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TECHNICAL NOTE

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Comparison of Modern Techniques for Saliva Screening*

ABSTRACT: Saliva stains present a unique challenge in the forensic setting, often challenging the analyst to weigh the value of presumptive indication of the fluid versus the potential for DNA analysis to yield identification information. There are many situations in which determining the presence of a body fluid is probative and further corroborates DNA evidence. That said, even a minute portion of sample consumed by a screening test could mean the difference between a full, partial, or null profile obtained through DNA analysis. The basis of presumptive testing or screening of saliva has historically been based on the presence of amylase, a component found in relatively high concentrations in human saliva versus other body fluids and substances. Though the current available methods for the screening of saliva in a forensic application have grown in number, the popularity of these methods seemingly has not. This study attempts to identify a specific and sensitive saliva screening test by comparing three modern techniques—the recently released SALIgAE®, Phadebas®, and starch-iodine mini-centrifuge test—on the basis of sensitivity, specificity, mixtures, and simulated casework samples while also considering sample consumption. The Phadebas® method for presumptive saliva testing detected dilutions of neat saliva down to 1:200 versus considerably less sensitive results with SALIgAE® and the starch-iodine mini-centrifuge test. Utilizing a screening test with a high degree of sensitivity, such as Phadebas®, allows an analyst to gain a maximum amount of information in the form of body fluid indication and DNA results because of the consumption of a small portion of sample.

KEYWORDS: forensic science, serology, presumptive test, saliva, amylase

The current available methods for the screening of saliva in a forensic setting have grown in number, but not necessarily in popularity. The analyst must often decide whether a screening method would be worth the consumption of sample that could be applied towards a more specific or probative method such as DNA analysis. This study attempts to identify a screening method for saliva that would be sensitive enough to enable the analyst to gain knowledge about the possible nature of the stain in addition to identifying information associated with a DNA profile. Sexual assaults often involve oral activity and an indication of the presence of saliva in these cases, with or without a DNA profile, could serve to corroborate other testimonial evidence. Furthermore, the use of a more sensitive and specific test for the detection of amylase could prevent the need for further testing if amylase is not identified, saving valuable time and money associated with DNA analysis.

Other obstacles in presumptive saliva testing include the innate inter- and intra-variability of salivary amylase levels in humans, difficulties in interpreting color change-based tests, body fluid specificity issues as well as relatively poor sensitivity in comparison to other body fluid screening tests for blood and semen. Amylase compounds are found in a wide variety of plants, micro-organisms, animals, and humans. There are detectable levels of α-amylase in the saliva of primates, pigs, rodents, and elephants (1). Of forensic importance is the α-amylase found in human saliva, which is an enzyme found in variable, but relatively high concentrations in saliva and rarely found at those concentrations in other fluids (2). The mechanism of a saliva screening test is based on the detection of α-amylase activity, which hydrolyzes or breaks down complex carbohydrates such as starch into a mixture of oligosaccharide products (3). Salivary α-amylase can also be found in other body fluids such as perspiration, tears, and breast milk (4). Another form of α-amylase, pancreatic amylase can be found in vaginal secretions, urine, serum of blood, fecal material, and seminal fluid (3).

The underlying assumption with any presumptive test is its ability to indicate the possible presence of the body fluid of interest with the knowledge that it can be found in other body fluids or substances. Casework samples such as swabs of bite marks or areas subjected to licking in sexual assaults can be tested for the possible presence of saliva through detection of α-amylase activity. This study examined two common screening methods, Phadebas® (Magle Life Sciences, Lund, Sweden) and starch-iodine mini-centrifuge test and comparing them to SALIgAE® (Abacus Diagnostic, Inc., West Hills, CA). The Phadebas® test utilizes a water insoluble starch that is covalently linked to a blue dye. A positive reaction is based upon α-amylase activity in the sample which hydrolyzes the starch-blue dye bonds leading to the release of blue dye into solution (1). The starch-iodine mini-centrifuge test is similar to its radial diffusion counterpart wherein a sample is incubated with starch solution to allow any α-amylase activity to proceed and is followed by the addition of iodine. The combination of starch and iodine creates a characteristic deep purple/blue color that is associated with the starch-iodine negative control and therefore a positive reaction is indicated by the disappearance of such color (4). The SALIgAE® test mechanism is proprietary (not disclosed) wherein a positive result is indicated by a yellow color change, but there is no color change associated with the negative control (5). The sensitivity was determined using two methods. First, a numerical sensitivity value in terms

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of International Units of Activity (IU/μL) was determined through the use of a commercially prepared standard for α-amylase and second with prepared dilutions from two sets of known saliva samples. Each screening method was also compared in terms of specificity with various body fluid comparisons, mixtures, and simulated casework samples. Finally, a holistic comparison of each method was discussed including other issues such as interpretation, sample consumption, and the simplicity of the test preparation as well as the cost effectiveness of each method employed.

