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CASE REPORT

CRIMINALISTICS

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False-Positive Results with Amylase Testing of Citrus Fruits

ABSTRACT: In a case of robbery in which the criminals passed through the garden adorned with calamondin trees (*Citrus madurensis)*, the investigators found in the grass six calamondin fruits, some undamaged, while others apparently bitten. The fruits were collected and sent to the laboratory for DNA analysis to verify the presence of saliva and robbers' DNA profile. A specific immunochromatographic strip test for saliva confirmed the presence of human salivary α-amylase, but similar positive results were also observed for intact calamondin and other citrus fruits. Further analysis with a specific automated amylase test confirmed the absence of amylase activity. DNA quantification and typing using a specific forensic kit revealed no human DNA presence in any fruits. This case report demonstrates for the first time the occurrence of false positives when human saliva is sought on citrus fruits.

KEYWORDS: forensic science, forensic genetics, α-amylase, RSID™-Saliva, citrus fruits, false-positive amylase

Accurate identification of a biological sample is important for crime scene reconstruction and for evidence admissibility in court. Saliva is the most common DNA source in crime scenes (1) and can be recovered on different surfaces after primary, secondary, and tertiary transfer (2). In our experience, cigarette butts, stamps, and balaclavas are the most common saliva forensic samples, but this biological fluid can be also recovered in other materials such as bitten objects. Sweet et al. (3) reported positive saliva results in a case of burglary recovering DNA from a piece of yellow cheese. Anzai-Kanto et al. (4) showed that the analysis of saliva left on skin can be integrated into criminal investigation and it may have great discriminatory power.

In humans, the α-amylases are enzymes produced mainly in salivary glands and pancreas and they catalyze the hydrolysis of α-1,4-glucosidic linkages in starch to form sugars. Traditionally, two distinct isoenzymes encoded by two separate gene loci on chromosome 1 (*Amy 1* and *Amy 2*) have been described, commonly known as S (salivary) and P (pancreatic). Human salivary α-amylase is the major protein component in human saliva and is found at very high levels (5). As α-amylases are secreted into the digestive system, small amounts diffuse into the bloodstream and are eliminated through urine and perspiration; these enzymes can also be recovered in other biological fluids such as serum, male, and female reproductive tissues and feces (6). There are different presumptive saliva identification systems that can be used to detect α-amylase activity (7,8), but they do not distinguish the two human α-amylase isoenzymes or plants, bacteria, and fungi α-amylase (9). RSID™-Saliva is the most specific system to detect saliva in forensic samples; it uses two antihuman salivary amylase monoclonal antibodies in a lateral flow immunochromatographic strip format (10). This test detects the *presence* but not the *activity* of human α-amylase; as the activity could be compromised in aged or degraded forensic samples, this test appears to be very important in forensic context.

When a forensic test is employed in practice, validation is a fundamental step. RSID™-Saliva species-specificity tests with a large number of pets and animal species show a positive signal just for rat (10) and gorilla (11) saliva. Possible false-positive results with detergents when performing RSID™-Saliva test were recently investigated resulting that the test does not falsely detect amylase in clothing (12).

We report a case of robbery in a villa in which the robbers passed through the garden adorned with calamondin trees (*Citrus madurensis)*, an attractive plant commonly used for ornamental purposes. calamondin is an acid fruit growing mostly on the Philippines Islands, although it is probably of Chinese origin; it is believed to be a natural hybrid relating to the kumquat. In the West, it is usually planted as an adorned tree, growing either in the ground or in a container. The classification of Calamondin as genus Citrus is due to Tanaka (13).

In this investigation, six calamondin's fruits were found on the garden grass; three of them presented presumptive bite marks. The fruits were collected and sent to the laboratory for DNA analysis to verify the possible saliva presence and robbers' DNA profile. Due to the yellow color of the flesh fruits, presumptive tests were not performed, and samples were directly analyzed for RSID™-Saliva. Positive results were obtained in all swabs collected. However, DNA quantification and sDNA typing gave steadily consistently negative results. To explicate these contradictory results, some undamaged *Citrus madurensis* fruits recovered from the crime scene were tested with RSID™-Saliva, obtaining a strong positive result. The absence of human saliva in the casework samples was verified using an automated method (14). Further investigations were then conducted on the

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flesh of other citrus fruits of *Fortunella* and *Citrus* genus and on two commercial citrus fruit juices to test the unexpected reactivity of this lateral flow immunochromatographic method specifically designated for human saliva. Surprisingly many different citrus fruits reacted positively.

