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PCR Applications in Identification of Saliva Samples Exposed to Different Conditions (Streptococci Detection based)

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Abstract: Oral streptococci represent about 20% of the total oral bacteria, so if it is possible to detect the presence of oral specific bacteria from a forensic specimen by Polymerase chain reaction, this could be used to verify the presence of saliva. Aim of this study is detection of *Streptococcus salivarius* which is one of the most common streptococci in oral bacteria and *Streptococcus mutans* which is common in cases of dental caries in various body fluids and skin swabs and assessment of which one of both organisms is more reliable in saliva identification, cross sectional study on Egypt population. Negative control samples (15 samples) were taken from various body fluids (urine, semen) and skin swabs. Mock forensic samples (85 samples) included fresh saliva, saliva, cotton fabrics contaminated with saliva, cigarette butts, bitten apple and semen mixed with saliva samples). DNA extraction was done using DNeasy blood and tissue kit (Qiagen, Tokyo, Japan). Polymerase chain reaction was done for DNA amplification using Polymerase chain reaction master mix then gel electrophoresis was done for samples qualification. Control bacteria were *S. salivarius* and *Streptococcus mutans*. *Streptococcus salivarius* was detected in 83.5% of all saliva contained samples and *S. mutans* was detected in 67% of saliva contained samples. Both bacteria were not detected in other body fluids and skin swabs, so *S. salivarius* is more reliable in saliva identification as well as differentiating it from other body fluids. Polymerase chain reaction is valuable in detection of saliva by detecting *S. salivarius*.

Key words: Saliva, identification, Polymerase Chain Reaction (PCR), *S. salivarius*, *S. mutans*, Egypt

INTRODUCTION

Differentiation of body fluids in forensic examination is important to determine the events that happened at the crime scene and detection of saliva is important for understanding the details of crime (Nakanishi *et al*., 2009).

Saliva is considered a common source of DNA retrieved from swabs of objects detected at the crime scene. However, in 57% of case reports done by the United Kingdom police force there was no enough DNA extracted from saliva stains, mostly caused by deficiency of pretesting (Hedman *et al*., 2011).

The primary steps in body Fluid identification are very important as knowing the type of body fluid is informative. But it may lead to loss of evidence if it is in a small amount because of the destructive nature of some tests (Virkler and Lednev, 2009).

Saliva stains appearance using ultraviolet light is bluish white but this method cannot differentiate it from other stains (Jones, 2005).

Enzyme α-amylase is one of the most common pres umptive tests for saliva identification. It is present in other body fluids but, more prevalent in saliva so it can't be considered as an exclusive test for saliva identification (Greenfield and Sloan, 2003).

Oral streptococci are one of the most available bacteria in human oral flora, *Streptococcus salivarius* is the commonest streptococcus in oral bacteria, Polymerase Chain Reaction (PCR) has been used to identify oral streptococcal bacteria such as *Streptococcus salivarius* and *Streptococcus mutans* (Chen *et al*., 2007). With respect to forensics, if oral specific bacteria could be detected by PCR from a forensic specimen, the presence of saliva can be confirmed (Nakanishi *et al*., 2009).

DNA profiling of oral bacteria derived from bite marks can be compared with that derived from the responsible individual helping in forensic identification where the individual DNA can't be extracted from oral contact site (Borgula *et al*., 2003).

This study is to prove a link between the presence of oral streptococci especially *S. salivarius* and *S. mutans* and saliva detection. Therefore it will be assessed if the detection of *S. salivarius* and *S. mutans* by PCR is sufficient to confirm saliva presence and differentiating it from other body fluids and skin swabs.

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MATERIALS AND METHODS

The present study was performed at the Unit of Biochemistry and Molecular Biology at The Medical Biochemistry Department, Faculty of Medicine, Cairo University, Cairo, Egypt.

Cross sectional study, mock samples were collected from 100 volunteer after informing them and getting their consent. Samples used in the study were divided into 2 main groups including:

- **Negative control specimens group:** Samples were obtained from 15 donors including semen, urine and skin swab samples which were obtained by wiping skin with a wet cotton swab, 5 samples for each type
- **Mock forensic specimens group (75 samples) including:** fresh saliva (5 samples), saliva stain samples which were obtained by licking filter paper (5 samples), Cigarette butts (20 samples) Bitten apples (20 samples), cotton swab contaminated with saliva (20 samples), Mixture stain of saliva and semen samples (15 samples)
- The bacterial strains used in this study were *Streptococcus salivarius* which was identified using API test from saliva swab culture on mitis salivarius agar (BHI, Difco Laboratories, Detroit, MI) and Reference strain *Streptococcus mutans* ATCC 25175 which was purchased from (Cairo MIRCEN, faculty of agriculture, ASU)
- For DNA extraction, we used 50 μL of body fluid, 10×10 mm of filter paper, bitten apple scrap or a swab in 50 μL of nuclease free water. Control bacteria were suspended in 50 μL of nuclease free water
- Samples were boiled at 98°C in micro-centrifuge tubes
- Samples were incubated with 100 μL of 200 U mL^{-1} mutanolysin (Sigma, St. Louis, MO) incubate at 50°C for 60 min
- Followed by treatment with 20 μL of Proteinase K (Qiagen, Tokyo, Japan) and 200 μL of Buffer AL (Qiagen, Tokyo, Japan), incubate at 56°C for 60 min.
- Add 200 μL ethanol (96-100%). Mix thoroughly by vortexing
- Pipet the mixture into a DNeasy Mini spin column in a 2 mL collection tube. Centrifuge at = 6000×g (8000 rpm) for 1 min
- Place the spin column in a new 2 mL collection tube. Add 500 μL buffer AW1. (Qiagen, Tokyo, Japan) Centrifuge for 1 min at = 6000×g. Discard flow-through and collection tube
- Place the spin column in a new 2 mL collection tube. Add 500 μL buffer AW2. (Qiagen, Tokyo, Japan) Centrifuge for 3 min at 20,000×g (14000 rpm)
- Remove the spin column carefully so it doesn't come into contact with the flow- through

Table 1: The oligonucleotide primers sequences used in the study

	Primer sequence
S. salivarius 544bp	Forward primer: MKK-F GTGTTGCCACATCTTCACTCGCTTCGG
	Reverse primer: MKK-R CGTTGATGTGCTTGAAAGGGCACCATT
S. mutans 433 bp	Forward primer: MKD-F GGCACCACAACATTGGGAAGCTCAGTT
	Reverse primer: MKD-R GGAATGGCCGCTAAGTCAACAGGAT

- Transfer the spin column to a new 2 mL micro centrifuge tube and add 50 μL buffer AE (Qiagen, Tokyo, Japan) for elution. Incubate for 1 min at room temperature. Centrifuge for 1 min at = 6000×g
- Amplification of DNA was done using PCR master mix (Qiagen, Tokyo, Japan) (Table 1)
- The amplified DNA was detected using agarose gel electrophoresis
- The DNA was visualized by placing the gel on an UV transilluminator. The EB intercalated into DNA and gave a bright pink band

RESULTS

In the present study it was proposed to analyze different types of saliva contained samples for detection of *S. salivarius* and *S. mutans* as indicators for the presence of saliva and differentiating it from other body samples.

The total number of saliva samples used for the present study was 85 samples, these saliva contained samples were derived from different sources as follows: 5 samples of fresh saliva, 5 samples of saliva stain, 20 samples of cotton, 20 samples of bitten apple, 20 samples of cigarette butts and 15 samples of semen mixed with saliva. Other 15 samples were taken from other body fluids (semen, urine) and skin swabs, 5 samples for each type as negative control. DNA was extracted from samples then PCR was done followed by agarose gel electrophoresis for qualitative analysis of data.

Table 2 shows the percentage of detection of *S. salivarius* from various body fluids samples and skin swabs using PCR test. *S. salivarius* was identified in 71 saliva samples (83.5%), whereas it was not detected in 14 samples (16.5%). On the other hand, the other body fluids including semen, urine and skin swabs showed negative results.

Table 3 shows the percentage of detection of *S. mutans* from various body fluids samples and skin swabs using PCR test.

S. mutans was identified in 57 saliva samples (67.1%), whereas it was not detected in 28 samples (32.9). On the other hand, other body fluids including semen, urine and skin swabs showed negative results.

Table 4 shows the analysis of variances (p = value) of detected *S. salivarius* and *S. mutans* from all saliva

Table 2: The percentage of detection of *S. salivarius* from various body fluid samples and skin swabs using PCR test

		S. salivarius		Percentage	
Sample	No.	Detected	Not detected	Detected	Not detected
All saliva samples	85	71	0	83.5	16.5
Semen	5	5	5	0	100.0
Urine	5	0	5	0	100.0
Skin swab	5	0	5	0	100.0

Table 3: The percentage of detection of *S. mutans* from various body fluids and skin swabs using PCR test

		S. mutans		Percentage	
Sample	No.	Detected	Not detected	Detected	Not detected
All saliva samples	85	57	28	67.1	23.9
Semen	5	0	5	0	100.0
Urine	5	0	5	0	100.0
Skin swab	5	0	5	0	100.0

Table 4: The analysis of variances (p_value) of detected *S. salivarius* and *S. mutans* from all saliva contained samples

