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TECHNICAL NOTE

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Developmental Validation of RSID™-Saliva: A Lateral Flow Immunochromatographic Strip Test for the Forensic Detection of Saliva

ABSTRACT: Current methods for forensic identification of saliva generally assay for the enzymatic activity of α-amylase, an enzyme long associated with human saliva. Here, we describe the *R*apid *S*tain *ID*entification (RSID™-Saliva), a lateral flow immunochromatographic strip test that uses two antisalivary amylase monoclonal antibodies to detect the presence of salivary amylase, rather than the activity of the enzyme. We demonstrate that RSID™-Saliva is accurate, reproducible, and highly sensitive for human saliva; RSID™-Saliva detects less than 1 μL of saliva. The sensitivity of RSID™-Saliva allows investigators to sample a fraction of a questioned stain while retaining the majority for DNA-STR analysis. We demonstrate that RSID™-Saliva identifies saliva from a variety of materials (e.g., cans, bottles, envelopes, and cigarette-butts) and it does not cross-react with blood, semen, urine, or vaginal fluid. RSID™-Saliva is a useful forensic test for determining which evidentiary items contain saliva and thus may yield a DNA profile.

KEYWORDS: forensic science, forensic biology, salivary alpha amylase, lateral flow strip test

The identification of human body fluids—blood, saliva, and semen—has long been important for forensic investigations. Body fluid identification can be used to (a) reconstruct what may have occurred during the crime and/or (b) to determine which items of evidence should be processed further for DNA-STR testing. Human saliva can be deposited at crime scenes, or on peripheral items that might have probative value, such as envelopes, aluminum cans, glass or plastic bottles, coffee mugs, or fabric and is generally a suitable biological source for obtaining a DNA profile. Current forensic methods for human saliva detection have significant drawbacks including lack of specificity, lack of sensitivity, and labor-intensive protocols. Furthermore, testing for biological fluids such as saliva with inefficient methods can consume a significant amount of a biological sample. A simpler, more specific, and more integrated method is needed; therefore, we chose to develop an immunochromatographic lateral flow assay directed against an antigen found in high quantity in saliva: α-amylase.

In normal individuals, saliva is produced in high quantity—estimated at the rate of *c.* 0.5–1.5 L per day—and is comprised of more than 99% water and <1% proteins and salts (1). Saliva is produced mostly from three major salivary glands: the parotid, the submandibular, and the sublingual glands, which together account for *c.* 90% of fluid production in the oral cavity. The most characteristic enzyme of saliva is α-amylase, which digests complex carbohydrates to maltose by cleaving α-1–4 glycosidic bonds (2). α-Amylase aids in the digestion of dietary starches and current crime laboratory methods used to identify saliva are activity-based. Given the ubiquity of α-amylase activity, which can be found in fungi, bacteria, and in pancreatic secretions (3–5), we sought a different approach to identify saliva: antigen identification.

Bio-marker identification for the forensic detection of body fluids is well established, e.g., PSA/p30/semenogelin for the presence of semen, and hemoglobin/glycophorin A for the presence of blood. Antigen detection for saliva has advantages over enzyme activity-based detection in sensitivity, specificity, and ease of use. For example, *Homo sapiens* possess two main isozymes of α-amylase, salivary and pancreatic (3,6) that activity-based detection methods cannot distinguish. Furthermore, activity-based tests cannot differentiate between the many nonhuman sources of this enzyme, such as bacterial, pancreatic, fungal, or nonhuman saliva.

When considering saliva-specific antigens that could be used to develop an antigen-based test, α-amylase has advantages including acceptance by the forensic community, and the availability of highly specific mouse monoclonal antibodies directed against the protein.

Here we present the developmental validation and testing of the *R*apid *S*tain *ID*entification Test for Saliva (RSID™-Saliva), a lateral flow immunochromatographic strip test (Independent Forensics, Hillside, IL) designed to detect the presence of human salivary α-amylase, an enzyme found at high levels in human saliva. The RSID™-Saliva test is accurate, reproducible, easy to use, and employs two antisalivary amylase monoclonal antibodies in a lateral flow format to detect the presence of salivary α-amylase protein, rather than the activity of the enzyme (7). Here, we detail studies on the sensitivity, body fluid specificity, species specificity, and stability of RSID™-Saliva. Additionally, we demonstrate the ability of the test to detect human saliva from a variety of substrates and surfaces that are typically encountered in forensic laboratory case-work. Importantly, RSID™-Saliva's detection limit of 1 μL of human saliva has been calibrated such that if the test is positive, it is likely that there is sufficient biological material present to yield a DNA profile. In addition, the high sensitivity of RSID™-Saliva minimizes consumption of biological material

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allowing the majority of each sample to be processed for DNA analysis.

Materials and Methods

Materials

Laminar flow immunochromatographic strip test production equipment was purchased from Biodot (Irvine, CA) and used according to manufacturer's recommendations. Test strip components, including glass fiber conjugate pads and cellulose wicks were purchased from Millipore (Billerica, MA). Test strip membranes were purchased from Whatman (Florham Park, NJ). Detection antibodies were labeled with colloidal gold (particle size: 40 nm) made by reduction of hydrogen tetrachloroaurate with sodium citrate. Goat antimouse IgG, used at the control line of RSID™-Saliva, was purchased from Sigma (St. Louis, MO).

RSID™-Saliva Buffer Components

RSID™-Saliva extraction buffer is designed to efficiently extract the protein α-amylase from questioned stains and swabs. RSID™-Saliva running buffer is designed to dissolve the antibody-colloidal gold conjugate from the conjugate pad, maintain an extract at the appropriate pH, and facilitate correct running of the test. Components of the extraction and running buffer include buffer and salts (Tris, NaCl, and KCl) for physiological stability, a chelating agent (ethylenediamine tetra-acetic acid) for stability, detergents and surfactants (Triton X-100 and Tween 20) for extraction efficiency and solubility maintenance, protein (bovine serum albumin) for reducing nonspecific adsorption and loss, and a preservative (sodium azide).