Materials and Methods

Study Parameters

Sensitivity—

1. Amylase standard, α-amylase, Type XIII-A from human saliva, Sigma (St. Louis, MO) (Catalog Number: A1031). Stock solution of 2.32 IU/μL was prepared by diluting with sterile, distilled deionized water (ddH_2O). The dilutions were allowed to dry completely at controlled humidity and room temperature on fabric cotton swatches. Sigma standard units were converted to IU/μL with 0.926 conversion factor. The Sigma unit is expressed as mg of maltose liberated from starch per 3 min under assay conditions. One can then reasonably assume that 1 mg of maltose liberated equals 1 mg maltose digested. Maltose has a molecular weight of 360.3 g/mol and 1 mg = 2.78 μmol. (1 mg altose) × (1000 μg/L mg) × (1 μmol/360.3 μg) = 2.78 μmol of maltose digested. Sigma uses 1 mg/3 min; 2.78 μmol/3 min = 0.926 μmol/min or 0.926 IU (e-mail communication with Dr. R.E. Gaensslen).
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 allowed to dry completely at controlled humidity and room temperature 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 mixtures. Sequestered neat saliva and blood were mixed in the following ratios: 1:2, 1:3, 1:5, and 1:10 in which saliva was the minor component and blood was the major component.
2. Saliva:semen mixtures. Sequestered neat saliva and neat semen were mixed in the following ratios: 1:2, 1:3, 1:5, and 1:10 in which saliva was the minor component and neat semen was the major component.

Simulated Casework Samples—

1. Swabs of the mouth area of water bottles collected immediately after use.
2. Swabs of the mouth area of soda cans collected immediately after use.
3. Cigarette butts.
4. Simulated sexual battery samples such as vulva, breast, and thigh swabs. These were collected by the individual, 8 h following the sexual event by rubbing a swab dampened with sterile ddH2O across the appropriate surface.

Amylase Presumptive Test Procedures

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

Mechanism of Action—Proprietary through Abacus Diagnostics, Inc.

Extraction of Sample—A 5 mm^2 size cutting removed from a stain or 1/6 of a swab was placed into a 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. Next, the extract was incubated at room temperature for 30 min. The extract should be colorless and if not, should be diluted until the extract becomes colorless. This is an important facet involved with interpreting the test results, especially with blood containing samples (5). For our experiments, the blood contaminated samples were diluted to *c*. 1.5 mL to achieve the colorless extract.

Test and Interpretation—Eight μL of sample extract was added to the test vial and the result read at 10 min. A yellow color change was a positive result while the lack of a visible yellow color change indicated a negative result for the presumptive presence of saliva. All experimental results were interpreted within the framework of valid positive and negative controls.

Starch-iodine Mini-centrifuge Test

Mechanism of Action—One diatomic iodine molecule binds per helical turn of linear amylose chains to produce a deep blue/purple color. Alpha-amylase is an endoenzyme and will hydrolyze internal α, 1-4 glycosidic bonds in a starch substrate to yield simpler sugars (4). Therefore, the progression of the color change from deep purple to yellow is proportional to the amount of α-amylase activity detected in the sample.

Sample and Test Preparation—Components: Starch solution: hydrolyzed starch (0.075% starch solution in ddH_2O) and iodine solution (resublimed iodine crystals, 0.05% 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 (1/6 swab, 5 mm^2 cutting), vortexed and incubated at 37°C for 20–30 min. An equivalent five drops of iodine was added following the incubation period. As a note to the reader, varying amounts of starch can be added to the sample depending on the amount of sample available for testing, while an equal amount of iodine should be added following the incubation period.

Interpretation—A deep blue/purple color indicates a negative result for the presumptive presence of saliva or absence of detectable α-amylase activity, whereas reddish/brown to yellow indicates enzymatic activity of α-amylase. All experimental results were interpreted within the framework of valid positive and negative controls.

