Comparison of Modern Techniques for Saliva Screening

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Abstract

Amylase is a component found in relatively high concentrations in human saliva, and is therefore typically used as the basis of body fluid screening for the possible presence of saliva in casework samples. The current available methods for the screening of saliva in a forensic application are growing in number, but not necessarily in popularity. The analyst must often decide whether a prescreening method would be worth the consumption of sample that could be applied for DNA analysis methods.

This study compared three modern techniques for saliva screening, the recently released SALIgAE®, Phadebas® and Starch-Iodine mini-centrifuge test based on common validation parameters including sensitivity, specificity, mixtures and simulated casework samples as well as further discussion concerning interpretation issues and sample consumption.

Materials and Methods

Validation Parameters

Sensitivity

1. Amylase standard, α-amylase, Type XIII-A: from Human saliva, Sigma (Catalog Number: A1031) Stock solution of 2.32 IU/µl prepared by diluting with sterile, distilled deionized water (ddH_2O). The dilutions were dried down on fabric cotton swatches.
2. Known saliva collected from a male and female donor in 1.5 ml tubes. Dilutions were prepared from the neat saliva and sterile ddH_2O. The Dilutions were dried down on cotton fabric swatches.

Specificity

1. SERI Stain Set (Catalog Number: R675): known human body fluid Samples on fabric swatches
2. Various animal saliva swab samples

Mixtures

1. Saliva: Blood (Dilutions of 1:2, 1:3, 1:5 and 1:10)
2. Saliva: Semen (Dilutions of 1:2, 1:3, 1:5 and 1:10)

Simulated casework samples

1. Swabs of the mouth area of water bottles, collected immediately
2. Swabs of the mouth area of soda cans, collected immediately
3. Cigarette butts
4. Simulated sexual battery samples such as vulva, breast and thigh swabs. These were collected 8 hours post event, applying a dampened swab with ddH2O and rubbed across skin surface.

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Amylase Presumptive Test Procedures

SALIgAE®: Abacus Diagnostics, Inc. (Catalog Number: 903295)

Sample Extraction and Preparation

Removed a $5mm^2$ size cutting from a stain or 1/6 of a swab and placed into an autoclaved 1.5 ml mini-centrifuge tube with 50 µl ddH_2O. The extract was vortexed and spun down in a centrifuge to aid the submersion of the cutting in extract solution. The extract was incubated at room temperature for 30 minutes. The extract should be colorless and if not, should be diluted until colorless which is extremely important with possible saliva: blood mixtures. For our experiments, the blood contaminated samples were diluted to approximately 1.5 ml.

Results Interpretation

Added 8 µL of sample extract and read the result at 10 minutes. A yellow color change was a positive result while no color change indicated a negative result. All experimental results were interpreted within the frame of valid positive and negative controls.

Starch-Iodine Mini-Centrifuge Test

Sample Extraction and Preparation

Starch Solution: Hydrolyzed Starch (.075% starch solution in ddH_2O)
Iodine Solution: Resublimed iodine crystals (.05% solution in ddH_2O) Test was performed in an autoclaved 1.5 ml mini-centrifuge tube. Five drops of starch solution were added to a sample of suspected saliva stain or swab (approximately 1/6 swab, $5mm^2$ cutting), vortexed and incubated at 37°C for 20-30 minutes. An equivalent 5 drops of iodine was added.

Results Interpretation

Deep blue/purple color indicates a negative result or absence of detectable α-amylase activity; while reddish/brown to yellow indicates enzymatic activity of α-amylase. All experimental results were interpreted within the frame of valid positive and negative controls.

Phadebas® Amylase Test: Magle Life Sciences (available directly through www.Phadebas.com)

Sample Extraction and Preparation

Phadebas® tablets were crushed and approximately 0.02 grams of crushed Phadebas® material were added to autoclaved 1.5 ml tubes. About 550 µl of sterile ddH_2O added to each tube and vortexes in order to make a slurry. $5mm^2$ cutting or approximately 1/6 swab was added to a 1.5 ml tube with 500 µl of sterile ddH_2O. Then, 100 µL of Phadebas® slurry was added to the sample slurry, vortexed and incubated at 37 °C for 30 minutes.
After incubation, the samples were vortexed again and centrifuged at 13000 RPM for 2 minutes.

