Forensic Science International 194 (2010) 67–71

Contents lists available at ScienceDirect

Forensic Science International

journal homepage: www.elsevier.com/locate/forsciint

The sensitivity and specificity of the RSID™-saliva kit for the detection of human salivary amylase in the Forensic Science Laboratory, Dublin, Ireland

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ARTICLE INFO

Article history:
Received 24 February 2009
Received in revised form 4 June 2009
Accepted 9 October 2009
Available online 20 November 2009

Keywords:
Saliva
Forensic science
Sexual assaults
Body fluids

ABSTRACT

We demonstrate here that the RSID™-saliva test can be used as a test for human salivary α-amylase on samples routinely examined in forensic casework. We show that the RSID™-saliva test detects salivary α-amylase at lower concentrations than the Phadebas® Quantitative test, that the RSID™-saliva test does not cross-react with forensically important human fluids and that the RSID™-saliva test can be successfully integrated into the whole swab semen extraction method.

1. Introduction

The forensic detection of human saliva can be a very powerful tool in the investigation of crime. In particular, in the investigation of cases of a sexual nature where the detection of saliva can demonstrate contact between the complainant and the accused. In cases of stranger sexual attacks the presence of human saliva can lead to a DNA profile from a suspect, as epithelial cells found within saliva are a potential source of DNA.

Salivary α-amylase is produced in the salivary glands and its physiological role is the digestion of starch, beginning in the mouth [1]. In humans two main isozymes of α-amylase exist, salivary α-amylase and pancreatic α-amylase. Both α-amylases's have been identified in many different body fluids [2–5]. Historically this has led to difficulties in reporting the presence of salivary α-amylase in forensic case work. Current methods available for the detection of saliva have a number of drawbacks, most importantly is specificity and sensitivity [6–8] and they can be difficult to integrated into DNA profiling techniques [8,9]. The presumptive identification of saliva has conventionally been preformed by the detection of amylase using techniques such as the Phadebas® paper assay [2] or Red-starch paper [9] followed by the Phadebas® Quantitative test. These systems rely on the ability to identify the enzyme activity of α-amylase, a constituent of saliva and cannot distinguish between these two different α-amylase isozymes or the α-amylase present in plants, bacteria and fungi [1]. The lack of mobility associated with these tests also limits their use to the laboratory. The current systems for salivary α-amylase detection in the Forensic Science Laboratory, Dublin are the presumptive Phadebas® paper assay followed by the Phadebas® Quantitative test (Magle Life Sciences, Lund, Sweden).

The RSID™-saliva test is a lateral flow immunochromatographic strip test designed to detect the presence of human salivary α-amylase. The test, which uses two anti-human salivary amylase monoclonal antibodies, detects the presence of salivary amylase, rather than the activity of salivary amylase as seen with other tests.

2. Materials and methods

2.1. Samples

Human saliva from three individuals was collected, combined and used within 12 h of collection. For sensitivity tests, serial dilutions of human liquid saliva were prepared using PBS (Sigma), or human blood or human urine as diluents for mixed body fluid tests. 50 μl was pipetted onto cotton swabs and allowed to dry. Semen and blood were obtained from a local hospital. Penile swabs of the coronal sulcus (the groove or furrow between the shaft and the head) of the penis and the glans (the head of the penis) were collected. Vulval swabs and faecal samples (anal swabs) and sweat samples (underarm swabs after exercise) were collected fresh and frozen until required. Vaginal secretions were sampled from female volunteers who wore a new pair of panties for their working day and visibly stained areas were excised and tested. Swabs of faecal material from the nappies of five children ranging in age from 8 months to 24 months were tested. Animal saliva samples (buccal swabs) from guinea pig, cat, dog, mouse and sheep were used for species specificity. Case work samples of vulva swabs from complainants processed through the full swab semen extraction method [10], were further tested for the presence of human salivary α-amylase. Controls included; positive

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doi:10.1016/j.forsciint.2009.10.009

human buccal swabs, negative unused swabs, swabs of fresh neat urine, swabs of semen or blood.