Phadebas® Amylase Test

Magle Life Sciences (available directly through http://www.Phadebas.com).

Mechanism of Action—Utilized water insoluble starch covalently linked to a blue dye. The hydrolysis of the blue dye linked

to starch substrate by α-amylase releases blue dye into solution which is detected following a centrifugation step.

Sample and Test Preparation—Phadebas® tablets were crushed and *c*. 0.02 g of crushed Phadebas® material was added to autoclaved 1.5 mL tubes. Next, 550 μL of sterile ddH_2O was added to each tube and vortexed to make a slurry. A 5 mm^2 cutting or *c*. 1/6 swab of sample 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 tube, vortexed, and incubated at 37°C for 30 min. After incubation, the samples were vortexed again and centrifuged at 13,000 rpm for 2 min.

Interpretation—The appearance of a blue color in the supernatant following the centrifugation step indicates α-amylase activity in the sample stain/swab and therefore a presumptive indication of saliva. A colorless supernatant solution indicated an undetectable level of α-amylase activity. All experimental results were interpreted within the framework of valid positive and negative controls.

Classification Guidelines for Results of Amylase Tests

The classification system for a positive, weak-positive, and trace-positive result was normalized as much as possible across the study parameters.

A positive classification yielded results similar to the appearance of the positive control of neat saliva. Weak-positive classification was based on a light or lighter color change for Phadebas® and SALIgAE®, whereas starch-iodine weak results were because of yellow-brown, reddish-brown, and light brown colors. Trace-positive classification was based on faint color changes to blue or yellow for Phadebas® and SALIgAE® tests and a dark brown color change for starch-iodine. Negative results matched the negative control in all cases.

Results

Sensitivity

International Units of Activity—According to the Whitehead and Kipps study, amylase concentration in human saliva ranges between 0.072 and 1.3 IU/μL with an average of about 0.35 IU/μL (6). With reference to Fig. 1, the sensitivity limit of each detection method was measured using α-amylase standard which demonstrated that SALIgAE® had a sensitivity down to 1.16 IU/μL whereas starch-iodine had a limit of 0.0116 IU/μL and Phadebas® had a limit of 0.0046 IU/μL. The sensitivity limit of 1.16 IU/μL for SALIgAE® is considerably higher than 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. Relating the amylase standard units to dilutions of saliva, the starch-iodine sensitivity limit was equivalent to 1:30 dilution of neat saliva whereas Phadebas® limit was equivalent to 1:75 dilution of neat saliva.

Known Saliva—The sensitivity for each method was also determined in relation to dilutions of sequestered neat saliva. Refer to Figs. 2 and 3 which demonstrate that both known saliva dilution sets demonstrated similar results across each detection method. SALIgAE® yielded results with a sensitivity to a dilution of 1:10, whereas starch-iodine results showed sensitivity to a dilution of 1:50 and Phadebas® demonstrated a sensitivity limit for dilutions as low as 1:200.

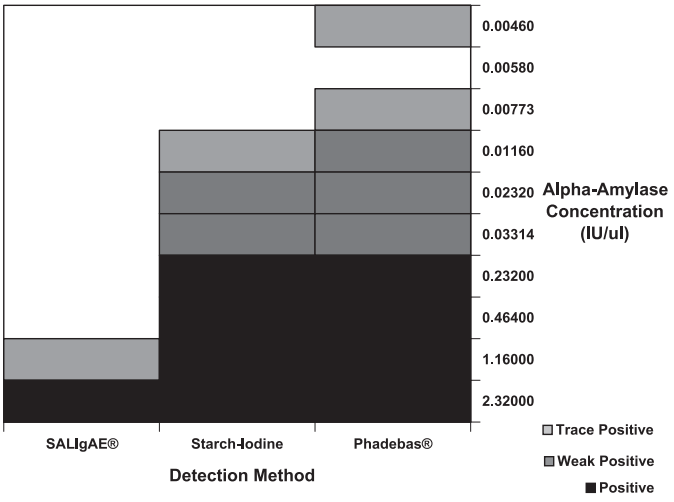

FIG. 1—*Amylase standard sensitivity. The color black corresponds to a positive result, dark gray to weak-positive-, and light gray to a trace-positive result. White corresponds to background as well as to 0.00580 for Phadebas®, indicating a negative result for the presence of amylase at this dilution of amylase standard. IU/μL = International units of activity per μL, concentration of α-amylase present in the sample.*

