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EFFECT OF WATER AND DETERGENT WASHINGS ON FORENSIC DETECTION OF SALIVA STAINS FROM COMMONLY USED NATURAL AND SYNTHETIC CLOTHES

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Abstract
Forensic science plays main role in criminal justice system. Mainly, homicide, sexual assault and burglary, permit the assembly of biological evidence. Blood, semen and saliva commonly obtain from the crime scene. Culprit often tries to remove these biological stains through washing out the crime scene or the items but previously it has demonstrated that DNA can still recover from laundered clothes. The purpose of this study was to observe the effect of water and detergent wash on clothes and to extract, detect and quantify the amount DNA from these clothes. In this study, natural and synthetic clothes gave positive results under various presumptive tests. Both types of clothes gave different ranges of mean diameter after water wash till three washings. Saliva was found retain from laundered natural clothes till ten washings after processing with detergent while the laundered synthetic clothes could not efficiently retain saliva after treatment with detergent. DNA extracted from clothes analyzed under UV spectrophotometer demonstrated that both natural and synthetic clothes could give the good amount of DNA that was enough to generate the DNA profile. Overall terry, cotton, linen, khaddar, denim and spandex clothes showed the efficient results for saliva detection while wool, polyester and nylon exhibited the poor results.

Keywords: Forensic detection, Saliva Stains, Clothes

INTRODUCTION

Forensic science comprises the physical, natural and social sciences to different disciplines of law (Maras and Miranda 2014). It is an approach that furnishes the knowledge and methodology to resolve legitimate queries and complications regarding individuals and societies (Shen and Vieira 2016). Forensic analysis has gained importance in various criminal activities, even minor evidence such as identification of fluid type and its source can help to lead the case beyond reasonable doubt, reinforcing the criminal justice system and reconstruction of the crime.

According to Locard's exchange principle, whatever a culprit or a victim touches consciously or unconsciously leaves behind a trace. The trace can be a biological fluid, fiber, footprint, fingerprint, tool mark and even a residue. These physical evidences are important and should be keenly observed and collected (Locard 2008). Similarly, forensic DNA analysis has made significant

progress over the previous three decades. Advancements in DNA profiling approaches have improved their discriminating strength, speed, and sensitivity, as well as the capacity to work with tough samples (Alketbi Salem, K. 2023).

Thus, a little evidence found from a crime scene can link the victim to the culprit. This evidence goes through various tests to be proved authentic and series of steps are taken to identify its source. After identification, a forensic report is written and submitted to the court for further proceedings (Cross 2017).

Forensic serology is an important branch of forensic science in which biological and other bodily fluids are detected, identified and examined. DNA that can be obtained from bodily fluids is helpful in recognition of victim and the suspect. (Virkler and Lednev 2009). When biological fluids leave the body, they can be contaminated by bacteria or agents such as sand or dust. Bodily fluids do not always present alone. These combinations can come from the same individual or various people, from the same fluid or two or more separate fluids, and from humans or animals. All of these factors can make detection, characterization, classification/differentiation, and individualization more difficult (Cano-Trujillo et al., 2023). Saliva is an essential biological fluid that a human secretes; moreover, it is the source of epithelial cells that assist to generate DNA profile. It is the compound of cells, enzymes, proteins and inorganic substances. It contains 99% water in existence of buccal epithelial cells, enzymes, salts, mucin and α-amylase (Virkler and Ledenev 2009, Li 2015). Enzyme, α-amylase (E.C 3.2.1.1; 1,4-a-D-glucan glucanohydrolase) contributes in hydrolysis of 1,4 glycosidic bonds in glycogen (starch related polysaccharides such as amylose and amylopectin) and few polysaccharides (Perry, Dominy et al. 2007).

An analyst focuses on saliva along with other bodily fluids to be found from the victim clothes and skin in sexual assault cases. The complexity of detection of saliva varies surface to surface. It is hard to perceive saliva from surfaces like, tissue papers, fabric clothes and bite marks on human skin because these surfaces absorb and evaporate saliva easily and rapidly due to their absorptive, textured and elastic nature. Moreover, dried saliva cannot be seen by naked eye which makes it more difficult to be collected and identified (Auvdel et al.1987).

Previous research showed that saliva was applied large cloth fragments of cotton, denim and polyester and washed with cold water in a washing machine. The results demonstrated that denim was the essential cloth that retained large amount of saliva in comparison with other clothes (Lotozynski, 2020). Another research was taken on various cotton and synthetic clothes; however both types of saliva containing clothes were treated with laundering at 40℃ and 60℃, that decreased the amount of extracted DNA from both type of clothes. Thus, it was observed that cloth type, washing conditions and number of cycles of washing reduced the amount of DNA, while storage time did not affect the DNA amount (Kulstein and Wiegand 2018).