Configuration of the Salivary Amylase Lateral Flow Test

The RSID-Saliva™ test is an immunochromatographic assay that uses two monoclonal antibodies specific for human salivary α-amylase. The system consists of overlapping components (conjugate pad, membrane, and wick), assembled such that the tested fluid is transported from the conjugate pad to the membrane and is finally retained on the wick (see Fig. 1). The conjugate pad and membrane are pre-treated before assembly such that the user need only add his/her extract in running buffer to initiate the test. Once the tested sample is added to the sample window, the running buffer and sample diffuse through the conjugate pad, which has predispersed colloidal gold-conjugated antihuman salivary α-amylase monoclonal antibodies. The sample redissolves the colloidal gold-labeled anti α-amylase antibodies, which will bind salivary α-amylase if it is present in the sample. Salivary α-amylase-colloidal gold antibody complexes are transported by bulk fluid flow to the membrane phase of the test strip. These complexes, if present, migrate along the membrane and are bound at the "test line" by the second anti-salivary α-amylase antibody, creating a red "line" (see Fig. 1; note that it depicts an RSID™-Saliva strip test that has already been developed with saliva present in the sample and therefore the test and control lines are visible on the membrane; test and control lines are not visible on an unused strip test).

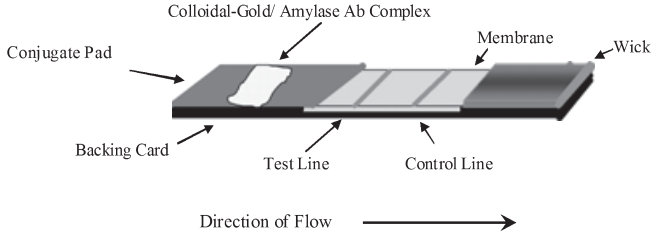

FIG. 1—*Schematic diagram of an assembled RSID™-Saliva strip test. The components of the RSID™-Saliva strip test encased within a plastic cassette are shown. The test consists of three main components (wick, membrane, and conjugate pad) adhered to a backing card. The colloidal-gold/amylase antibody conjugate is dispersed on the conjugate pad, and the test and control line antibodies are striped on the membrane. The direction of bulk liquid flow is indicated.*

Uncomplexed colloidal gold-labeled mouse antibody will progress along the membrane and be bound by antimouse antibody at the "control line," again creating a red line. A red line at the "test" position indicates the presence of human saliva, while a red line at the "control" position indicates that the strip test is working as designed. When performed correctly and functioning properly, all RSID™-Saliva test strips should produce a line at the control position.

The control line is made by "striping" goat antimouse antibody onto the membrane component of the lateral flow strip test; the deposited antibody will retain colloidal-gold anti-α-amylase mouse monoclonal antibody that migrates past the test line. The line closest to the sample well is the test line and indicates that human α-amylase is present in the sample. The test line is made by "striping" a mouse monoclonal anti-α-amylase antibody onto the membrane component of the strip test; complexes of colloidal gold-labeled anti-α-amylase mouse monoclonal antibody that are formed in solution upon addition of the sample to the sample well and have progressed through the conjugate pad and membrane (or allowed to wick up the conjugate pad when the strip is tested outside of a plastic housing, e.g., in a 12 × 75-mm test tube) will be retained at the test line. A red control line must be visible at 10 min after sample addition in order to interpret results.

Quantification of the Salivary Amylase Lateral Flow Test

In order to maintain test-to-test consistency and to reduce operator-induced bias, strip test results were quantified by comparing the intensity of the control and test lines to a reference set of red lines drawn with increasing intensity (with line no. 10 exhibiting the darkest red color). In addition, a digital picture of the results was also recorded: both quantitative and pictorial results are presented. Note: RSID™-Saliva is *not* a quantitative test for the amount of saliva present in a given sample.

Specimens

Human saliva, blood, and urine samples were obtained voluntarily from laboratory staff and deposited on sterile cotton swabs in aliquots of 50 μL. Unwashed semen was obtained from a local sperm bank and deposited on sterile cotton swabs in aliquots of 50 μL. Human breast milk samples were obtained from SRI (Richmond, CA). Briefly, human breast milk was collected from lactating mothers in a manner that would preclude contamination and deposited on sterile cotton swabs and air dried. Human fecal samples were obtained from a library of body fluid samples obtained under institutional review board supervision. Postcoital vaginal swabs were obtained voluntarily from laboratory staff. Animal saliva samples were kindly provided by the Brookfield Zoo (Brookfield, IL).

Preparation of Body Fluid Extracts

For body fluid extracts (saliva, semen, blood, urine, and breast milk), 50 μL of fluid was deposited on a sterile cotton swab and

allowed to air-dry. The cotton batting was removed using laboratory clean technique and placed in a 1.5-mL microcentrifuge tube and extracted in 1 mL of RSID™-Saliva extraction buffer for 1 h at room temperature. Assuming 100% extraction efficiency each microliter of extract will contain 50 nL (0.05 μL) of whole fluid. Oral swab extracts were made by swabbing the inside of an individual's cheek for 10 sec with a cotton swab, and extracting the swab in 1 mL RSID™-Saliva extraction buffer for 1 h at room temperature. Negative control extracts were made in an identical manner, but omitting the addition of body fluid to the swab before extraction.

Unless otherwise specified, experimental samples were prepared by combining the noted volume of extraction solution with sufficient running buffer to produce a final volume of 100 μL (extract sample volume + RSID™-Saliva running buffer = 100 μL). Most samples were tested on strips placed in cassettes, but for photographic clarity, some experiments were performed in 12 × 75-mm test tubes; in all cases results were recorded 10 min after sample addition.