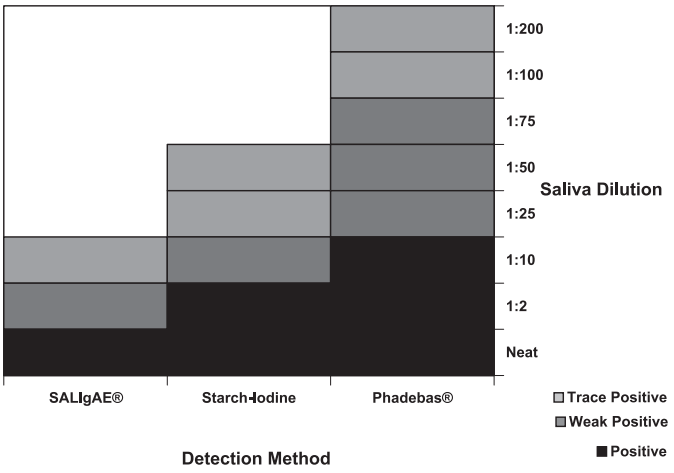

FIG. 2—*Known saliva sensitivity (Set 1). The color black corresponds to a positive result, dark gray to weak-positive-, and light gray to a trace-positive result.*

SALIgAE® sample vials from the sensitivity trials were kept for a period of time following the completion of the test (10-min mark). Of interest, those sensitivity vials containing known amounts of saliva demonstrated increases in yellow color intensity beyond the 10-min mark whereas the negative control vials for SALIgAE® never deviated from the clear color expected (personal observation). This suggests that the test is sensitive enough to detect the saliva present in the sample, just not within the 10-min time limit (data not shown).

Specificity

Table 1 demonstrates the specificity results for each detection method. The SERI stain set samples, including sperm positive, sperm negative, vaginal swab, and 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

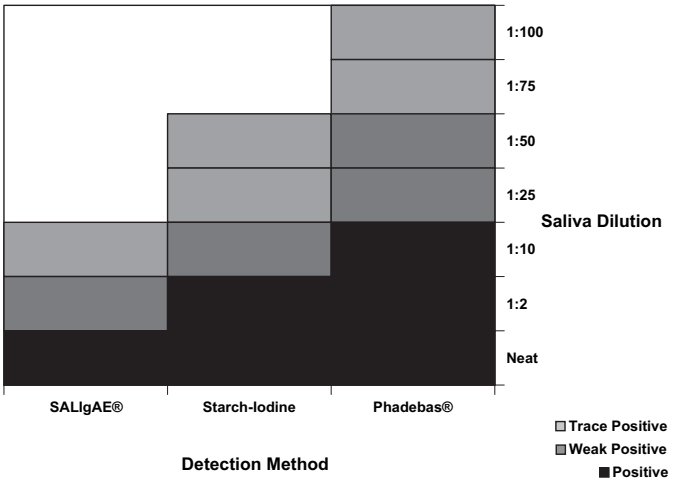

FIG. 3—*Known saliva sensitivity (Set 2). The color black corresponds to a positive result, dark gray to weak-positive-, and light gray to a trace-positive result.*

TABLE 1—*Specificity results.*

	SALIgAE®	Starch-Iodine	Phadebas®
SERI blood stain	Negative	INC*	INC*
SERI breast milk	Negative	Trace positive	Weak positive
Saliva swabs, guinea pig	Positive	Positive	Positive
Saliva swabs, pot belly pig	Negative	Trace positive	Negative
Saliva swabs, rat	Weak positive	Positive	Positive

*INC, Inconclusive results due to the interfering presence of blood in the sample extract before test results were to be read.

whereas the SALIgAE® method yielded a negative result, which was diluted to 1.5 mL to achieve a colorless extract. The SERI breast milk stain demonstrated negative, trace-positive-, and weak-positive results with SALIgAE®, starch-iodine, and Phadebas®, respectively. The guinea pig and rat saliva yielded a positive result with starch-iodine and Phadebas® and a weak-positive result with SALIgAE®.

Mixtures

Referring to Table 2, all three saliva screening methods yielded positive results for the indication of saliva in the set of dilutions with blood or semen. With respect to the recommended protocol for SALIgAE®, a second set of tests was run for saliva:blood mixtures at the 1:5 and 1:10 dilutions to test the mixtures at a colorless state because of much greater proportion of blood in the mixture.

