.

Procedures

The study participants were refrained from eating or drinking for 1 hr prior to the study so as not to interfere with biomarker evaluation. They were randomly assigned to one of two training modalities: anaesthetized porcine live tissue (LT) model or high-fidelity patient simulator (HFPS; CAESARTM, CAE Healthcare, Saint-Laurent, Quebec, Canada). The participants had never been exposed to these modalities prior to this study. Med Techs were initially trained in Tactical Combat Casualty Care life-saving procedures on either the LT or HFPS model and then evaluated on that modality in a controlled operating room (OR) environment. Following the testing in the OR, the study participants proceeded to a field setting and were reevaluated in a simulated combat environment. All participants were trained and tested on the same life-saving skills. The testing in the OR and field lasted approximately 2 hr. For the purpose of this study, we focused on the comparison of different analytical methods of salivary amylase measurements in two modality groups (LT and HFPS) before and after OR testing and field testing.

Measurement of Salivary Amylase

Two salivary amylase biosensors, each comprising a hand-held reader and disposable saliva collection strip, were purchased from Nipro Corporation and used according to the manufacturer's instructions. The collection strip consists of a collector pad at its tip and a reagent paper containing a chromogenic substrate for salivary amylase. The reader is a miniaturized optical platform to quantify amylase via colorimetric detection of the reaction product of amylase substrate. The participants were instructed not to drink and eat food for 1 hr prior to the measurement and allow saliva to pool on the floor of the mouth for about 30 sec. They were then instructed to place the end of the strip inside the mouth under the tongue for 30 sec (under-the-tongue method). They were asked not to chew or otherwise manipulate their tongues or mouth while collecting saliva with the test strip. The saliva-containing strip was immediately inserted into the reader to transfer the saliva absorbed in the collector pad to the reagent paper to initiate the enzymatic reaction. The amylase activity was measured by the reflectance light intensity of the reaction product. Each biosensor was operated by the same person.

In addition, saliva was immediately collected using Salivette (SARSTEDT Inc., Montreal, Quebec, Canada). Additional salivary samples were collected the day before and the day after the study. The participants were instructed to chew on a synthetic swab gently for 45 sec and then to allow the swab to soak in their mouths for another 45 sec. The swab was spat into a Salivette and was immediately centrifuged at $2,700 \times g$ for 5 min to yield clear saliva. The saliva samples were frozen and stored at -70°C until analyses were conducted by two methods described below.

The frozen saliva samples collected using Salivette from two healthy controls were thawed at room temperature, vortexed, and centrifuged at $1,500 \times g$ for 15 min. An aliquot of saliva in a volume ranging from 5 to 30 μl was pipetted onto the test strip of the hand-held biosensor and measured for amylase activity using the biosensor to verify the effect of saliva volume on its accuracy (pipetting method) and determine the appropriate volume used for subsequent analysis of collected saliva in the pipetting method for comparison with other methods. The intra- and intermeasurement coefficients of variation for the amylase biosensor were 8.4% and 8.1%, respectively, as reported (15).

The same biosensor was used to measure salivary amylase before and after the OR testing and field testing for the same participant using the under-the-tongue method and pipetting method.

In addition, the same saliva sample was analyzed for amylase activity using a standard laboratory kinetic

enzyme assay kit (Salimetrics, LLC, State College, PA) as per manufacturer's instructions (assay method). The assay possesses intra- and interassay coefficients of variation of $\leq 7.2\%$ and $\leq 5.8\%$, respectively, according to the manufacturer's technical note.

Data Analysis

Data were analyzed for normality using a Kolmogorov–Smirnov test and log-transformed if not normally distributed. One-way analysis of variance (ANOVA) was applied to assess variables (e.g., pipetting volumes) affecting the biosensor accuracy and reproducibility, and to compare salivary amylase activities measured by the three methods. Salivary amylase levels measured by the under-the-tongue method were nonnormally distributed and log-transformed for ANOVA (20). Stress-induced increases of amylase activity were also assessed before and after the testing in the OR and in the field-testing scenarios for the different groups, respectively, using the method as reported by Strahler et al. (21). *t*-Tests were used to examine the significance of the increased amylase activity and compare the under-the-tongue method and pipetting method with the assay method, respectively. Correlations between the two biosensor methods and the assay method were analyzed using a Spearman nonparametric analysis and Bland–Altman difference mean plot. An increase in variability of the differences with the magnitude of the measurement was observed and thus data were log-transformed for creating the Bland–Altman plot as recommended in the literature (22). Therefore, some analyses were conducted using the data that were log-transformed, but in other analyses, the absolute value was used. All data were presented as mean $\pm$ standard error of mean (SEM) unless indicated otherwise. We used IBM SPSS Statistics 20 program to perform all statistical analyses with the level of significance at $P < 0.05$, however due to the small group sizes, $P < 0.10$ was identified as a trend toward statistical significance.