Preparation of Mock Casework Extracts

Laboratory volunteers consumed the contents of a commercial soda (packaged in an aluminum can) and a commercial cup of coffee (packaged in a Styrofoam cup with a plastic lid). The aluminum cans and plastic coffee lids were sampled using standard forensic practice: areas on the cans and lids that would have had oral contact were swabbed repeatedly with a moistened, sterile cotton swab. Swabs were subsequently air-dried in a protected environment and extracted in 300 μL of RSID™-Saliva extraction buffer for 1 h at room temperature. Twenty-five microliters of this extract was removed for RSID™-Saliva testing while the remaining extract (including the swab batting which was subsequently removed using a Spin-Eze basket) were processed for DNA extraction and multiplex STR analysis. A positive control saliva swab was processed in an identical manner as a control sample.

Three cigarette butts (samples 1, 2, and 3) collected from an outdoor smoking area were sampled by removing approximately half the circumference of the filter paper and extracting the fragmented cigarette paper in 200 μL of RSID™-Saliva extraction buffer; an aliquot (25 μL) of each extraction was used for testing with RSID™-Saliva while the majority of the extract was processed for DNA-STR analysis.

DNA Extraction and STR DNA Analysis

DNA was extracted from swabs of a plastic coffee lid and aluminum soda can, and from cigarette butt paper using a Chelex extraction protocol. The extracted DNA was amplified using Identifiler (Applied Biosystems, Foster City, CA) following a low copy number protocol. The amplification reactions were run on an ABI Prism 310 Genetic Analyzer and analyzed with GENESCAN (v. 3.7) and GENOTYPER (v 3.7) using an allele threshold of 75 relative fluorescent units.

Results and Discussion

Sensitivity Testing of RSID™-Saliva

The sensitivity of RSID™-Saliva was assessed by testing various volumes and dilutions of human saliva extract (prepared as described in Materials and Methods). Our results with RSID™-Saliva using this fluid standard were consistent, reproducible, and essentially independent of the source of saliva. Various volumes of saliva extract, made as described above, were brought to a final volume of 100 μL with RSID™-Saliva running buffer (e.g., 10 μL of extract + 90 μL of running buffer = 100 μL total) and the entire volume (extract + plus running buffer) loaded into the sample well of an RSID™-Saliva cassette (Fig. 2*A*). In some experiments, strips were tested in 12 × 75-mm test tubes, again using a 100 μL (final volume) of extract and running buffer. Results obtained from strips in cassettes or in test tubes were identical (Fig. 2*B*); strips in test tubes were easier to photograph and were used for many figures. Results of all experiments were scored relative to a standard intensity chart at 10 min.

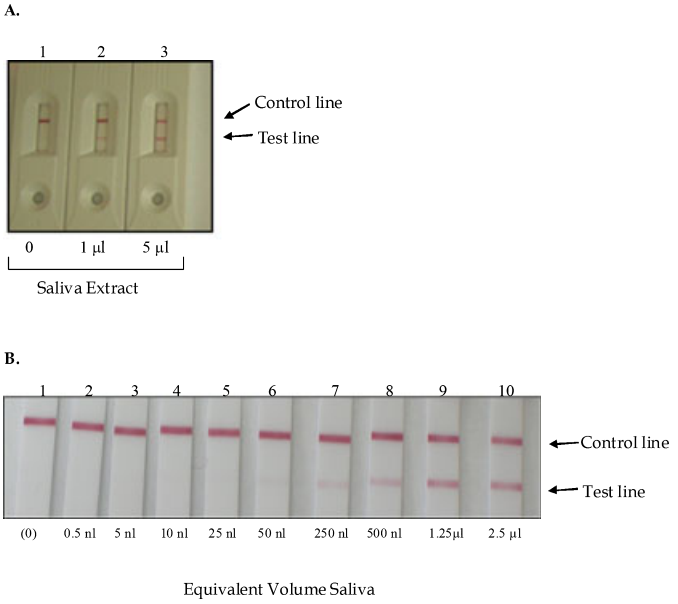

FIG. 2—*Sensitivity of RSID™-Saliva, saliva extract. (A) Strips 1–3: 0, 1, and 5 μL of saliva extract was tested with RSID™-Saliva. The position of the control and test lines are indicated. Results recorded 10 min post sample addition. (B) A series of dilutions of saliva extract were tested with RSID™-Saliva. The following equivalent volumes of saliva were tested: lanes (1) 0 nL; (2) 0.5 nL; (3) 5 nL; (4) 10 nL; (5) 25 nL; (6) 50 nL; (7) 250 nL; (8) 500 nL; (9) 1.25 μL; (10) 2.5 μL. The position of the control and test lines are indicated. Results recorded 10 min post sample addition.*

Using saliva extract prepared and tested as described, the RSID™-Saliva limit of detection was *c.* 50 nL of human saliva (Fig. 2): test line intensity scores at 10 min of *c.* 0, 4, and 8, were recorded for 0, 1, and 5 μL of saliva extracts, respectively, equivalent to 0, 50, and 250 nL of human saliva (Fig. 2*A*). Additional tests with every production lot of RSID™-Saliva were consistent with this low limit of detection and control line appearance (data not shown). Results from independent production lots demonstrated further that control line intensities remained constant (intensity scores of *c.* 8 or 9), indicating consistent lot to lot test performance (data not shown) and a consistent sensitive limit of detection for RSID™-Saliva.