2.2. Methodologies

The manufacturers (single tube-Stain ID Integrated into STR analysis, RSID™-saliva, April 2007) protocol was used initially in this investigation (described as Section 2.3). Two other methods (Sections 2.3 and 2.4 described below) were developed after difficulties were identified with the above protocol for some of the forensic samples examined in this paper. Body fluids (liquid saliva diluted in PBS, urine or blood) were prepared at the following dilutions: 1/10, 1/100, 1/200, 1/300, 1/400, 1/500, 1/600, 1/700, 1/800, 1/900, 1/1000.

2.3. Method 1

The single tube-Stain ID Integrated into STR analysis protocol.

50 μl of the body fluid preparations were pipette onto swabs and left to air-dry. Swab (one half) was placed into 1.5 ml microcentrifuge tubes. 300 μl of RSID™-saliva extraction buffer was added to the tube contained the cutting and agitated by vortexing for approximately 15 s. Samples were incubated for 1–2 h at room temperature to extract. 20 μl of the extracted solution was then added to a new microcentrifuge tube containing 80 μl of running buffer (20% final volume of extracted sample). This solution (100 μl) was then loaded on to a RSID™-saliva cassette. Results were read at 10 min.

2.4. Method 2

Swabs (one half) were placed into 1.5 ml microcentrifuge tubes. 300 μl of RSID™-saliva extraction buffer was added to the tube containing the swab cutting and agitated by vortexing for approximately 15 s. Samples were incubated for 1–2 h at room temperature. 12 μl of the extract solution was then added to a new microcentrifuge tube containing 108 μl of running buffer (10% final volume of extracted solution). 100 μl was then loaded on to a RSID™-saliva cassette. Results were read at 10 min.

2.5. Method 3

The whole swab semen extraction method [10] used in the Forensic Science Laboratory generates a 300 μl final volume of supernatant. 40 μl of this supernatant was pipetted into 1.5 ml microcentrifuge tubes containing 260 μl RSID™-saliva extraction buffer. Samples were incubated for 1–2 h at room temperature. 12 μl of the extract solution was then added to a new microcentrifuge tube containing 108 μl of running buffer. 100 μl was then loaded on to a RSID™-saliva cassette. Results were read at 10 min.

We compared the sensitivity and the robustness of the RSID™-saliva kit against the Phadebas® Quantitative test (Magle Life Sciences) as per the manufacturers protocol. Samples were analysed using the Perkin–Elmer Lamda 35 UV/vis spectrophotometer. The Phadebas® Quantitative test is considered to give a positive result for salivary α-amylase activity at OD 620_{nm} >0.3 (Metropolitan Police Manual, 1973) [11].

2.6. Reading results

100 μl of sample are pipetted into the sample window (S), and results read at 10 min. The presence of two red lines, one in the test area 'T' and one in the control area 'C' indicates a positive result. A red line in the control 'C' area only indicates a negative result. The absence of a red line at the 'C' indicates an invalid test (Fig. 1).

3. Results and discussion

3.1. The sensitivity of the RSID™-salvia test

Swabs of liquid saliva/PBS serial dilutions up to a 1000-fold dilution extracted through Methods 1 and 2 resulted in a limit of detection of up to a 500-fold dilution and a limit of detection to a 100-fold through Method 3. Parallel samples extracted through Methods 1, 2 and 3 assayed using the Phadebas® Quantitative test resulted in a limit of detection of up to a 100-fold dilution (Table 1). Pang and Cheung [8] has shown that RSID™-Saliva kit can detect up to a 10,000-fold dilution (0.1 nl/μl) of commercially lyophilized human saliva and up to a 20,000-fold dilution (0.5 ng/μl) human salivary amylase respectively. This equates to a limit of detection from the liquid saliva combined from three individuals in this study to approximately 5 nl/μl (1/500) of human saliva or 50 ng/μl (1/500) of human salivary amylase for the RSID™-saliva test compared to approximately 9 nl/μl (1/100) of human saliva or 90 ng/μl (1/100) of human salivary amylase for the Phadebas® Quantitative test. Our results are in agreement with Pang and Cheung [8] and demonstrate that the RSID™-saliva test is more sensitive detecting Human salivary amylase than the Phadebas® Quantitative test. No variation in the level of sensitivity of the RSID™-saliva test was observed when similar dilutions were tested using Method 1, on nylon, denim or cotton fabrics (data not shown). Gutowski and Henthorn [12] have reported variations in the detectable levels of salivary amylase activity on dried saliva stains on different fabrics, using the Phadebas® Quantitative test.