RESULTS

Sixty measurements for salivary amylase activity were performed on site using the under-the-tongue method and 100 measurements were conducted using the pipetting method with four and five failures for each of the methods, respectively. According to the company's error codes, the failures seen in the under-the-tongue method were due to no detection of amylase activity, while the failures in the pipetting method were due to the incorrect operation of the biosensor.

Figure 1 shows amylase activity measured using the under-the-tongue method before and after the OR testing and field testing. There were no significant differences in

Fig. 1. Salivary amylase levels measured by the under-the-tongue method in the living animal (LT) and high-fidelity patient simulator (HFPS) groups at pre- and post-OR testing and field testing. Data represent mean $\pm$ SEM ($n = 4-6$ for the OR testing and $8-10$ for the field testing).

amylase activity after the OR testing for the LT group ($P = 0.345$) and HFPS group ($P = 0.385$), and after the field testing for the two groups (LT, $P = 0.659$; HFPS, $P = 0.140$). Similarly, one-way ANOVA test showed no significant differences among the groups at pre- and post-testing in OR ($F_{3,16} = 0.955$, $P = 0.438$) and in field ($F_{3,33} = 1.977$, $P = 0.137$).

As observed in this study, for a few subjects it appears that insufficient saliva was collected by the strip using the under-the-tongue method resulting in the failure of the measurement. We thus analyzed the collected salivary samples using the same biosensors, but by pipetting an exact amount of sample onto the test strip instead of sampling directly with the strip under the tongue. The samples were collected from two healthy subjects and contained a low (< 50 U/ml) and high amylase level (> 100 U/ml) as predetermined by the assay method. This allowed us to confirm if sampling volume was an important variable. We also compared the two biosensor methods with the standard assay method to validate the results obtained by the biosensors.

Figure 2 shows the salivary amylase activity measured using the biosensor with different pipetting volumes for the two healthy subjects. For a low amylase activity level (< 50 U/ml), the biosensor was unable to analyze the sample due to the error indicating no amylase detected when $5 \mu\text{l}$ aliquots of saliva were used. For those assays using $10 \mu\text{l}$ aliquots pipetted onto the test strip, and then inserted into the biosensor reader for a reading, only one of the three attempts was successful. Readings with no errors were achieved when 20 and $30 \mu\text{l}$ volumes were used with no significant difference in the measurements between the two volumes.

Fig. 2. Effect of sampling volume on the measurement of salivary amylase activity by the biosensor. Data represent mean $\pm$ SEM ($n = 3$). Subject 1 had a low level (<50 U/ml) and subject 2 had a high level of amylase activity (>100 U/ml), respectively, as determined by the assay method.

For a high amylase activity level (>100 U/ml), the biosensor provided the measurement at all volumes used, but there were significant differences between the readings at 5 and 10 μ l and those at 20 and 30 μ l. There were no differences among the low volumes (5 and 10 μ l) and high volumes (20 and 30 μ l). Based on these results, the volume of 20 μ l was used in the pipetting method for subsequent analysis of collected saliva.

Figure 3 shows the correlations between the under-the-tongue method, pipetting method, and assay method. A strong correlation between the pipetting method and

Fig. 3. Correlations between the saliva amylase activities measured by different methods: assay method, under-the-tongue method, and pipetting method. The scatter plot shows a clear positive correlation between the assay method and pipetting method, but not equivalent to each other, and no correlation between the assay method and under-the-tongue method.

Fig. 4. Correlations of the saliva amylase activities between the assay method and pipetting method with each individual biosensor. The scatter plot shows two clear positive correlations between the assay method and pipetting method, which are not equivalent to each other. The amylase activities measured by biosensor 1 are higher than those by biosensor 2.

assay method for the amylase activity was found ($r = 0.807$, $P < 0.0001$), but not between the under-the-tongue method and assay method ($r = 0.113$, $P = 0.407$). The values obtained from the pipetting method and assay method were consistently higher than those obtained from the under-the-tongue method.