In order to more precisely determine the limit of detection of RSID™-Saliva and to demonstrate that RSID™-Saliva can detect a broad range of saliva volumes (0–50 μL), positive control extract was tested corresponding to 0–2.5 μL of saliva (Fig. 2*B*). A clear positive signal at the test line can be observed for extract volumes corresponding to 50 nL, 250 nL, 500 nL, 1.25 μL, and 2.5 μL equivalent volume of saliva (Fig. 2*B*, strips 6–10, respectively); for clarity, these experiments were performed in 12 × 75-mm test tubes. The intensity of the test line increased with increasing volumes of extract tested. This also demonstrates that RSID™-Saliva can detect saliva over a range of more than 50-fold: from 50 nL to

2.5 μL of saliva. Also, Fig. 2*B* confirms the detection limit of RSID™-Saliva to be *c.* 50 nL (0.05 μL), which is equivalent to *c.* 1/1000th of a drop.

By comparing RSID™-Saliva results using saliva extracts and commercially available semipurified salivary α-amylase (Sigma Chemical Co., St. Louis, MO), we sought to determine the amount of α-amylase in human saliva; while the levels of enzymatic activity of α-amylase in saliva is cited extensively in the scientific literature, the *amount* of the enzyme in saliva is not well documented. Using RSID™-Saliva and semi-purified material, we estimate that human saliva contains *c.* 1 ng salivary α-amylase per microliter of saliva (data not shown). A common observation with many strip tests is that authentic body fluids react differently than semi-purified or purified biomarkers when tested on lateral flow devices. Based on similar observations that extracts from swabs absorbed with whole saliva produce stronger results with RSID™-Saliva than solutions of purified components, our estimate of the concentration of α-amylase in saliva cannot be refined further. We hypothesize that the increased sensitivity seen from saliva extracted from swabs as compared with solutions of semi-purified α-amylase preparations is due to the retention of inhibitors on the cotton batting, and the protein complexity of authentic saliva. Our consistent observation of reduced background and enhanced sensitivity of RSID™-Saliva when actual saliva extracts were tested as compared with semi-purified α-amylase preparations demonstrates the importance of using samples that better approximate real world evidence for the evaluation and testing of RSID™-Saliva. The use of saliva extracts more closely resembles "authentic" samples likely to be encountered in case work, and is therefore a better preparation for laboratory validation studies.

Specificity of RSID™-Saliva: Testing Noncognate Body Fluids

In order to evaluate potential cross-reaction or inhibition of RSID™-Saliva, extracts from human body fluids (saliva [Sa], blood [Bl], semen [Se], and urine [Ur], prepared as described above) were tested on RSID™-Saliva (Fig. 3*A*). Individual extracts of saliva, blood, semen, and urine reacted as expected with only saliva extracts providing a positive result (Fig. 3*A*, strips 2–5). Combinations of extracts with or without saliva were also tested; only the mixture containing all four body fluid extracts gave a positive signal (blood, semen, urine, and saliva;

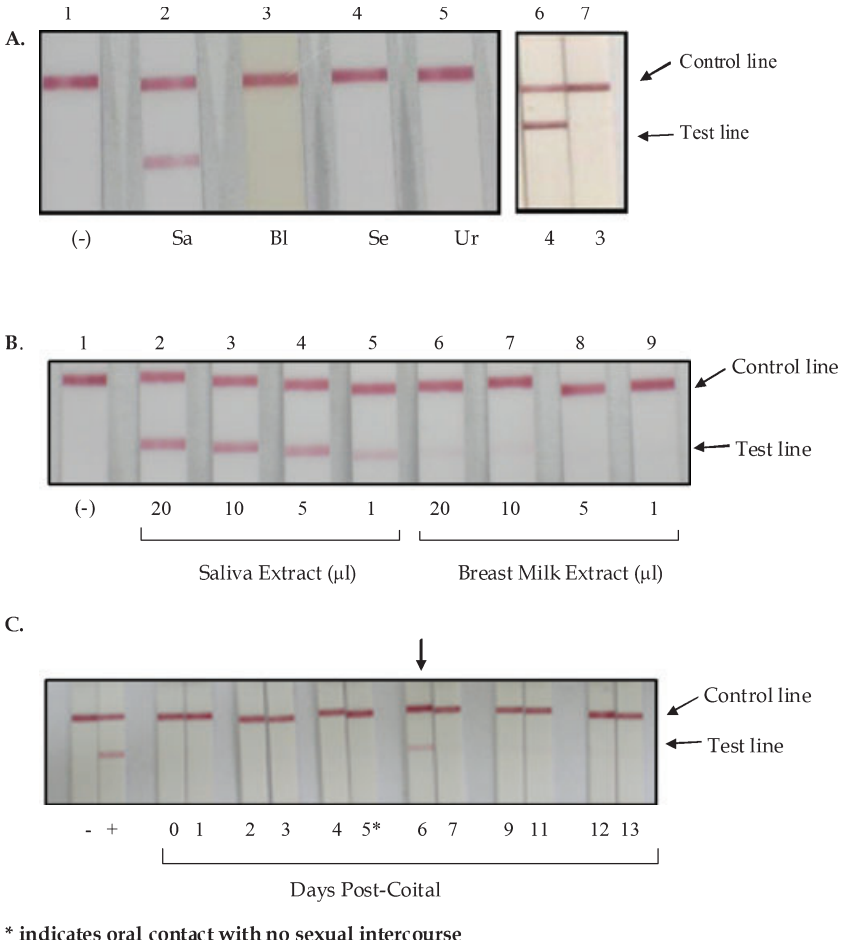

FIG. 3—*Specificity of RSID™-Saliva. (A) Testing noncognate body fluids: 25 μL each from extracts of saliva (Sa, lane 2), blood (Bl, lane 3), semen (Se, lane 4), and urine (Ur, lane 5) swabs were tested alone, or as a mixture (4, lane 6). A mixture of 25 μL each of urine, semen, and blood extracts was also tested (3, lane 7). Lane 1 is a negative control. (B) Cross-reactivity with human breast milk: 20, 10, 5, and 1 μL saliva swab extract were tested on RSID™-Saliva (lanes 2–5); 20, 10, 5, and 1 μL breast milk swab extract were analyzed side by side for comparison (lanes 6–9). Lane 1 is a negative control. (C) Testing extracts from vaginal swabs: 20 μL extract from vaginal swabs obtained at days 0–7, 9, 11–13, postcoital were tested on RSID™-Saliva. Arrow designates positive signal from RSID™-Saliva on postcoital day 6, following oral contact reported on day 5. Lanes 1 and 2 are negative and positive controls (5 μL saliva extract).*