Another study showed that the cold-water washing could detach the fresh saliva stains effortlessly. Immunoassay furnished only 5% poorly constructive consequences. Presence of saliva stains on natural fibers could generate better results because of their rough surface while synthetic and semi-synthetic fibers were unable to give any useful results due to their plane surfaces saliva detached easily (Mussabekova 2017). For another research, five different clothes; cotton, polyester, denim, spandex and rayon were taken for identification of undiluted detergents contain amylase. Each set of clothes washed with detergents at warm-cold temperature that made the clothes to give undetectable amount of amylase. Out of five detergents, three gave intense blue color, while other two gave less intense color change, whereas, undiluted detergents did not give positive results under RSID amylase test (Feia and Novroski 2013).

An earlier research used Polilight to recover blood, semen and saliva. Saliva was stored at 41℃, on cotton swabs and colored fabrics and allowed to dry for 24 hours. Cloth color seemed to influence the stain appearance, however, dark colored clothes were hard to observe. Absorbance of the material was another factor influenced the detection. Neat saliva and diluted stains (1:10) on white nylon were weakly visible with Polilight. After 1:2 diluted saliva stains were not visible under natural light. Saliva stains placed on nylon were detected by tape lift when viewed under Polilight,

gave positive results. Later on, a full DNA profile was generated from the stain (200µl in amount). Phadebas paper test did not appear more reliable for DNA profiling. Thus, Polilight can be considered as essential tool for detection of biological fluids (Vandenberg and van Oorschot 2006).
Due to fluorescence property, various detection tests such as alternative light source (ALS) or fluorescence spectroscopy, ultraviolet light sources, different chemicals and lasers are also used (Auvdel 1987, Virkler and Lednev 2009, Lee and Khoo 2010). Saliva stains are harder to distinguish due to presence of low amount of solid particles and bluish-white spot occurs when placed under UV but it does not separate it from other fluids. Radial gel diffusion is used during routine saliva cases, in various agencies of Pakistan, due to its non-destructive ability (Draz, Ali et al. 2019). Conventionally, the drawback of this technique is the expensive reagents that can only be limited for research purposes thus cannot be used in regular teaching labs. This drawback has now replaced by using low-cost agar and starch as supporting material and amylase substrate respectively (Virkler and Lednev 2009, Farias, Carvalho et al. 2010).
A forensic scientist may find mixed quantity of different fluids at crime scene so it becomes difficult to choose a test for a specific fluid. One of the problems faced during saliva identification is to source of saliva. In this way, a range of confirmatory tests must be applied to the stain to identify the fluid present in sample, however these confirmatory tests are also costly and can only limited for higher researches. Amylase activities can be observed in humans, animals, fruit and vegetable extracts and sometimes it become harder to distinguish them with a naked eye unless a highly specific and sensitive test is used. Each method having its own strengths and limitations because these tests can be more sensitive than specific and they are limited to some wavelength, exceeding from limit provides inappropriate results but previous researches shows that these presumptive test can generate DNA profile and are useful to solve the crime (Barbaro, Cormaci et al. 2015).
Recently, a research was taken to recover nuclear and mitochondrial DNA through immunochromatographic tests. In this study, 50 µl saliva sample was applied on different types of clothes (denim, cotton, and polyester). The samples stored at R.T for upto 180 days and recovered through swabbing and analyzed by Seratec amylase and Seratec saliva tests. DNA was extracted by extraction buffer, applied through silica based methodology as well as quantified by fluorescent and human-specific quantifications. Then STR profiling and mtDNA sequencing was carried out through a laboratory. The total DNA was recovered and results were valid even after 6 months deposition of saliva (C. Zapico, S., & Roca, G. (2023).
In this study, recovery of saliva stains after water and detergent washing was done using different presumptive and confirmatory tests. Moreover, DNA quantity obtained after washing was also determined using spectrophotometer.

MATERIALS AND METHODS

Sample collection and preparation

Total 20 different types of clothes, 10 natural (Malmal, Pashmina, Mali, Silk, Khaddar, Cotton, Crepe, Linen, Wool and Terry) and 10 synthetic (Velvet, Nylon, Polyester, Jamawar, Net, Chiffon, Georgette, Crinkle, Denim and Spandex) were collected from a market in Lahore and stored at room temperature without providing any sterilized condition and prior washing. Approximately, 1-2ml fresh saliva was collected each time from a single donor, in 5ml falcon tube. Clothes were cut in smaller fragments (13×18cm) and 100µl saliva was applied to each cloth, then these stained clothes were allowed to dry at room temperature and stored in small individual centrifuge tubes.