Although both biosensors using the pipetting method exhibited similar extent of the correlation with the assay method ($r = 0.889$ and 0.951), their correlation coefficients were different (Fig. 4). The readings from one biosensor were consistently higher than the readings from the other, and were less consistent with the values obtained from the assay method. In addition, when the pipetting method was compared with the assay method for each individual biosensor, the correlation between the two methods was better than the pooled data from the two biosensors together ($r = 0.889$ and 0.951 vs. $r = 0.807$).

To assess agreement between the methods, Bland–Altman difference mean plots were used (Fig. 5). The plots showed a larger mean difference between the under-the-tongue method and assay method when compared to the pipetting method (-0.36 vs. 0.20), and a larger confidence interval for the limits of agreement (-1.16 to 0.45 vs. -0.13 to 0.53).

Figure 6 compares the increases in amylase activities measured by the three methods before and immediately after the LT testing and HFPS testing in OR. The under-the-tongue method showed no significant increase before and after the LT testing ($P = 0.361$) and HFPS testing ($P = 0.691$) in the OR setting with large individual variations. The pipetting method detected a significant increase in amylase activities before and after the LT testing in OR

Fig. 5. Difference in amylase activity between the under-the-tongue and assay measurements (A) and between the pipetting and assay measurements (B) against their averages after log-transformation with 95% confidence intervals (mean $\pm$ 1.96 SD).

$P = 0.047$), but not after the HFPS testing ($P = 0.515$). The assay method showed a trend toward significance for changes before and after the LT testing ($P = 0.073$), but not the HFPS testing ($P = 0.157$) in OR.

Comparing the under-the-tongue method to the assay method showed no significant differences in the increased amylase activity in either LT group ($P = 0.485$) or HFPS group ($P = 0.534$). Comparing the pipetting method to the assay method also did not show any significant differences in the two groups (LT, $P = 0.479$; HFPS, $P = 0.913$).

Fig. 6. Increases in salivary amylase activity measured by different analytical methods before and immediately after the LT testing and HFPS testing in the OR setting. Data represent mean $\pm$ SEM ($n = 4-10$). The increased amylase activity was calculated as the amylase activity after the OR testing minus the corresponding value before the OR testing. *Significant increases from the pre-OR testing, $P < 0.05$, # $P < 0.1$.

Fig. 7. Increases in the salivary amylase activities after the field testing measured by the under-the-tongue method, pipetting method, and assay method. Data represent mean $\pm$ SEM ($n = 8-10$). The increased amylase activity was calculated as the amylase activity after the field testing minus the corresponding value before the field testing. *Significantly increased amylase activity, $P < 0.1$.

Figure 7 summarizes the changes in the amylase activity level after field testing on either LT or HFPS. The under-the-tongue method showed no changes in amylase activity in the LT group ($P = 0.875$) and HFPS group ($P = 0.168$). The pipetting method showed an increased amylase

activity as the assay method with a similar significant level for the LT group ($P = 0.056$ and 0.055 , respectively), but no increased amylase activity in the HFPS group ($P = 0.117$ and 0.250).

A *t*-test showed no differences in the increased amylase activity between the under-the-tongue method and assay method ($P = 0.155$) or between the pipetting method and assay method ($P = 0.660$) for the LT group and no differences between the under-the-tongue method and assay method ($P = 0.961$) and between the pipetting method and assay method ($P = 0.880$) for the HFPS group.

DISCUSSION

The biosensor (Nipro Corporation Cocoro Meter) employed in this study has been developed and refined over the past few years (13, 15, 19). It has been used in a number of studies to provide close to real-time measurement of salivary amylase levels as a biomarker for stress from academic examination (18), a trier social stress test (23), a flight task (24), a simulated space mission (25), and surgeries (26, 27).

To our knowledge, this study is the first application of the biosensor to objectively assess the stress experienced by medical technicians during training and testing in a simulated battlefield scenario. In our study, we encountered more failures and inconsistencies as compared to those reported in the literature (15, 18). These failures could have been in the methods used to collect the samples. The strip pads might not have been saturated with saliva for some individuals with little saliva production due to dry mouth after stressful conditions. This can cause a problem since the test strip needs to be saturated with saliva to collect a fixed volume of sample ($23\ \mu\text{l}$) for an accurate and reproducible result (18). This explanation is supported by the present results using the pipetting method confirming the effect of salivary volume on the biosensor readings. The same pipetting method with $20\ \mu\text{l}$ volume has been used previously showing excellent inter- and intraobserver reliability and correlation with a conventional laboratory enzymatic analysis (28). However, the variability between the two biosensors has been observed. This is in agreement with the literature (15) and could be due to inherent problems with the equipment.