The level of detection from liquid saliva/blood mix increased to a 1000-fold dilution (Table 2). The high protein concentration of blood has been attributed to a reduction in the measurable activity of amylase [13]. However given blood homeostasis is tightly controlled; the natural buffering capacity of blood appears to improve the sensitivity of the RSID™-saliva test.

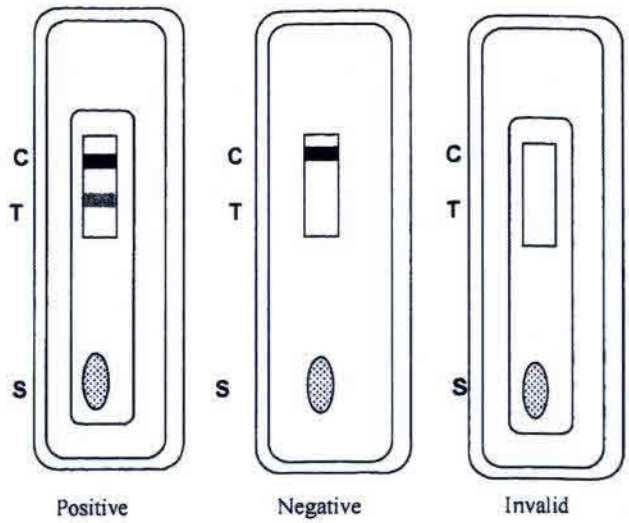

Fig. 1. Three RSID™-saliva cassettes showing the expected results from a positive reaction, negative reaction and an invalid reaction for salivary amylase. In cases where the operators disagreed on the presence or absence of a red line in the test area 'T' the test was identified by a 'R' in the results sheet.

Table 1
The limit of detection of the RSID™-saliva test versus the Phadebas® Amylase assay.

Diluent (PBS)	RSID™-saliva result methods			Phadebas® OD 620_{nm} result methods			
	1	2	3	1	2	3	
Pos. buccal	+	+	+	5.02	3.80	1.03	(+)
Neg. (PBS)	–	–	–	0.00	0.00	0.00	(–)
1/10	+	n.t.	+	4.60	n.t.	4.73	(+)
1/100	+	+	+	0.49	0.50	4.52	(+)
1/200	+	+	–	0.04	0.02	0.15	(–)
1/300	+	+	–	0.07	0.16	0.20	(–)
1/400	+	+	–	0.14	0.21	0.08	(–)
1/500	+	+	–	0.07	0.16	0.08	(–)
1/600	–	–	–	0.05	0.11	0.07	(–)
1/700	–	–	–	0.04	0.11	0.12	(–)
1/800	–	–	–	0.02	0.09	0.10	(–)
1/900	–	–	–	0.00	0.00	0.06	(–)
1/1000	–	–	–	0.00	0.00	0.04	(–)

Serial dilutions of liquid saliva were prepared in PBS tested as per Methods 1, 2 and 3. (+) positive result, (n.t.) not tested, (–) negative result, OD 620_{nm} (+>0.3).

Table 2
Serial dilutions of liquid saliva were prepared in human blood tested as per Method 2.

Diluent (blood)	RSID™-saliva
Pos. (buccal)	+
Neat blood	–
1/100	+
1/200	+
1/300	+
1/400	+
1/500	+
1/600	+
1/700	+
1/800	+
1/900	+
1/1000	+

(+) Positive result; (–) negative result.

3.2. Body fluid specificity

3.2.1. Urine and sweat

Positive results were recorded from neat urine samples and from sweat samples from two individuals using Method 1 (Table 3). No positives results were observed when these samples and individuals were retested using Method 2 (Table 4). Pang and Cheung [8] reported positive reactions to male and female urine but not from sweat. The RSID™-salvia test relies on the conjugation of two monoclonal antibodies with their respective antigens in the test cassettes. Antibody–antigen conjugations are very sensitive to environmental factors including pH levels. Urine contains large amounts of excess water, excess salt and uric acid and small amount of urea is excreted (along with sodium chloride and water) in sweat [14]. The optimum pH for normal human homeostasis ranges from 7.35 to 7.45, where as the pH of urine and sweat may range from 4.5 to 8. The pH levels of these two body fluids may interfere with the antibody–antigen conjugation reaction, the pH of the running buffer or lead to the denaturation of the antibody (Dr. Karl Reich, Personal communication, IFI, 2008). To eliminate the pH effect we reduced the final volume of the extracted solution from 20% final volume (Method 1) to 10% final volume in the running buffer (Method 2).