Washing of clothes with surfactant

After preservation of neat saliva containing clothes (that did not go through washing process), other two sets of clothes were spotted with fresh saliva (100µl on each cloth) that allowed to dry at room temperature. Later on, one set clothes was treated with water upto three washings (until clothes gave the negative results) while the other set integrated with surfactant (Ariel) upto ten washings

(until clothes gave negative results). Like neat saliva containing clothes, these clothes were preserved in individual centrifuge tubes and all tubes were stored in refrigerator to avoid fungal growth on clothes.

Detection of saliva stains through ALS

Alternate light source (ALS) and radial gel diffusion test were used for detection of saliva stains on clothes. All preserved clothes (neat and those treated with water and surfactant) visualized under alternate light source (ALS), OZSTOCK® 51 LED UV Flashlight of wavelength 395nm. Moreover, various mobile camera filters were used to enhance the quality of pictures of saliva containing clothes. The extreme dark area was provided separately to each cloth to observe the pattern and luminescence produced by saliva containing clothes.

Measurement of amylase activity through radial gel diffusion test

For determination of amylase activity, the presumptive radial gel diffusion test was performed. 0.1M phosphate buffer was prepare by adding 2.7g anhydrous NaH2PO4, 3.9g anhydrous Na2HPO4, 0.2g NaCl and 500 ml distilled water in reagent bottle and PH (6.9) was maintained. For gel preparation, 20ml of phosphate buffer, 0.4g agarose and 0.02g soluble starch were mixed in a reagent bottle and heated for 30 seconds. After cooling at room temperature, mixture was poured in the petri plates. After 20 minutes, liquid got solidify, holes were punched into the gel and petri plates were labeled.

2µl of positive control (neat saliva), negative control (DEPC water) and dilutions of neat saliva (1:100 and 1:500) along with those all prior stored samples applied in each hole separately. Petri plates were sealed and incubated at 37℃ for 16 to 24 hours. Later on, the gel in each petri plate was stained with iodine development solution that was prepared with 4.95g potassium iodide (KI) and 7.62g iodine (I2) in 90ml of distilled water (Draz, Ali et al. 2019).

Quantification of DNA obtained from saliva stained clothes

Ethanol precipitation method was used to extract the DNA from the samples. Samples were transferred to each separate costar, 500µl lysis buffer (mixed 2g of SDS, 0.292g EDTA and 0.12g tris in 100ml of distilled water) and 5-10µl protenase K was added to each costar, centrifuged them at 10,000 rpm for 2 minutes. Solution carrying costars were incubated at 56℃ for 1 to 3 hours. Then 500µl PCI was added in each costar, inverted them for 3-5 minutes and centrifuged at 10,000 rpm and 4℃ for 10 minutes. The supernatant was transferred to individual centrifuge tubes and chilled isopropanol and 3M solution of sodium acetate mixed in the tubes, kept them in refrigerator for 1 hour and then centrifuged for 10 minutes at 10,000 rpm and 4℃. Pellet was decanted and 250µl ethanol (70%) was added to each tube to dissolve the pellet. After centrifugation at 12,000 rpm and 4℃ for 10 minutes, supernatant decanted from each tube and pellet was air dried. Lastly, 30µl of DEPC water added in all tubes and stored at -20℃.

The extracted DNA was visualized through agarose gel electrophoresis. For preparation of gel, 50ml of 1X TBE buffer (10.8g tris, 5.5g boric acid and 4ml disodium EDTA (Na2EDTA) dissolved in 900ml distilled water in reagent bottle, pH was maintained to 8.1 and solution was made upto 1000 ml), 0.5g agarose and 2.5µl of ethedium bromide mixed in a flask. Solution heated for 30 seconds for proper mixing, poured in the gel tray and an appropriate comb was inserted to create wells. After solidification of gel, the comb was removed and the gel tray was placed in the gel tank. 3µl of the each preserved DNA sample and 2µl of loading dye (for each sample) was mixed properly through a pipette (range=10µl) and loaded into the wells. The tank tightly closed with the lid, power (85-92 V) was supplied to run the gel for 30-40 minutes. When the gel covered the appropriate distance, the lid was removed and the gel carefully placed under UV light to observe the DNA bands.