Materials and Methods

RSID™-Saliva Test Assay

The Rapid Stain Identification Test for saliva (Independent Forensics, Lombard, IL) is an immunochromatographic assay designed to detect the presence of human α-amylase using two mouse monoclonal antibodies (11). One antibody is conjugated to colloidal gold and is deposited on a conjugate pad beneath the sample window. The second antibody is stripped onto the "Test line" on a membrane attached to the conjugate pad. The "Control line" on the membrane consists of anti-mouse IgG antibody, and it is used as a positive control. The tested fluid and Running Buffer diffuse through a sample window, and a complex antigen-antibody/colloidal gold will form if human salivary amylase is present. Two lines appear for a positive result, whereas just one line (control) appears for a negative result. The results of the test were based on the independent interpretation of two analysts.

Casework Samples

All calamondin's fruits collected by investigators were analyzed. In the supposed bite marks of each fruit, a sterile dry cotton swab was applied to absorb the biological material. Sterile cotton swabs were also used on the undamaged fruits flesh, previously washed with sterile distilled water, and cut with sterile blades. Cotton swabs were extracted in 200 μL RSID™ Saliva Extraction Buffer for 2 h at room temperature. Twenty microliters of the extract was added to 80 μL of RSID™ Running Buffer and then loaded onto the sample well. The test results were scored after 10 min.

Simulated Forensic Samples

Lemon *(Citrus limonum),* orange (*Citrus aurantium),* grapefruit (*Citrus grandis),* mandarin *(Citrus reticulata),* Satsuma Miyagawa *(Citrus unshiu),* kumquat *(Fortunella ovale),* and lime *(Citrus aurantifolia)* were tested with RSID™-Saliva. In addition, two commercial *Citrus aurantium* and *Citrus grandis*—100% fruit juices without additional constituents—were analyzed.

Each citrus fruit was washed externally with sterile distilled water and cut with sterile blades. Hundred microliters of each juice was aspirated with sterile pipettes from the deeper part of each fruit, added to 200 μL of RSID™ Saliva Extraction Buffer in a tube, and extracted at room temperature for 2 h. Twenty microliters of extract was finally added to 80 μL of Running Buffer and run into the sample cassette well. Stains on cotton from simulated samples were also prepared and dried for 2 days at room temperature before extraction (as above). To verify whether the rinds of the fruits could give a similar reaction of the juices/pulps, the outer surface of the fruits was swabbed with sterile dry swabs and run in the RSID cards after extraction. The test results were scored after exactly 10 min.

Fifty microliters of human saliva spotted on sterile cotton was used as positive control. The swab was extracted in 200 μL of RSID™ Saliva Extraction Buffer for 2 h at room temperature. Twenty microliters of the extract was added to 80 μL of RSID™ Saliva Running Buffer.

A negative control was generated by adding 20 μL of Extraction Buffer to 80 μL of Running Buffer.

Both positive and negative controls were loaded into RSID cassettes and scored after 10 min.

Clinical Chemistry Systems

A Siemens Healthcare Diagnostics instrument (Tarrytown, NY) was used for the automated analyses of the samples (14). The AMYLAS method uses ethylidene-blocked p-nitrophenyl-maltoheptaoside as substrate in association with α-glucosidase enzyme used to release p-nitrophenol. The terminal glucose of the substrate is chemically blocked preventing cleavage by the indicator enzymes. The instrument was initially calibrated using RSID® Saliva Extraction Buffer; 100 μL of extracts was analyzed automatically by Siemens system.

DNA Analysis

DNA was extracted from forensic samples by using QIAamp® DNA Micro Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Quantification was performed with Quantifiler® Human DNA Quantification Kit and PCR amplification by using AmpFlSTR® Identifiler® PCR Amplification Kit (Applied Biosystems). ABI Prism 310 (Applied Biosystems) and GeneMapper® v3.2 software (Applied Biosystems Foster City, CA) were used for analyzing profiles.