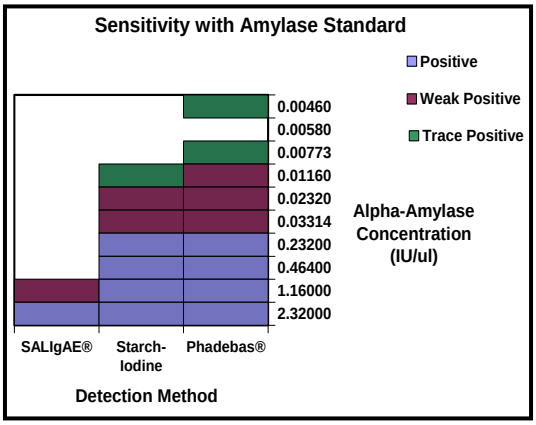
Results Interpretation

The appearance of a blue color in the supernatant following the centrifugation step indicates α-amylase activity in the sample stain or swab. A colorless supernatant solution indicated an undetectable level of α-amylase activity. All experimental results were interpreted within the frame of valid positive and negative controls.

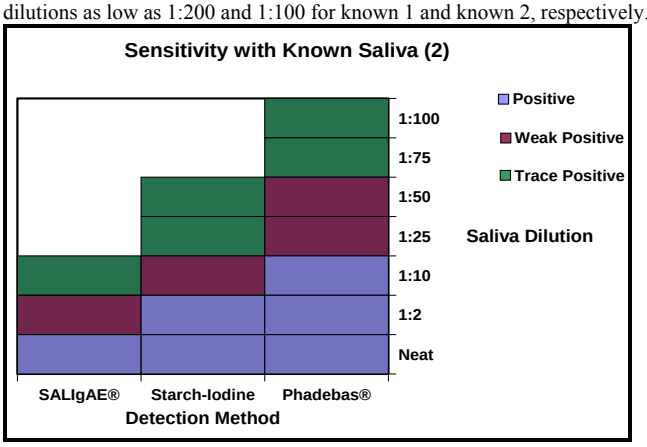
Results

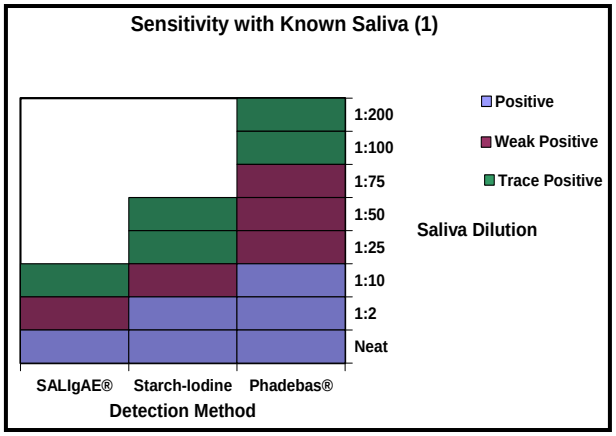
Sensitivity

According to the Whitehead and Kipps[10] study, amylase concentration in human saliva ranges between 0.072-1.3 IU/µl with an average of about 0.35 IU/µl. Relating these numbers to the sensitivity limits detected using the Sigma α-amylase standard for each method demonstrated that SALIgAE® had a sensitivity limit of 1.16 IU/µl, Starch-Iodine 0.0116 IU/µl and Phadebas® 0.0046 IU/µl. The sensitivity limit of 1.16 IU/µl for SALIgAE® is well above the average of 0.35 IU/µl for human saliva, in other words, the SALIgAE® test was unable to detect average levels of amylase in human saliva. The Starch-Iodine sensitivity limit is equivalent to 1:30 dilution of neat saliva while Phadebas® limit is equivalent to a 1:75 dilution of neat saliva.



The sensitivity for each method was also determined in relation to dilutions of sequestered neat saliva. Both known saliva dilution sets demonstrated similar results. SALIgAE® demonstrated a sensitivity to a dilution of 1:10, Starch-Iodine results showed a sensitivity to dilution of 1:50 and Phadebas® demonstrated a sensitivity limit for dilutions as low as 1:200 and 1:100 for known 1 and known 2, respectively.





Specificity

Results for amylase detection in the SERI stain set samples of sperm positive, sperm negative, vaginal swab, male and female urine yielded negative results for all three detection methods. The SERI blood stain was not interpretable for the Starch-Iodine and Phadebas® tests. The blood stain extract for SALIgAE® was diluted to 1.5 ml in order to make the extract colorless and yielded a negative result. The SERI breast milk stain demonstrated negative, trace and weak positive results with SALIgAE®, Starch-Iodine and Phadebas®, respectively.