Spectrophotometer was used to detect the absorbance of each sample at specific wavelengths (260 and 280nm). The dilutions of DNA samples were made (5µl DNA in 1495µl of distilled water) in

centrifuge tubes and these tubes preserved for 24 hours in refrigerator. After cooling at room temperature, the diluted samples were poured in a cuvette and placed inside the spectrophotometer. Absorbance of all samples was observed individually at 260nm and 280nm wavelengths by keeping each sample carrying the cuvette at same level in spectrophotometer. All the values were noted carefully, however, the value of DNA concentration was identified through a formula:

DNA concentration= (A260-A280) X dilution factor x 50 µg/ml

The mean value of diameters and standard deviation of the samples and controls were measured and compared (in millimeters mm) to determine the possible levels of amylase activity. All the cloth samples were tested after various time intervals (24 hours, 36 hours and 48 hours) and mean distribution of diameter and standard deviation was recorded by repeating the test five times per sample given the samples same environmental conditions (Draz, Ali et al. 2019). Graph Pad Prism and 2-way Anova method was used to generate graphs.

RESULTS

Three sets of all twenty (ten natural and ten synthetic) saliva containing clothes (first set of clothes was containing neat saliva, second set was treated with water for three washings while the third set was treated with detergent (Ariel) wash till three washings while five cloth samples gave positive results till ten washings= total 190 samples) were quantitatively analyzed after various presumptive tests to determine the amount of saliva that persist after washing of these clothes. Radial gel diffusion test, and ALS (alternate light source) were performed to detect saliva presence in clothes, following these tests, absorbance of samples was statistically observed through UV analysis.

Detection of saliva using alternate light source (ALS)

All saliva containing clothes were analyzed under alternate light source (ALS), at wavelength 395nm. Those clothes treated with water wash and surfactant wash gave negative results. When tested on plain clothes, ALS did not give false positive results for any cloth. Thus, neat saliva containing natural and synthetic clothes gave positive results. **Table 1** demonstrates all clothes on which saliva stains were visible and invisible. These clothes were analyzed in dark however a proper background was provided according to color of clothes to enhance detection. Texture, color and printed design of cloth as well as background color (black or white) on which the cloth was analyzed, greatly influenced the detection of saliva, dark colored clothes were more visible at light background and vice versa, while, printed clothes were difficult to analyze. Furthermore, various mobile filters also enhanced the quality of the picture that made the presence of saliva on picture even clearer. **Figure 1** shows the saliva containing clothes that gave positive results, thus saliva was exhibiting luminescence (bluish white glow) and pattern on variety of clothes under ALS.

Table 1. Visual detection of saliva containing natural and synthetic clothes under alternate light source (ALS)

Visual detection of saliva on Natural clothes under ALS		
No of Samples	**Cloth Type**	**Neat**
1	Malmal	NV*
2	Pashmina	NV
3	Mali	NV
4	Silk	NV
5	Khaddar	V*
6	Cotton	V
7	Crepe	V
8	Linen	NV

9	Wool	NV
10	Terry	NV
Visual detection of saliva on Synthetic clothes under ALS		
No of Samples	**Cloth Type**	**Neat**
11	Velvet	NV
12	Nylon	V
13	Polyester	NV
14	Jamawar	NV
15	Net	NV
16	Chiffon	V
17	Georgette	V
18	Crinkle	V
19	Denim	V
20	Spandex	V

*NV represents that saliva was not visible while V represents that saliva was visible

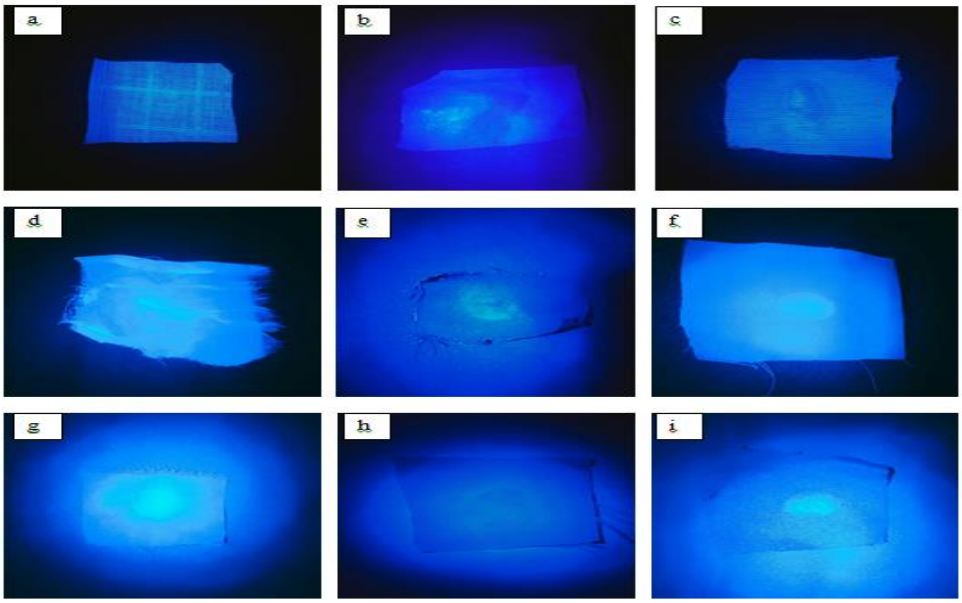


Figure 1. Saliva containing natural and synthetic clothes glowing under alternate light source (ALS).