Fig. 3*A*, strip 6), while the mixture of blood, semen, and urine produced only a band at the control line with no visible signal at the test line (Fig. 3*A*, strip 7). Again, strips were analyzed in 12 × 75-mm test tubes for photographic clarity; identical results were obtained with strips held in plastic cassettes (data not shown). Sufficient volumes of extract, 25 μL of each extract equivalent to 1.25 μL of each body fluid, were tested to insure that even low levels of cross-reactivity would be observed, if present. For comparison, a negative control was included in the experiment (Fig. 3*A*, strip 1). As an additional test of specificity, extracts of saliva, blood, semen, and urine were combined at different ratios (1:1, 1:3, and 3:1) and tested with RSID™-Saliva. Again, RSID™-Saliva did not cross-react with mixed extracts from urine, blood, or semen at any ratio tested (data not shown). Taken together, these experiments demonstrate that RSID™-Saliva does not cross-react with the body fluids tested. The presence of semen, blood, and urine does not interfere with the detection of saliva, an important issue as multiple body fluids are often present on evidence collected at crime scenes.

Specificity of RSID™-Saliva: Detection of Salivary α-Amylase in Human Breast Milk and Fecal Samples

It is well documented that human breast milk contains low levels of salivary α-amylase that is probably present as an aid to carbohydrate digestion in infants (8,9). Therefore, we tested if human breast milk would give a positive signal with RSID™-Saliva. Samples of human breast milk (50 μL) (kindly provided by SRI and described in Materials and Methods) were extracted and various volumes of breast milk extract—1, 5, 10, and 20 μL, equivalent to 0.05, 0.25, 0.5, and 1.0 μL of human breast milk—were analyzed with RSID™-Saliva and compared side by side with equivalent volumes of authentic human saliva (Fig. 3*B*, strips 1–9).

As expected, RSID™-Saliva demonstrates a weak positive result with extracts prepared from human breast milk (Fig. 3*B*, strips 6–8). By comparing equivalent volumes of saliva and human breast milk (Fig. 3*B*, strips 2–5), we estimate that breast milk is at least 20-fold less reactive on RSID™-Saliva than authentic human saliva (Fig. 3*B*, strips 2 and 6, strips 3 and 7).

As the majority of saliva is swallowed, we expected RSID™-Saliva to detect salivary α-amylase in fecal samples. Six fecal samples from a human stain library were extracted in 1 mL RSID™-Saliva extraction buffer for 1 h at room temperature and 5, 20, and 100 μL of extract were analyzed with RSID™-Saliva. One-hundred microliters of extract from each of the six samples showed a weak positive while the other extract volumes were negative (data not shown). In the same experiment, 1 μL of saliva extract (50 nL of equivalent saliva) produced a strong positive, indicating that saliva is many times more reactive on RSID™-Saliva test strips than fecal samples. Because of the unknown amount of fecal matter present on the swabs, direct quantitative comparison with α-amylase levels in saliva is not possible. This finding must be considered when anal swabs from sexual assault evidence kits are tested with RSID™-Saliva.

A significant disadvantage of using α-amylase as a forensic indicator for saliva is the distribution of this enzyme in human breast milk and feces, thereby making any test using α-amylase a presumptive test. When using RSID™-Saliva, some conclusions based on the signal intensity must be carefully considered. Fecal swabs tested on RSID™-Saliva only generate a weak RSID™-Saliva positive, as do human breast milk samples. A weak RSID™-Saliva positive signal can indicate either minimal amounts of saliva, a fecal sample or breast milk sample, or inefficient sample extraction. RSID™-Saliva cannot overcome the biological distribution of α-amylase, but as the relative concentration of α-amylase varies considerably between these three body fluids, a strong positive RSID™-Saliva result indicates, but does not prove, the presence of saliva.

Specificity of RSID™-Saliva: Testing Extracts from Vaginal Swabs

The ability to detect human saliva from sexual assault evidence is an important issue for forensic scientists. Therefore, we tested the ability of RSID™-Saliva to reliably identify saliva from a series of vaginal swabs obtained from a subject with a well-defined sexual contact history. Postcoital swabs collected at 0–7, 9, and 11–13 days following intercourse without a condom, were extracted with RSID™-Saliva extraction buffer and analyzed with RSID™-Saliva test strips. Contact history included both semen deposition (day 0) and oral contact (day 5). To increase the stringency of the test, swabs were extracted in 300 μL of extraction buffer and 20 μL of this extract was combined with 80 μL of RSID™-Saliva running buffer and then tested on RSID™-Saliva test strips.

The results clearly demonstrate that in this sample set, RSID™-Saliva does not cross-react with postcoital vaginal swab extracts as no signal was observed from samples taken 0–4, or 5 days post intercourse (Fig. 3*C*, strips 0–5, respectively). However, oral contact on day 5 was confirmed using RSID™-Saliva when day 6 vaginal swabs were tested (Fig. 3*C*, strip 6 designated by arrow). No other RSID™-Saliva positive samples were observed from this experimental series, demonstrating the specificity of RSID™-Saliva; RSID™-Saliva results correlated precisely with the known sexual history of the samples. The lack of cross-reactivity of the vaginal fluid extracts observed in this experiment is representative of results seen with over 20 additional subjects, in which no signal was detected in extracts from vaginal swabs with no reported presence of semen (data not shown). This supports the conclusion that RSID™-Saliva does not cross-react with vaginal fluid.