The average of salivary amylase activity at pretesting in our study was higher than in a large healthy young Japanese male population (68.5 ± 49.0 vs. 17.48 ± 11.70) obtained using the same method (29). Our value is also above the salivary amylase level reported in a healthy population under a stressful condition where participants were restricted from any body movement for 10 min (30). The reasons for such discrepancies are not clear, but could reflect age, race, gender, or stress-related differences between study populations (21, 31). The large individual dif-

ference and skewness of the data obtained by all three methods are in accordance with others results (11, 29).

The enzymatic kinetic assay method has been considered as a standard method for measuring salivary amylase in biobehavioral research (11, 12). Therefore, we compared the biosensor-based under-the-tongue method and pipetting method with the standard assay method to validate the results obtained from the biosensor.

In our study, we did not see any correlation between the under-the-tongue method and assay method for measuring salivary amylase. The lack of correlation between the two methods can be at least partially explained by the difference in the saliva sampling location and is consistent with the literature given the different sources of salivary samples (32). The former only measured the salivary amylase under the tongue, while the latter two methods measured the whole saliva collected by chewing on a swab. We also illustrated some of the inconsistencies in the under-the-tongue method. This may be ascribed to the variations in the amount of saliva collected by the strip and the exact location of the strip placed under the tongue. On the other hand, the salivary amylase activity measured by the pipetting method can be correlated with that obtained from the standard assay kit for the same sample, although not as closely as what was reported in the literature (15). In addition, the inaccuracy and variation of the biosensor could also contribute to the differences in the amylase activity values obtained by the two methods.

The stress levels were further analyzed using the change in the amylase activity in two groups (LT and HFPS) in OR and a simulated battlefield. The pipetting method and assay method were able to detect a significant increase in the amylase activities after the OR testing and field testing in the LT group. The increase in the amylase activity is consistent with other studies investigating the stress response in army nurses during combat casualty simulation (33) and human-based anesthesia simulator training (34), where the amylase activity was measured by the assay method. Comparison of the values obtained using the three methods for each testing condition verified the largest variation in the measurement by the under-the-tongue method.

As salivary amylase is produced by different salivary glands in the mouth including the parotid, submandibular, and sublingual glands (35), the quantitative estimates of salivary amylase activity provided by three methods varied according to the specific source of the saliva. The under-the-tongue method measured the saliva mainly from the sublingual gland. The saliva we collected using the Salivette swab and measured by the pipetting method and assay method includes combined fluids from all three glands.

Our findings demonstrated that the under-the-tongue method was less consistent with the standard assay

method than the pipetting method. This inconsistency can be attributed to the variation in the amount of saliva collected by the under-the-tongue method and the source of the saliva. This is in agreement with the literature indicating saliva collection location and disparities as a major cause of measurement variability (32) and the relatively imprecise under-the-tongue measurement compared to the biosensor measurement obtained with the whole mouth saliva (36).

In addition, the salivary amylase activity may be also influenced by salivary flow rate depending on the collection techniques and oral fluid types (35). Under stress, activation of the sympathetic nervous system can decrease the salivary flow rate (37) leading to a confounding effect on amylase concentration (38). This effect may be more profound for the under-the-tongue saliva collected by the test strip than the stimulated whole saliva collected by the swab (39).

Our study did not support measuring salivary amylase under the tongue using the biosensor, however, it demonstrated the usefulness of this particular biosensor by the pipetting method. Alternatively, new electrochemical biosensors have been reported for the determination of salivary amylase in humans (40), but have not been evaluated for stress assessment in any laboratory and field settings.

The current study has several limitations. First, as aforementioned, this study was part of a larger study and measured salivary samples collected under the tongue and in the whole mouth. Further study using the same sample collection may be necessary to verify our findings. A second limitation of this study is the relatively small sample size. Larger sample size may allow us to further compare the sensitivity of different analytical methods for detecting salivary amylase response to the medical training on different modalities.

CONCLUSION

The performance of the biosensor for directly measuring salivary amylase under the tongue in our field study was not as good as that reported in the literature. The performance was improved with the pipetting method and was comparable with the standard assay method. Further refinement of the hand-held biosensor is desirable to improve its validity in field research examining the biological response to stress during military training.

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