a) Saliva spotted Khaddar cloth, showing a pattern on viewing under alternate light source (ALS). b) Cotton cloth containing saliva glowing under ALS. c) Crinkle cloth containing saliva showing a pattern under ALS. d) Saliva containing chiffon cloth illuminating under ALS. e) Saliva stain glowing under ALS on denim cloth. f) Saliva stain glowing under ALS on crepe cloth. g) Georgette cloth stained with saliva illuminating under ALS. h) Saliva stain showing pattern on nylon cloth under ALS. i) Spandex cloth containing saliva, glowing under ALS.

Detection of amylase through radial gel diffusion

All 20 saliva containing and plain clothes (containing no saliva) were analyzed through radial gel diffusion test thus there was no false positive result for any cloth. Neat saliva containing clothes distributed the higher mean diameter in comparison to the diameters after water and detergent wash. **Figure 2**, s1, s2, s3 and s4 display the salivary amylase activity of positive controls and different types of clothes. Below **Table 2** describes the individual mean distribution of amylase activity and standard deviation of neat saliva containing clothes before and after water wash. Thus, The mean distribution and standard deviation of each cloth was observed repeatedly through radial gel diffusion test after 24, 36 and 48 hours, however after these time intervals clothes did not show

much deviation regarding diameter, almost same level of amylase activity was observed in each cloth after repeated analysis. Below **Table 3**, s4 and s5 show the standard deviation and mean distribution of salivary amylase after surfactant wash, however the diameters of surfactant treated clothes were smaller in comparison to those treated with water wash, moreover, amylase activity on clothes remained same after repeated analysis. The **Figure 3** below, demonstrates the mean diameter ranges of natural and synthetic clothes after water and surfactant wash. After water wash of natural clothes, terry cloth showed the highest range of mean diameter for neat saliva as well as after 3rd water wash while malmal cloth gave the lower mean diameter for neat saliva. Linen and cotton clothes restrained the higher amount of saliva after 1st water wash while after 2nd water wash cotton cloth again showed the higher peak of mean diameter. On the other hand, wool cloth gave the negative results after 1st water wash.After water wash of synthetic clothes, shows that velvet, jamawar, net, chiffon, crinkle and spandex distributed almost equal higher ranges of mean diameter while georgette cloth gave the minimum mean diameter range for neat saliva containing clothes. After 1st water wash chiffon cloth gave the higher mean range for diameter while polyester cloth distributed the minimum mean diameter range. Nylon cloth distributed the higher mean diameter range after 2nd water wash whereas polyester gave negative results for 2nd water wash whereas velvet, nylon, net and chiffon clothes gave negative results for 3rd water wash.Surfactant wash of natural clothes demonstrate that cotton, khaddar, linen and terry clothes gave the positive results upto ten washings, while malmal cloth gave positive results upto seven washings, however, such clothes can be beneficial for forensic purpose to generate a genetic profile. According to the figure, cotton cloth overall gave the highest ranges of mean diameter throughout the 10 washings while mali and crepe gave negative results throughout all washings.Surfactant wash of synthetic clothes demonstrate that velvet, jamawar, net, chiffon, crinkle and spandex cloth distributed the higher ranges of mean diameter for neat. Neat saliva containing synthetic clothes distributed the higher ranges mean diameter in comparison to those treated with surfactant wash. Other clothes like velvet, nylon, chiffon, georgette, denim and spandex gave negative results throughout.