Sample	No.	Detected *S. salivarius*	Detected *S. mutans*	p_value
Saliva contained samples	85	71	57	0.011

p_ value = 0.011, Statistical Tests used were: chi-square test, Kruskall-Wilis test

Table 5: The percentage of detection of *S. salivarius* and *S .mutans* from different forensic samples

		S. salivarius		*S. mutans*	
Sample	No.	Detected	Percentage	Detected	Percentage
Fresh saliva	5	5	100	3	60
Licked filter paper	5	4	80	3	60
Semen mixed with saliva	15	11	73.3	3	66.7
Cotton	20	17	85	14	70
Bitten apple	20	17	85	14	70
Cigarette butts	20	17	85	13	65

Table 6: The significant difference between different forensic groups

Specie	N	Mean rank	Chi-square	p_value
S.salivrius[a]				
Saliva	5	50.00	2.232	0.816
Filter paper	5	41.50		
Semen mixed with saliva (mix)	15	38.67		
Cotton	20	43.63		
Bitten apple	20	43.63		
Cigarette butts	20	43.63		
Total	85			
S. mutans[b]				
Saliva	5	40.00	0.417	0.995
Filter paper	5	40.00		
Semen mixed with saliva	15	42.83		
Cotton	20	44.25		
Bitten apple	20	44.25		
Cigarette butts	20	42.13		
Total	85			

p<value[a] = 0.816, p<value[b] = 0.9

contained samples. There was a statistically difference between the number of saliva contained samples in which *S. salivarius* was detected and the number of samples in which *S. mutans* was detected (p = value = 0.011).

Table 5 shows the percentage of detection of *S.salivarius* and *S.mutans* from different forensic samples. *S.salivarius* was identified in all fresh saliva (100%), 4 samples of saliva stain (80%), 11 samples of semen mixed with saliva (73.3%),17 samples of cotton contaminated with saliva (85%),17 samples of bitten apple (85%) and 17 samples of cigarette butts (85%). whereas, *S.mutans* was identified in only 3 samples of fresh saliva (60%), 3 samples of saliva stain (60%), 10 samples of semen mixed with saliva (66.7%), 14 samples of cotton contaminated with saliva (70%), 14 samples of bitten apple (70%) and 13 samples of cigarette butts (65%).

Table 6 shows p-value that was 0.81 in *S. salivarius* that is not significant at 0.05, but the highest detection was in the fresh saliva with mean rank 50.00 followed by cotton, bitten apple and cigarette butts with the same mean rank 43.63. The p value was 0.99 in *S. mutans* that is not significant at 0.05 but the highest detection was in the cotton contaminated with saliva and the bitten apple with mean rank 44.25. Statistical Tests used are: chi-square test, kruskall-wilis test.

Figure 1 and 2 showing the PCR products for *S. salivarius* and *S. mutans* identification in different saliva contained forensic samples including filter paper, fresh saliva, cigarette butts, bitten apple and cotton Species specific PCR products were successfully identified in the saliva contained forensic samples and no nonspecific bands were observed.

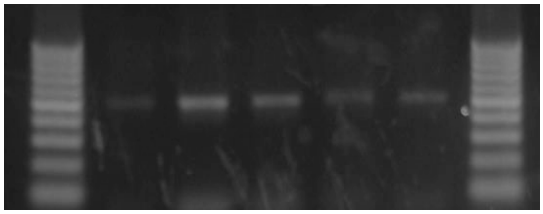

Fig. 1: An agarose gel electrophoresis shows PCR products of *Streptococcus salivarius* strain in different saliva contained samples, Lane M: DNA marker with 100 bp, Lane 1, 2, 3, 4 and 5: PCR products of *Streptococcus salivarius* strain in filter paper, fresh saliva, cigarette butts, bitten apple and cotton, respectively

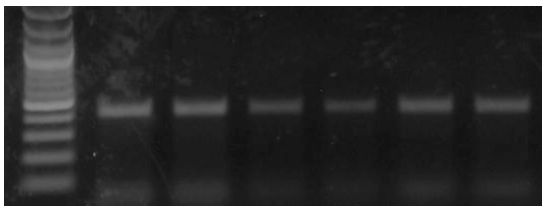

Fig. 2: An agarose gel electrophoresis shows PCR products of *Streptococcus mutans* strain in different saliva contained forensic samples, Lane M: DNA marker with 100 bp, Lane 1, 2, 3, 4, 5 and 6: PCR products of *Streptococcus mutans* strain in filter paper, fresh saliva, cigarette butts, bitten apple, cotton and semen mixed with saliva, respectively.