These data indicate that using mock sexual assault samples, RSID™-Saliva does not cross-react with semen or vaginal fluid and can easily and specifically detect saliva from collected vaginal swabs. It should be noted that we have demonstrated body fluid specificity using RSID™-Saliva for only the tested human body fluids of semen, saliva, urine, blood, and vaginal fluid as well as detection of α-amylase in breast milk and fecal samples. We have not tested RSID™-Saliva on samples obtained from cadavers or other decomposing specimens; forensic lore states that cadaver samples present particularly difficult body fluid identification issues.

Species Specificity of RSID™-Saliva: Testing of Animal Samples

Saliva swabs from various animal species, both exotic and companion animals, were kindly provided by the Brookfield Zoo. Extracts were prepared as described in Materials and Methods, and 25 μL of each extract was tested with RSID™-Saliva. No cross-reactivity was observed with saliva from the following animals: dog, opossum, guinea pig, woodchuck, cow, domestic cat, domestic rabbit, tokay gecko, cuckoo, mongoose, chameleon, domestic pig, llama, sheep, horse, goat, grey gull, ferret, hedgehog, skunk, lion, tiger, rhinoceros, marsh snake, Sykes monkey, Capuchin monkey, tamarin, and marmoset. A positive signal was obtained from the saliva of gorilla (data not shown).

High Dose Hook Effect

The high dose hook effect can induce a false negative result on some lateral flow immunochromatographic strip tests when high levels of target antigen are present in the tested sample. The false negative result due to the high dose hook effect occurs when the amount of target antigen in the sample is sufficiently high that a significant amount of target antigen remains unbound by the colloidal gold-labeled antibody in the conjugate pad. Free antigen then migrates to the membrane ahead of the labeled antibody-antigen complexes, thereby occupying the bound antibody on the test line with unlabeled antigen and leaving no sites for the gold-labeled antibody-antigen complexes. By blocking the test line with unlabeled antigen, the test result appears negative. Most forensic laboratory personnel are familiar with high dose hook effects and test a dilution of the questioned stain extract to insure that the observed result is a true negative, and not due to a high dose effect. We evaluated RSID™-Saliva with increasing amounts of saliva extract to evaluate RSID™-Saliva's response to high levels of antigen. Positive control extracts of 0, 5, 25, 50, 75, and 100 μL were prepared and run on RSID™-Saliva (Fig. 4*A*, strips 1–6, respectively). Note that at all extract volumes tested (even the equivalent of 5 μL saliva), there is no decrease in the intensity of the test line (all test lines scored 9), demonstrating a complete lack of high dose hook effect for RSID™-Saliva at all tested concentrations.

This observation led us to more stringent tests of high dose hook effect in which the concentration of the positive control extract was increased by decreasing the extract volume from 1 mL to 400 μL (2.5× more concentrated), and finally to extracting the positive control swab in 200 μL in order to produce a more concentrated extract (5× more concentrated than standard positive control). In addition, larger volumes of these more concentrated extracts were used on RSID™-Saliva test strips. These experimental approaches for preparing highly concentrated saliva extracts were designed to demonstrate the functional upper limit of saliva detection by RSID™-Saliva. For these experiments, strip tests were run in 12 × 75-mm test tubes (Fig. 4*B*). A standard positive control swab with 50 μL of saliva was extracted in 400 μL of extraction buffer and 50 μL and 150 μL of this extract was run on RSID™-Saliva strips (Fig. 4*B*, strips 2 and 3). For comparison, 20 μL of a sham extract was included as a negative control (Fig. 4*B*, strip 1). A fresh positive control swab was extracted in 200 μL and the entire extract was tested with RSID™-Saliva. Again no evidence of a high dose hook effect was observed (Fig. 4*B*, strip 4). It is important to note that the tested volumes of 150 and 200 μL are significantly above the recommended run volume, and that unless special precautions are taken, testing these volumes on a RSID™-Saliva strip test in a plastic cassette will cause the test to fail. These increased volumes of highly concentrated saliva extract were tested numerous times with the same result: no evidence of a high dose hook effect was observed with no reduction of the test line intensity (data not shown). Users of RSID™-Saliva can expect no false negative results due to high dose hook effects.

The lack of high dose hook effect will facilitate the integration of RSID™-Saliva into DNA forensic laboratory protocols, as a wide range of saliva concentrations, and stain sizes, can be tested without performing dilutions of questioned stain extracts.