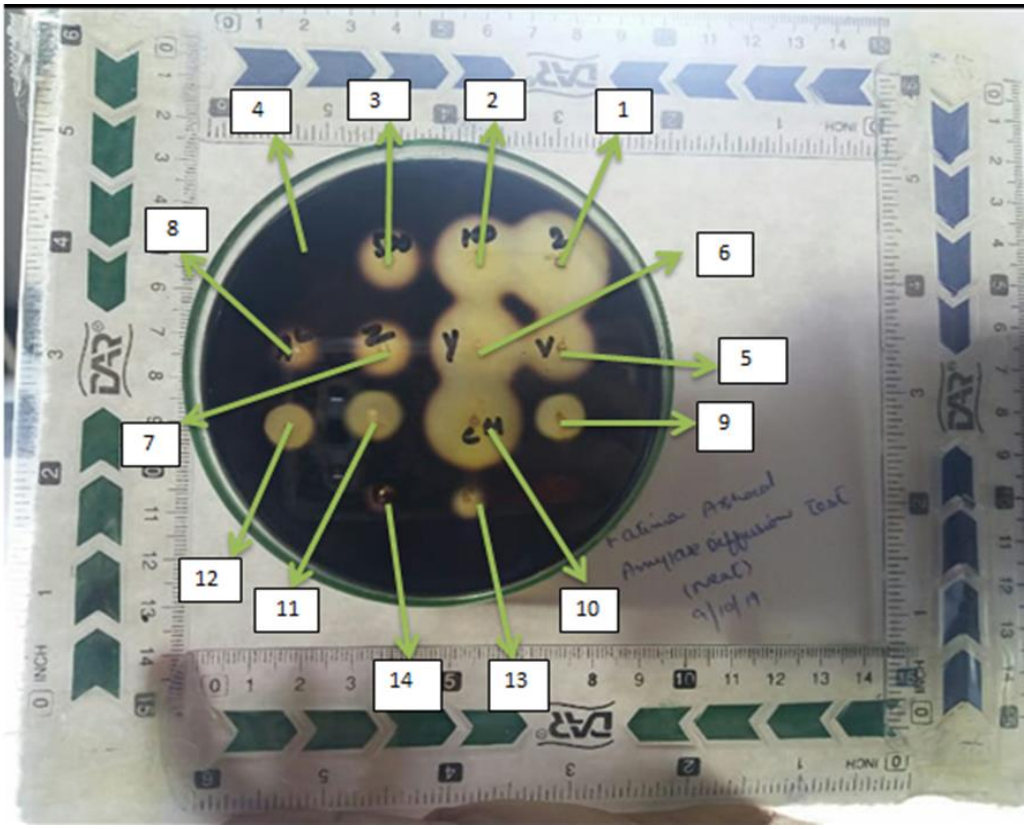


Figure 2: Identification of amylase activity of saliva on neat clothes

(Controls) Well No 1: Neat; Well No 2: 1:100; Well No 3: 1:500; Well No 4: DEPC water (Samples/ Clothes type) Well No 5: Denim; Well No 6: Spandex; Well No 7: Terry; Well No 8: Khaddar; Well No 9: Chiffon; Well No 10: Cotton; Well No 11: Mali; Well No 12: Nylon; Well No-13: Crepe; Well No 14: Jamawar

Table 2: Mean distribution and standard deviation of salivary amylase for saliva containing clothes after 1st, 2nd and 3rd water wash

Water based detection									
Neat				**1st water wash**		**2nd water wash**		**3rd water wash**	
Sr. No	**Clothing Type**	**M.D***	**S.D***	**M.D**	**S.D**	**M.D**	**S.D**	**M.D**	**S.D**
A	Malmal (Ntrl)*	6.5	0.289	4.5	0.115	3.5	0.083	3.3	0.167
B	Pashmina (Ntrl)	8.5	0.289	5.5	0.289	4.7	0.144	0	0
C	Velvet (Syn)*	8	0.577	6.3	0.726	3.7	0.144	0	0
D	Mali (Ntrl)	8.5	0.521	5.3	0.441	3.7	0.167	0	0
E	Nylon (Syn)	7	0.577	6	0.577	5.5	0.289	0	0
F	Polyester (Syn)	6.5	0.289	4	0.577	0	0	0	0
G	Jamawar (Syn)	9	0.577	6.5	0.289	4	0	3.2	0.145
H	Net (Syn)	8.7	0.333	5.8	0.333	4.7	0.441	0	0
I	Chiffon (Syn)	8.2	0.167	7	0.289	4.5	0.289	0	0
J	Georgette (Syn)	6	0.577	6.8	0.167	3.8	0.167	3.2	0.167
K	Silk (Ntrl)	8.8	0.441	6.3	0.601	3.2	0.167	0	0
L	Khaddar (Ntrl)	9	0.577	6	0.577	4.3	0.167	3.8	0.4
M	Cotton (Ntrl)	9.3	0.441	7.7	0.3	6.3	0.441	5.2	0.177
N	Crepe (Ntrl)	8.7	0.441	5.3	0.333	3.2	0.167	0	0
O	Linen (Ntrl)	9.3	0.333	8.2	0.167	4.5	0	4.2	0.167
U	Crinkle (Syn)	8.7	0.667	6.5	0	3.7	0.144	3.3	0.144
V	Denim (Syn)	6.7	0.167	5	0.577	5.5	0.289	3.3	0.333
X	Wool (Ntrl)	9.7	0.882	5.7	0.167	0	0	0	0
Y	Spandex (Syn)	8.8	0.726	6.2	0.601	4.2	0.167	3	0
Z	Terry (Ntrl)	10.2	0.441	6.3	0.167	5.8	0.167	5.8	0.167