DISCUSSION

The results were statistically analysed By SPSS program. Sample size used was 85 of saliva contained mock forensic samples including (fresh saliva, saliva stain, cigarette butts, cotton mixed with saliva, semen mixed with saliva and bitten apple) and 15 samples of semen, urine and skin swabs, 5 samples each type. It shows that *S. salivarius* was identified in 83.5% of saliva contained samples tested in this study; it was identified in 71 samples of them and was not detected in 14 samples. *S. mutans* was identified in 67.1% of saliva contained samples; it was identified in 57 samples of them and was not detected in 28 samples. Both of the organisms were not detected in other body fluids including semen, urine and skin swabs.

The detection of oral streptococci in forensics was used to assess the presence of bite marks. But it didn't discuss the identification of saliva. So if the presence of oral-specific bacteria could be assessed by PCR in forensic specimens, the presence of saliva can be confirmed (Rahimi *et al*., 2005).

Oral streptococci can be identified by colony morphology on mitis-salivarius agar plates and then confirmed by biochemical, immunological and genetic tests. But this requires considerable time and skills and no sure results. So, molecular tests using a Polymerase Chain Reaction (PCR) and a DNA probe had been developed (Igarashi *et al*., 2000).

Similar results were obtained by Nakamishi *et al*. (2009) where saliva, semen and urine samples were collected from 20 healthy donors and saliva stain samples were made by licking filter paper. Skin bacteria were collected from 20 healthy donors by wiping the skin with a wet cotton swab and forensic samples were used to test the forensic application of this method including (10 of cigarette butts, 10 of cotton gauzes wiping licked skin and a mixture stain of semen and saliva (10:1 by volume) on filter paper were obtained. PCR-based identification of streptococci was done. The results were that *S. salivarius* and *S. mutans* were not detected in semen, urine, or skin surface samples, but they were present in saliva. The PCR results of *S. salivarius* and *S. mutans* detection were 100% and 90% respectively. These results suggest that *S. salivarius* is more reliable than *S. mutans* as a target species for the identification of liquid saliva. Also, the detection rate of *S. mutans* was only 60% in the saliva stains. So, PCR-based identification of these streptococci is sufficient to demonstrate the presence of saliva.

Power *et al*. (2010) investigated the use of direct DNA extraction from mixed blood/saliva stains on fabrics. DNA was amplified from very small amounts of saliva stains also amplification of oral microflora from mixed blood/saliva stains was done. The results were that streptococcal DNA was detectable in saliva applied to fabric swatches for up to 62 days after seeding, demonstrating that the bacterial DNA can persist for protracted periods. Also, results were obtained from mixed blood/saliva stains even in the presence of a 10-fold excess of blood, so the technique could be used to detect traces of saliva in expirated bloodstains. So, detecting DNA from streptococci could be used as a test for saliva identification in forensic samples as it doesn't present in other bodily fluids.

Nakamishi *et al*. (2011) study was detecting *Streptococcus salivarius* using loop-mediated isothermal amplification (LAMP). The LAMP primer set was designed using *S. salivarius*-specific sequences of glucosyl transferase K. The sample was prepared by boiling and mutanolysin treatment only and the entire

analytical process was completed within 2.5 h. The cut-off value was set at 0.1 absorbance units, measured at 660 nm. *S. salivarius* was identified in all saliva samples, but was not detected in other body fluids or on the skin surface. Using this method, *S. salivarius* was successfully detected in various forensic samples. Therefore it was suggested that this approach is useful for the identification of saliva in forensic practice.

Donaldson *et al.* (2010) also distinguished expirated bloodstains from impact spatter. This study developed a Polymerase Chain Reaction (PCR) method that detects DNA from oral streptococci as a sensitive tool to detect the presence of saliva. The PCR method was very specific to human oral streptococci, with no PCR product being made from human DNA or DNA from other microbes that were tested. It was also sensitive, detecting as little as 60 fg of target DNA. The PCR amplification gave product with 99 out of 100 saliva samples tested. PCR was not inhibited by the presence of blood and could detect target DNA in expirated bloodstains in a range of materials and for up to 92 days after deposit on cardboard or cotton fabric. the PCR method could discriminate three forensic samples that contained expirated blood from four that did not. This study showed that bacteria present in the oral cavity can be detected in bloodstains that contain saliva.

CONCLUSION AND RECOMMENDATIONS

- *S. salivarius* and *S. mutans* were not detected in semen, urine, or skin surface samples, but they were present in liquid saliva, saliva stain and all forensic specimens containing saliva
- *S. salivarius* is more reliable than S. mutans as a target species for the identification of saliva
- These oral bacteria can provide a sensitive marker for saliva differentiating it from other body fluids
- These oral bacteria can be detected in bloodstains that contain saliva and therefore can be used as a marker in forensic work to differentiate mouth expirated bloodstains from other types of bloodstains
- PCR-based identification of these streptococci is sufficient to demonstrate the presence of saliva

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