Serum amylase has been show to be higher in pregnant females in particular in the 2nd and 3rd trimesters when compared it men and non-pregnant females [15]. One female participant who donated a urine and a sweat sample was in her third trimester (Table 4). No human salvia was observed in the urine or sweat samples from this female.

All other samples in this study were processed using Method 2 unless otherwise indicted.

Human saliva was detected using both the RSID™-saliva test and Phadebas® Quantitative in one semen sample (Table 5).

Table 3
Swabs of human sweat and urine from different individuals tested for the presence of saliva as per Method 1. Included was neat RSID extraction and running buffer.

Body Fluid	RSID™-saliva
Pos. buccal	+
Sweat ♂	–
Sweat ♂	+
Sweat ♀	–
Sweat ♀	–
Urine ♂	–
Urine ♂	–
Urine ♀	+
Urine ♀	–
RSID extraction buffer	–
RSID running buffer	–

(+) Positive; (–) negative.

Table 4
Swabs of human sweat and urine from different individuals tested for the presence of saliva as per Method 2.

Body fluid	RSID™-saliva
Pos (buccal)	+
Urine ♂	–
Urine ♂	–
Urine ♂	–
Urine ♂	–
Urine ♀	–
Urine ♀	–
Urine ♀ (*)	–
Urine ♀ (#)	–
Urine ♀	–
Sweat ♂	–
Sweat ♂ (*)	–
Sweat ♂	–
Sweat ♂	–
Sweat ♂	–
Sweat ♂	–
Sweat ♂	–
Sweat ♂	–
Sweat ♀	–
Sweat ♀	–

(+) Positive; (–) negative; (#) sample from pregnant participant. Note the negative result from urine sample (*) and sweat sample these samples gave the initial positive result when processed using Method 1.

Table 5
Semen samples tested for the presence of saliva as per Method 2. Samples positive for the presence of salvia were tested using the Phadebas® Quantitative test.

Sample	RSID™-saliva	Phadebas® 620_{nm}
Pos. (buccal)	+	2.23 (+)
Semen 1	–	0.0 (–)
Semen 2	–	0.0 (–)
Semen 3	+	2.5197 (+)
Semen 4	–	0.0 (–)

(+) Positive; (–) negative; OD 620_{nm} (+>0.3).

Seminal fluid has been reported as having elevated levels of α-amylase [2–4]. The level of amylase activity detected in this semen sample (OD 620_{nm} = 2.52) was higher than the level of amylase activity detected from the control buccal swab (OD 620_{nm} = 2.23). Hochmeister [5], has reported that the presence of amylase may be due to the use of saliva as a lubricant during masturbation. No human salivary amylase was detected on penile, anal or vaginal swabs from different individuals or vaginal discharge (gusset/crotch fabric from panties) (Table 6) in this study.

Table 6
Vulval, penile and anal swabs and the excised fabric from the gussets/crotches of panties from different individuals tested for the presence of saliva as per Method 2.

Sample	RSID™-saliva
Pos. (buccal)	+
Vaginal	–
Vaginal	–
Penile	–
Penile	–
Penile	–
Penile	–
Gusset/crotch	–
Gusset/crotch	–
Gusset/crotch	–
Anal♀	–
Anal♂	–
Anal♂	–

(+) Positive; (–) negative.

Table 7
Swabs of faecal material from nappies of infants tested for the presence of saliva as per Method 2, and the Phadebas® Amylase assay.

Diluent	RSID™-saliva	Phadebas® 620 nm	Age (months)
Pos (buccal)	+	1.06 (+)	
Neg	–	0.00 (–)	
WJ	+	4.99 (+)	16
Cian	+	2.19 (+)	8
NC	+	4.26 (+)	4
MB	–	0.0 (–)	24

(+) Positive; (–) negative; OD 620_{nm} (+ >0.3).