*Dia represents the Diameter of Amylase activity while M.D represents the Mean Distribution of Salivary Amylase and S.D represents the Standard Deviation of Salivary Amylase.
*Ntrl represents the natural clothes while Syn represents the synthetic clothes.

Table 3: Mean distribution and standard deviation of salivary amylase for saliva containing clothes treated with surfactant upto three washings

Surfactant based detection									
Surfactant wash				**1st wash**		**2nd wash**		**3rd wash**	
Sr. No	**Clothing Type**	**M.D***	**S.D***	**M.D**	**S.D**	**M.D**	**S.D**	**M.D**	**S.D**
A	Malmal (Ntrl)*	4.5	0.289	4.2	0.601	3.3	0.333	2.8	0.441
B	Pashmina (Ntrl)	3.5	0.289	2.7	0.441	0	0	0	0
C	Velvet (Syn)*	0	0	0	0	0	0	0	0
D	Mali (Ntrl)	0	0	0	0	0	0	0	0
E	Nylon (Syn)	0	0	0	0	0	0	0	0
F	Polyester (Syn)	4	0.289	2.7	0.333	0	0	0	0
G	Jamawar (Syn)	3.5	0.289	2.8	0.167	0	0	0	0
H	Net (Syn)	2.5	0.289	2	0	0	0	0	0
I	Chiffon (Syn)	0	0	0	0	0	0	0	0
J	Georgette (Syn)	0	0	0	0	0	0	0	0
K	Silk (Ntrl)	2.7	0.433	0	0	0	0	0	0
L	Khaddar (Ntrl)	4.9	0.083	4.6	0.3	4.2	0.167	3.2	0.145
M	Cotton (Ntrl)	6.7	0.145	6	0.289	5.5	0.5	5.2	0.441
N	Crepe (Ntrl)	0	0	0	0	0	0	0	0
O	Linen (Ntrl)	5.6	0.208	5.3	0.333	4	0.289	3.7	0.233
U	Crinkle (Syn)	3	0.289	0	0	0	0	0	0
V	Denim (Syn)	0	0	0	0	0	0	0	0
X	Wool (Ntrl)	2.7	0.441	0	0	0	0	0	0
Y	Spandex (Syn)	0	0	0	0	0	0	0	0
Z	Terry (Ntrl)	5.8	0.115	5.3	0.333	4.7	0.167	3.7	0.167

*****M.D represents the Mean Distribution of Salivary Amylase and S.D represents the Standard Deviation of Salivary Amylase.
*Ntrl represents the natural clothes while Syn represents the synthetic clothes