TABLE 2—*Mixture study results.*

	SALIgAE®	Starch-Iodine	Phadebas®
Saliva: semen (1:2)	Positive	Positive	Positive
Saliva: semen (1:3)	Positive	Positive	Positive
Saliva: semen (1:5)	Positive	Positive	Positive
Saliva: semen (1:10)	Positive	Positive	Positive
Saliva: blood (1:2)	Positive	Positive*	Positive
Saliva: blood (1:3)	Positive	Positive*	Positive
Saliva: blood (1:5) #1	Positive	Positive*	Positive
Saliva: blood (1:5) #2**	Weak positive	N/A	N/A
Saliva: blood (1:10) #1	Positive	Positive*	Positive
Saliva: blood (1:10) #2**	Trace positive	N/A	N/A

*Positive results indicate that the sample extract with starch had a yellow tint before the addition of iodine solution.

** Saliva: Blood mixtures #2 were diluted to 1.5 mL before adding 8 μL of extract to the SALIgAE® test vial

TABLE 3—*Casework sample results.*

	SALIgAE®	Starch-Iodine	Phadebas®
Water bottle swab 1	Weak positive	Weak positive	Positive
Water bottle swab 2	Weak positive	Trace positive	Weak positive
Soda can swab 1	Positive	Positive	Positive
Soda can swab 2	Trace positive	Weak positive	Weak positive
Cigarette butt 1	Positive	Positive	Positive
Cigarette butt 2	Positive	Positive	Positive
Vulva swab	Weak positive	Weak positive	Weak positive
Thigh swab	Weak positive	Weak positive	Positive
Breast swab	Weak positive	Weak positive	Positive

Casework Simulated Samples

Of specific interest for the casework simulated samples were the thigh and breast swabs which were collected 8 h postincident and yielded results across each detection method with Phadebas® showing a greater change in color intensity. Refer to Table 3 for a summary of the remaining results for casework type samples.

Discussion

Results Discussion

The SALIgAE® detection method consistently demonstrated an inability to detect saliva down to similar sensitivity levels yielded by both Phadebas® and starch-iodine presumptive saliva tests. SALIgAE® was at least a factor of five less sensitive compared to the other two methods in each set of data. Possible reasons for this marked decrease in sensitivity could be because of protocol modifications in reference to SALIgAE®'s technical information sheet, issues with small working volumes, or poor sample extraction. Through observation, the SALIgAE® test was further limited by the recommendation that the results be determined at the 10-min mark.

A major challenge to presumptive saliva testing has been because of the contaminating presence of blood in a saliva mixture or alone through specificity testing. The SALIgAE® test protocol indicated that any blood-contaminated extract should be diluted until it is colorless, as the test results are based on a yellow color change. The blood-containing samples were diluted to *c*. 1 and 1.5 mL before adding the 8 μL to the test vials. The further dilution of the 1:5 and 1:10 (mixtures of saliva to blood ratio) seemed to only affect the color intensity of the positive result in comparison to the undiluted saliva (blood mixture). Furthermore, the SERI Blood Stain standard was negative at 10 min while the starch-iodine and Phadebas® methods were inconclusive because of the interfering presence of blood and the corresponding color of the sample extract.

The negative result for SERI Breast Milk standard, which is known to contain detectable levels of α-amylase with SALIgAE® could be attributed to the comparatively low sensitivity discussed earlier. This negative result in combination with comparatively poor sensitivity concerning the α-amylase standard raise the possibility that SALIgAE® may not detect α-amylase activity as the nature of the mechanism is unknown. The test may be screening for another component of saliva although the consistent results of the sensitivity data between the α-amylase standard and known saliva dilutions would suggest that the detection of α-amylase activity is at least a part of the mechanism.

Specificity results for other body fluids yielded expected results across each presumptive method. Animal saliva samples containing α-amylase from guinea pig and rat (1) also demonstrated positive

results with each method. The differences between the detection methods were reflected in the strength of the positive which could also be attributed to the sensitivity of the method.