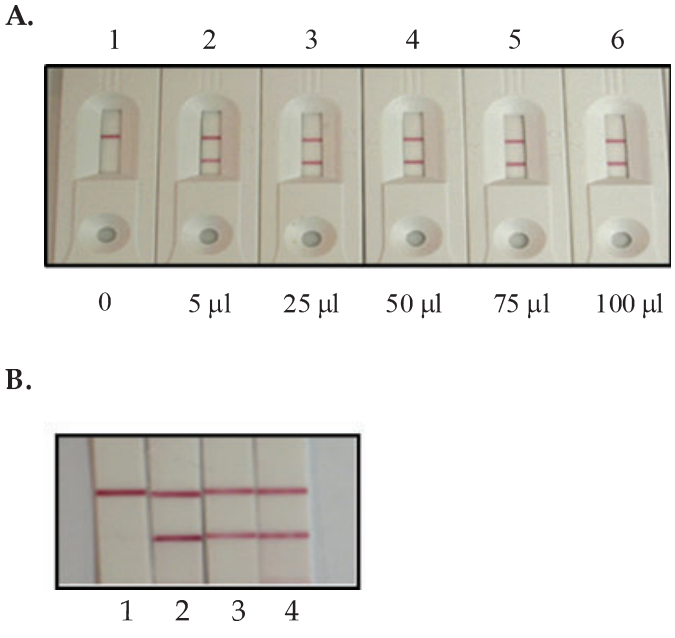

FIG. 4—*Lack of high dose hook effect of RSID™-Saliva. (A) Volumes (0, 5, 25, 50, 75, and 100 μL) of a standard positive control saliva extract—50 μL saliva on a cotton swab extracted in 1 mL RSID™-Saliva extraction buffer—were tested on RSID™-Saliva (strips 1–6, respectively). (B) Saliva (50 μL) deposited on a cotton swab was extracted in 400 μL RSID™-Saliva extraction buffer and 50 and 150 μL of extract were tested (strips 2 and 3, respectively). Saliva (50 μL) deposited on a buccal swab was extracted in 200 μL RSID™-Saliva extraction buffer and all the liquid extract (*c. *150 μL) was tested (strip 4). For comparison, a negative control was included (strip 1).*

Stability Testing of RSID™-Saliva

We have previously demonstrated that RSID™-Saliva is both specific and sensitive for human saliva detection; here the stability of the assembled strip tests is investigated by performing saliva detection with RSID™-Saliva strips that have been stored at 37°C and subjected to a heat shock at 56°C. These conditions were chosen to test for accelerated degradation of the components (storage at 37°C) and for stability in extreme shipping conditions (heat shock to 56°C).

Extracts prepared from positive control swabs were tested on RSID™-Saliva strips stored at 37°C for 30 days; 0, 5, and 25 μL of positive control extract (equivalent to 0, 0.25, and 1.25 μL of saliva) were tested with RSID™-Saliva and compared with strips stored at room temperature. In addition, stored strips were exposed to 56°C for 30 min, and then tested with all three extract volumes. No difference in sensitivity or background was observed at any extract volume tested on the strips stored under high temperature conditions, demonstrating the ability of RSID™-Saliva to withstand prolonged storage or exposure to higher temperatures (data not shown).

The ability of RSID™-Saliva to detect saliva on evidence stored or maintained at high temperatures is an important consideration as the environmental conditions of crime scenes are not controlled. Adverse environmental conditions can affect the stability of biological evidence as high temperatures (especially over prolonged periods of time) can affect the integrity of biological samples. To address this possibility, samples that were known to contain saliva (a plastic water bottle, an aluminum soda can, and a buccal swab) were stored at 37°C for 2 weeks. The beverage containers were swabbed, all samples were extracted with RSID™-Saliva extraction buffer, and 20 μL of each extract was tested with RSID™-Saliva.

No effect on the sensitivity of samples stored at 37°C for 2 weeks, as compared to samples stored at room temperature was observed (data not shown). These experiments indicate that