3.2.2. Faecal matter

Swabs of faecal material from the nappies of infants aged 4–24 months gave positive results for both the RSID™-saliva test and the Phadebas® Quantitative test, with up to a four fold increase in amylase activity (Table 7) compared to the control buccal swabs. Pang and Cheung [8] reported limit of detection for human pancreatic amylase was about 2000 ng. Given the normal reported range of salivary amylase is between 0.2 and 6.4 mg/ml. One would have to expect a 200-fold increase in the normal production of pancreatic amylase in casework samples for a positive RSID™-saliva test result [8]. However pancreatic amylase in casework samples may come about due to the presence of faecal material. Faecal stains are visible and can be recognised visually and by smell and have been shown as unsuitable for testing with presumptive tests such as Phadebas® paper [2,3]. Faecal stained material must be given special consideration when using these tests.

3.2.3. Species specificity

No salivary amylase was detected from the animal saliva sampled in this study (Table 8), including mouse. However Pang and Cheung [8] reported a positive reaction for rat saliva using the RSID™-saliva test. Both the rat (*Rattus norvegicus*) salivary amylase gene AMY1A and the mouse (*Mus musculus*) salivary amylase AMY gene have homology with the human salivary amylase gene AMY1A, HGNC: 474 (source: NCBI-ENTREZ).

Table 8
Animal saliva tested for the presence of saliva as per Method 2.

Sample	Result
Human buccal	+
Donkey buccal	–
Cat buccal	–
Mouse buccal	–
Sheep buccal	–
Dog buccal	–

(+) Positive; (–) negative.

Table 9
Extracts from vulva swabs from casework tested for the presence of saliva as per Method 3.

Sample	RSID™-saliva	Phadebas® 620_{nm}	Time interval (h)
Pos. (buccal)	+	2.6 (+)	–
Case 1	+	0.67 (+)	6.3
Case 2	+	3.14 (+)	2.15
Case 3*	+	n.t.	5.5
Case 4*	+	0.81 (+)	24
Case 5*	–	0.0 (–)	24
Case 6*	–	0.0 (–)	33
Case 7*	–	0.0 (–)	20

(+) Positive; (–) negative; n.t. = not tested, OD 620_{nm} (+>0.3); Time interval; the time between the alleged offence and the medical examination, (*) complainants did not have a clear recollection of the specific events of the alleged offence due to drink or drugs.

3.2.4. Case work samples

Five vulval swabs processed through Method 3 were taken from cases where the complainant had alleged vaginal–oral contact (cunnilingus) (Table 9). Of interest is the time interval between the alleged offence and the medical examination of the complainant. The results from cases 5 to 7, may be due to the time interval between the alleged offence and the medical examination or that no cunnilingus took place. Keating and Higgs [16] showed that of 400 casework swabs, 32% of vaginal swabs of which 60 were external vaginal swabs were positive for amylase. The likelihood of obtaining a positive result reducing after 9 h but a positive result was recorded up to 55 h after the alleged offence.

4. Summary

We have validated the RSID™-saliva kit in the Forensic Science Laboratory for the detection of salivary α-amylase on samples routinely examined in forensic casework. Forensic analysis of casework samples in the Forensic Science Laboratory, Dublin is governed by the case assessment and interpretation model as described by Cook [17,18]. The forensic scientist must balance the probabative value of samples submitted for analysis against the condition and origin of the sample, any time interval associated with the sample and the results obtained from the type of forensic tests applied to these samples. In the case of the RSID™-saliva test, the scientist must weight up the likelihood of a false-positive result from samples not due to salivary amylase, the likelihood of a positive reaction due to salivary amylase, and the likelihood of obtaining a result at all. We show that the RSID™-saliva detects salivary α-amylase at lower concentrations than the Phadebas® Quantitative test, that the RSID™-saliva test does not cross-react with other forensically important human body fluids and that the RSID™-saliva test can be successfully integrated into the whole swab semen extraction method.

Acknowledgement

The authors wish to thank all the staff in the Forensic Science Laboratory who volunteered samples.

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