Protocol Modifications and Sample Consumption

An extraction volume of 50 μL was applied to both cuttings and swabs for preparation of samples for the SALIgAE® test. In the opinion of the authors, SALIgAE®'s recommended volume of 30 μL was not enough to saturate or submerge the cutting or swab, which could reduce extraction efficiency. Possible ways to reduce this volume to the suggested protocol could have been to cut the sample into smaller sizes for extraction or apply a spin basket to remove any liquid from the material. Another change is concerned with the use of about 1/6 of a swab instead of consuming the recommended 1/2 of a swab. In addition to the dilution of extraction volume, a reduction in sample size for that extraction could potentially raise an issue concerning the relative sensitivity of SALIgAE®. The protocol for SALIgAE® was modified to keep the sample size as well as the amylase or saliva concentration the same across each presumptive test method. Consistency between the three methods was considered to be more important for an accurate comparison of sensitivity and specificity results rather than strict adherence to the recommended protocol. In terms of casework application, sample conservation could mean the difference in yielding an interpretable profile through DNA analysis.

Interpretation Issues

Color-based presumptive testing relies on the ability to attribute a certain color with a positive or negative result. While the starch-iodine test interpretation was straightforward at high concentrations of α-amylase activity (obvious yellow color change), the test was challenging to interpret for weak to trace positives. The transition from the negative color of deep blue/purple to yellow is dependent upon the amount of α-amylase activity present in the sample and therefore the test yields a range of colors from yellow, yellow-red, reddish-brown, and light brown for positive results. Additional issues with starch-iodine interpretation concerned the inconclusive results with blood-containing samples in which the reddish-brown color of the blood interfered with the interpretation of the test.

Phadebas® demonstrated the most objective interpretation in combination with highest sensitivity. Interpretation of SALIgAE® test results was relatively straightforward as well, but the ease increased as the intensity of the yellow color change changed beyond the 10-min mark. Both Phadebas® and SALIgAE® methods have a clear negative control which allowed an easier determination of a color change from clear to either blue or yellow.

Simplicity

The easiest test to perform was starch-iodine because of minimal sample preparation and short incubation period. SALIgAE® required some additional sample preparation, tube labeling, and transferring than the starch-iodine. Phadebas® required the preparation of Phadebas® slurry in addition to a few more pipetting steps along with a 2-min final centrifugation step. In terms of simplicity, SALIgAE® and Phadebas® were very comparable.

Cost-effectiveness

The most cost-effective test for saliva presumptive testing is the starch-iodine method. Though the exact number of tests per dollar has not been calculated, the reagents required for the in-house preparation of both starch and iodine solutions are typically readily available in most forensic labs and last longer than tablets or kits because of the long shelf-life of the reagents themselves before preparation into solutions. Additionally, the mini-centrifuge method presents a smooth transition from the radial diffusion method with little or no additional cost, decrease in time for plate development, and more rapid indication of saliva presence.

Phadebas® method presents another cost-effective option for saliva presumptive testing. An order of fifty tablets is US$113.00 (7), but our laboratory uses *c.* 0.02 g/test giving *c.* 550 tests per fifty tablets with an average of about five tests per dollar.

SALIgAE® test kits are valued at $47/10 vials (8) with *c.* one test for $5.

Probable Sources of Error

In addition to points already discussed such as protocol modifications, there are intrinsic errors in any laboratory experiment such as measurement and pipette error. It is possible that the dilutions prepared as stains on the fabric swatches did not contain equal quantities of saliva or amylase. Also a possibility is that the cuttings taken to compare each method were not of the exact size although the cuttings were taken from the same area for each test preparation to minimize such variation.

Conclusion

Short tandem repeat-polymerase chain reaction analysis has afforded forensic biology the advantage of yielding DNA results from extremely small stains. A goal of any physical evidence examination through serological methods is to perform presumptive testing to identify the stains that have the greatest potential to yield probative DNA profiles. The probative information is not only limited to DNA results but also with the indication of the body fluid present. Still, methods used for the search for biological stains have to continually balance effectiveness, sensitivity, and specificity against the consumption of potentially useful sample for further testing.

From this study, certain modifications to the SALIgAE® protocol concerning extraction method, sample volume addition, and length of time for color development could vastly improve the sensitivity of the test. The issues with starch-iodine concerning interpretation of gradated color changes will persist as long as this form of the test is used and is best at determining high levels of α-amylase with an obvious yellow color change.

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. Any disadvantages of test in relation to sample preparation were far outgained by the ease of interpretation and the clarity of results. The sensitivity of Phadebas® affords the analyst the opportunity to determine the possible presence of saliva with minimal sample consumption which enables additional testing on the sample such as DNA analysis.

Disclaimer

Neither the authors nor the Miami-Dade Police Department Crime Laboratory Bureau endorses one specific product over any other for the purposes of saliva screening.

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