Results

All casework samples from calamondin's fruits were strongly positive for RSID™ Saliva test; positive and negative controls resulted as expected.

Table 1 shows simulated forensic samples data. All tested citrus varieties showed positive results with RSID™ Saliva, with some differences in reactivity. For example, orange (*Citrus aurantium*) showed lower reactivity if compared with grapefruit (*Citrus grandis*) and lime (*Citrus aurantifolia*). However, test positivity strongly depends on pH and sample concentration.

Antibody–antigen conjugations are very sensitive to environmental factors including pH levels, as reported by Casey et al. (9) for urine and sweat. Thus, in the same way, pH levels of the juices may interfere with the antibody–antigen conjugation reaction, with the pH of the Running Buffer or can lead to antibody denaturation. In the simulated forensic samples, pH levels varied from 2.3 of the lemon to 3.8 for mandarin.

For example, using 40 μL of lemon (*Citrus limonum*) extract, a failure of RSID-Saliva test occurred, with no visible lines in

TABLE 1—*RSID-™ Saliva results using different* Citrus*,* Fortunella *and juice fruits (scale +++/−).*

Sample	Color	RSID-™ Saliva
Lemon *(Citrus limonum)*	Light yellow	++
Orange *(Citrus aurantium)*	Light orange	+
Grapefruit *(Citrus grandis)*	Light yellow	+++
Mandarin *(Citrus reticulate)*	Light orange	+
Satsuma Miyagawa *(Citrus unshiu)*	Light orange	+++
Lime *(Citrus aurantifolia)*	Light yellow	+++
Kumquat *(Fortunella ovale)*	Light orange	−
Orange juice *(Citrus aurantium)*	Dark orange	−
Grapefruit juice *(Citrus grandis)*	Pink	−

correspondence of both control and test position. This may occur because of many factors as high "Hook effect" dose, sample acidity, degree of fruit ripeness, or concentration of such chemical components. To better clarify the factors that can affect RSID immunochemical reaction, two solutions were prepared: a juice dilution 1:1 with distilled water and a juice with pH acidity leaded up to 3.0. Testing the solutions, positive results were observed for both control and sample lines, suggesting that both pH acidity and Hook effect can interfere with RSID immunochemical reaction.

The kumquat fruit *(Citrus aurantifolia*) gave negative result with RSID™ Saliva test. The two commercial citrus orange and grapefruit juices were clearly negative with RSID™ Saliva test.

Examining simulated forensic stains on cotton fabric with the RSID™ Saliva test yielded similar results to that obtained with fresh fruit extracts. Swabbing the rinds of the fruits, negative results for all simulated samples were obtained.

The absence of human amylase in the juices was obvious because the samples were collected from the deeper part of the fruits. Further analyses confirmed the absence of both amylase activity and human DNA. When the same extracts from these fruits were tested with a specific automated amylase test commonly used to detect amylase activity in fluids, negative results were constantly obtained (data not shown). Quantification by real-time PCR in all tested samples constantly confirmed human DNA absence. PCR amplification using specific forensic kit validated for human identification followed by capillary electrophoresis using an analytical threshold of 50 RFU gave negative results.

Discussion

In forensic practice, the reliability and specificity in differentiating between human and non-human biological samples play a crucial rule. Saliva screening using amylase can assist in choosing the possible samples for DNA analysis, saving time and cost. Without pretesting, over 50% of presumed crime scene saliva samples can be expected to yield insufficient DNA amounts (1, 15). More importantly, false-positive results using an appropriate test must be taken into account by investigators and any possible source of error should be highlighted. In addition, it is widely known that errors of consequence due to mistakes or bias could have a strong relapse in judicial systems (16).

The obtained results show that different citrus varieties result in false positives when examined with a lateral flow immunochromatographic strip test, commonly used in the forensic practice to detect the presence of human saliva.

The RSID™-Saliva strip test has proven to be substantially more specific than presumptive tests like Phadebas® amylase test, and until now no false results with other substances other than rat and gorilla saliva samples were reported. RSID™-Saliva test still continues to be probably the best compromise between cost, speed, and specificity to prove the presence of human saliva in forensic samples. However, the limit of this test when searching for human saliva traces on citrus fruits or related products must be taken into account.

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