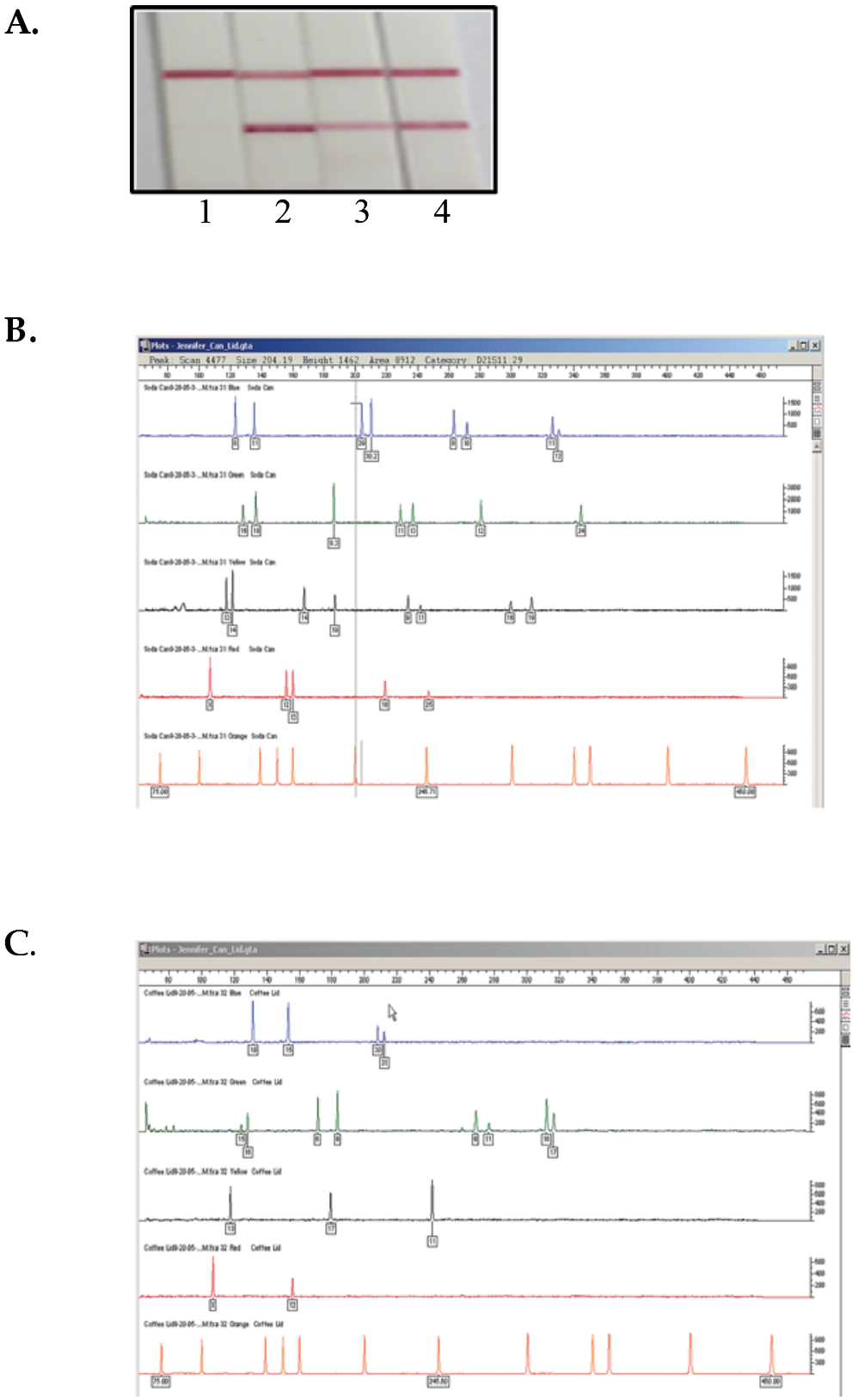

FIG. 5—*Testing of mock forensic samples with RSID™-Saliva: plastic coffee lid and aluminum soda can. (A) Extract (25 μL) from swabs of a plastic coffee lid and aluminum soda can were tested with RSID™-Saliva (strips 3 and 4, respectively). A negative control and 20 μL from saliva extract were included as a positive control (strips 1 and 2, respectively). (B) The electropherogram is shown revealing a complete DNA-STR profile (15 loci + amelogenin) acquired from the aluminum can sample. The DNA amplification reactions were run on an ABI Prism 310 Genetic Analyzer and analyzed with* GENESCAN *(v. 3.7) and* GENOTYPER *(v 3.7) using an allele threshold of 75. (C) The electropherogram is shown revealing a partial DNA-STR profile (10 loci + amelogenin) acquired from the plastic coffee lid. The amplification reactions were run on an ABI Prism 310 Genetic Analyzer and analyzed with* GENESCAN *(v. 3.7) and* GENOTYPER *(v 3.7) using an allele threshold of 75.*

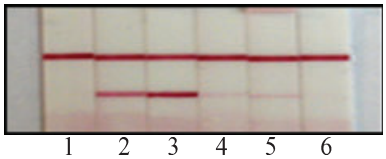

FIG. 6—*Testing of mock forensic samples with RSID™-Saliva: cigarette butts. Extract (25 μL) from three cigarette butt filter papers was tested with RSID™-Saliva (strips 4–6, respectively). A 5 and 25 μL saliva extract was included as a positive control (strips 2 and 3, respectively). Strip 1 is a negative control.*

RSID™-Saliva can detect saliva from biological evidence subject to higher temperatures, e.g., evidence collected during the summer months.

Detection of Saliva from Mock Casework Samples

We have established that RSID™-Saliva can detect saliva from laboratory-prepared control samples and from sexual assault-like evidence. Here, we demonstrate the ability of RSID™-Saliva to detect saliva from samples likely to be encountered in forensic laboratory case work including an aluminum soda can, a coffee cup lid, and cigarette butts. In addition, we show that RSID™-Saliva testing can be integrated into DNA-STR analysis and suggest laboratory protocols such that saliva detection can be conveniently performed prior to DNA-STR analysis.

Samples from a plastic coffee lid and an aluminum soda can were prepared as described in Materials and Methods. Extracts from these samples were scored at an intensity of *c.* 6 and 7, respectively, using RSID™-Saliva (Fig. 5*A*, strips 3 and 4). DNA extraction, multiplex PCR and STR analysis on an ABI310 Genetic Analyzer (see Materials and Methods for details) gave a complete DNA-STR profile (15 loci + amelogenin) from the aluminum can (Fig. 5*B*) and a partial DNA-STR profile (10 loci + amelogenin) from the coffee lid (Fig. 5*C*). Some individual variation in STR profile intensity was seen from sample to sample, and was attributed either to swabbing technique or drinking patterns. However, these results demonstrate the ability to obtain both body fluid testing data and DNA-STR results from a single swab. No effort was taken to perform additional concentration steps on the extracted DNA. These data are representative of many samples (several plastic bottles, water bottles, and envelopes) tested with RSID™-Saliva in which a DNA profile using Identifiler (ABI) was obtained following a positive RSID™-Saliva result (data not shown).

Forensic laboratories often process cigarette butts found at crime scenes; therefore, we tested this sample type using RSID™-Saliva. Three cigarette butts were processed as described in Materials and Methods (designated samples 1, 2, and 3). Positive control saliva extracts gave normal band intensities: *c.* 6 and 8 for 5 and 25 μL of saliva extract, respectively (Fig. 6, strips 2 and 3), samples 1 and 2 gave intensity scores of *c.* 2 and 3, respectively, low but clearly above background levels (Fig. 6, strips 4 and 5, respectively). Sample 3 was negative with RSID™-Saliva (Fig. 6, strip 6).

The ability of RSID™-Saliva to detect saliva from samples likely to be encountered in forensic laboratory casework is essential for laboratory validation studies and is a demonstration of the usefulness of RSID™-Saliva as a viable forensic tool for saliva detection. RSID™-Saliva can detect saliva from cigarette butt paper and extracts of swabs used to sample aluminum cans and plastic coffee lids. Furthermore, DNA-STR profiles were obtained from extracts of these samples that tested positive with RSID™-Saliva, demonstrating the ease of integrating this lateral flow strip test into forensic DNA laboratory work flow and procedures. The correlation of positive RSID™-Saliva results with STR analysis from these mock forensic case samples will enable analysts to efficiently triage crime scene evidence and to choose the best sample(s) to process for DNA-STR analysis.

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