The dog, cat, pot belly pig and ferret saliva samples were negative with each method while the guinea pig and rat saliva yielded a positive result with Starch-Iodine and Phadebas® and a weak positive with SALIgAE®.

Mixtures

Two sets of mixtures were analyzed; saliva with blood and saliva with semen. Each method yielded positive results for each dilution examined (1:2, 1:3, 1:5 and 1:10). In respect to the recommended protocol for SALIgAE®, a second set of tests were run with the 1:5 and 1:10 Saliva: Blood mixtures to dilute them with 500 µl of ddH_2O in order to ensure the extract was colorless.

Discussion

Protocol Modifications for SALIgAE®

- Extraction volume of 50µl used, recommended 30µl
- 30µl not enough to submerge nor saturate the cutting or swab
- Suggested use of ½ of a swab replaced with 1/6 swab to reduce sample consumption
- Potential to raise an issue concerning the relative sensitivity of SALIgAE®
- Goal for the study was consistency across the three methods for accurate comparison of study parameters

Results Discussion

Sensitivity

- **SALIgAE®** results for the three sets of sensitivity data were consistently lower than the sensitivity for both Phadebas® and Starch-Iodine presumptive saliva tests
- **SALIgAE®** was at least a **factor of five less sensitive** compared to the other two methods
- Protocol modifications could be partly responsible, as well as:
  - Small working volumes
  - Poor sample extraction
  - Results reading at 10 minutes

Specificity

SALIgAE®

- The blood containing samples were diluted to approximately 1.5ml before adding the 8µl volume to the test vials
- SERI Blood Stain standard was negative at 10 minutes
- SERI Breast Milk standard was **negative** at 10 minutes
  - Breast milk known to contain α-amylase
  - Negative result possibly due to low sensitivity of test
  - Proprietary mechanism: may not be detecting α-amylase activity

Starch-Iodine and Phadebas®

- Inconclusive with blood containing samples
- Phadebas® was the only detection method to yield a positive result with SERI Breast Milk standard (known to contain detectable levels of α-amylase)

Each Method

- Expected positive results with guinea pig and rat[4]

Interpretation Issues

- **Phadebas®** demonstrated the most objective interpretation in combination with highest sensitivity
- **SALIgAE®** interpretation straightforward due to drop off in sensitivity *Intensity differences and further development of reaction passed 10 minutes- while the negative controls never developed color
- **Starch-Iodine test** interpretation difficult to interpret for weak to trace positive reactions
- Transition from the negative color of deep blue/purple to yellow is related to the amount of α-amylase activity present in the sample
- Test yields a range of colors from yellow, yellow-red, reddish-brown and light brown for positive results
- **Phadebas®** was the most time consuming

Simplicity

- Starch-Iodine required little or no sample preparation merely addition of drops of substrate with a short incubation time
- SALIgAE® required some additional sample preparation and tube labeling than Starch-Iodine
- Phadebas® required the preparation of Phadebas® slurry plus more pipetting steps and a centrifugation step

Conclusion

- ❖ STR-PCR analysis has afforded forensic biology the advantage of yielding results from extremely small stains
- ❖ The goal of any physical evidence examination through serological methods is to identify stains with the highest degree of reliability and sensitivity
- ❖ The overall effectiveness of a presumptive method should balance sensitivity and specificity with the consumption of a potentially useful sample for further analysis
- ❖ Certain modifications to the SALIgAE® protocol concerning extraction method and length of time for color development could vastly improve the sensitivity of the test
- ❖ Starch-Iodine interpretation issues due to gradated color changes will persist as long as this form of the test is used and this method is best at indicating high levels of α-amylase (an obvious yellow color change for positive)
- ❖ The Phadebas® method for presumptive saliva testing consistently demonstrated its ability to detect saliva with a relatively high degree of specificity at lower limits of detection than the other two methods examined in this study
- ❖ The advantages of the Phadebas® test, due to the clarity of interpretation and sensitivity, far outweigh the additional labor with test preparation
- ❖ The sensitivity of Phadebas® affords the forensic scientist the opportunity to decrease sample consumption with a higher expectation of yielding results that indicate the presence of saliva

Disclaimer

Neither this paper, nor the Miami-Dade Police Department Crime Laboratory Bureau, in any way endorses the use of a specific product over any other product available for amylase or saliva presumptive testing.

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