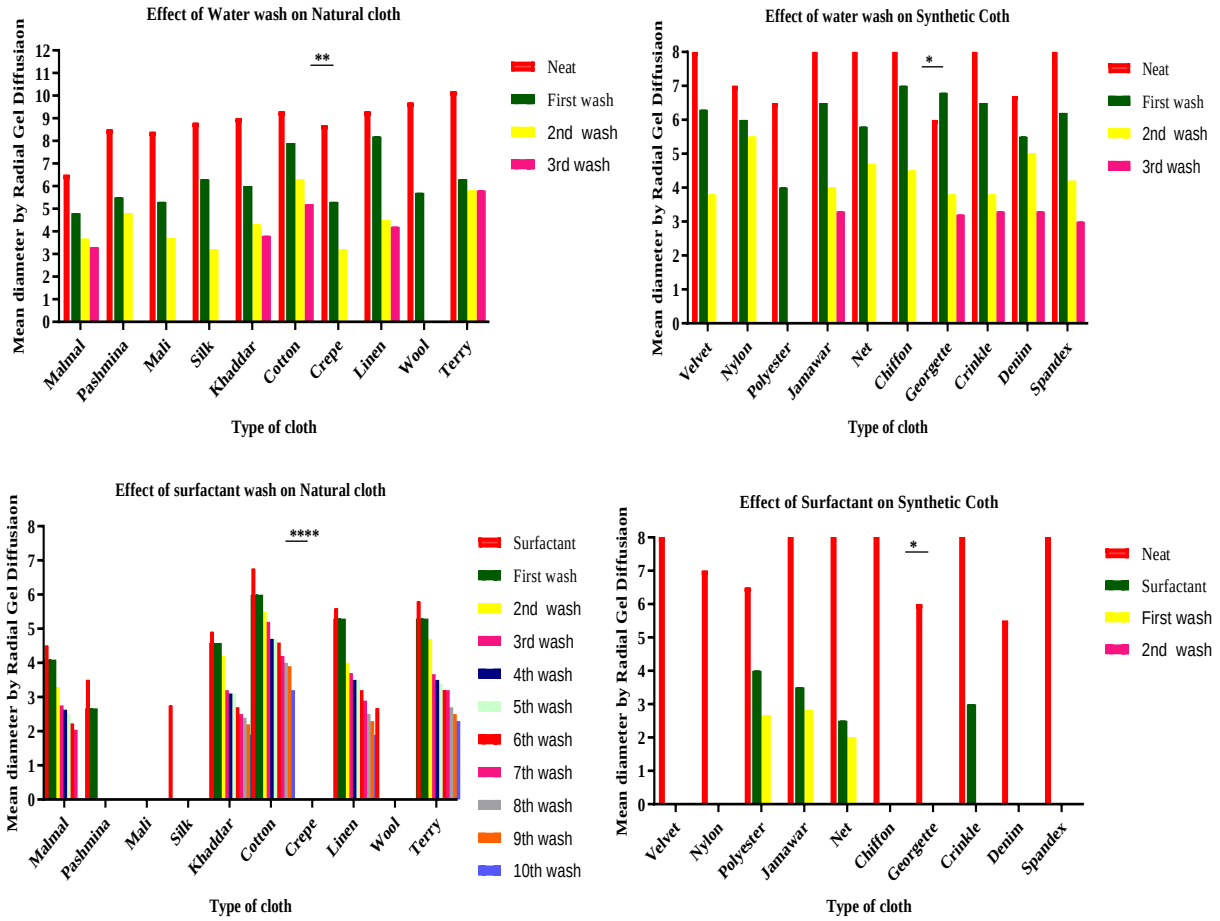


Figure 3: Comparison of mean diameter distributed by saliva containing natural and synthetic clothes, before and after water and surfactant wash

Recovery of DNA from saliva stains after washing treatment

The preserved DNA was further treated with agarose gel electrophoresis method for visual detection. **Table 4** and s6 exhibit the detection of DNA from natural and synthetic clothes after water and surfactant wash. Below **Table 4** demonstrates that linen, terry, denim and spandex cloth restrained the detectable amount of DNA upto three water washings, while khaddar, cotton, velvet, nylon and jamawar clothes could hold back the DNA till first water wash. Malmal, pashmina, silk, wool, net, chiffon and georgette could give the detectable amount of DNA only for neat saliva; on the other hand mali, crepe, polyester and crinkle did not give any detectable amount of DNA. The below labeled **Figure 4** shows the various positive bands of DNA samples with an orange glow. DNA extracted from various types of clothes containing neat saliva as well as after treatment with water and surfactant wash, was loaded in the wells for visual detection of DNA from the samples.

Table 4: Visual detection of extracted DNA from natural and synthetic clothes after water wash

Visibility of extracted DNA from natural clothes treated with water wash					
No of Samples	**Cloth Type**	**Neat**	**1st wash**	**2nd wash**	**3rd wash**
1	Malmal	D*	ND*	ND	ND
2	Pashmina	D	ND	ND	ND
3	Mali	ND	ND	ND	ND
4	Silk	D	ND	ND	ND
5	Khaddar	D	D	ND	ND

6	Cotton	D	D	ND	ND
7	Crepe	ND	ND	ND	ND
8	Linen	D	D	D	D
9	Wool	D	ND	ND	ND`
10	Terry	D	D	D	D
Visibility of DNA extracted from synthetic clothes treated with water wash					
No of Samples	**Cloth Type**	**Neat**	**1st wash**	**2nd wash**	**3rd wash**
11	Velvet	D	D	ND	ND
12	Nylon	D	D	ND	ND
13	Polyester	ND	ND	ND	ND
14	Jamawar	D	D	ND	ND
15	Net	D	ND	ND	ND
16	Chiffon	D	ND	ND	ND
17	Georgette	D	ND	ND	ND
18	Crinkle	ND	ND	ND	ND
19	Denim	D	D	D	D
20	Spandex	D	D	D	D

*D represents that DNA detected (band appear) while ND represents no DNA detected (no band appear)

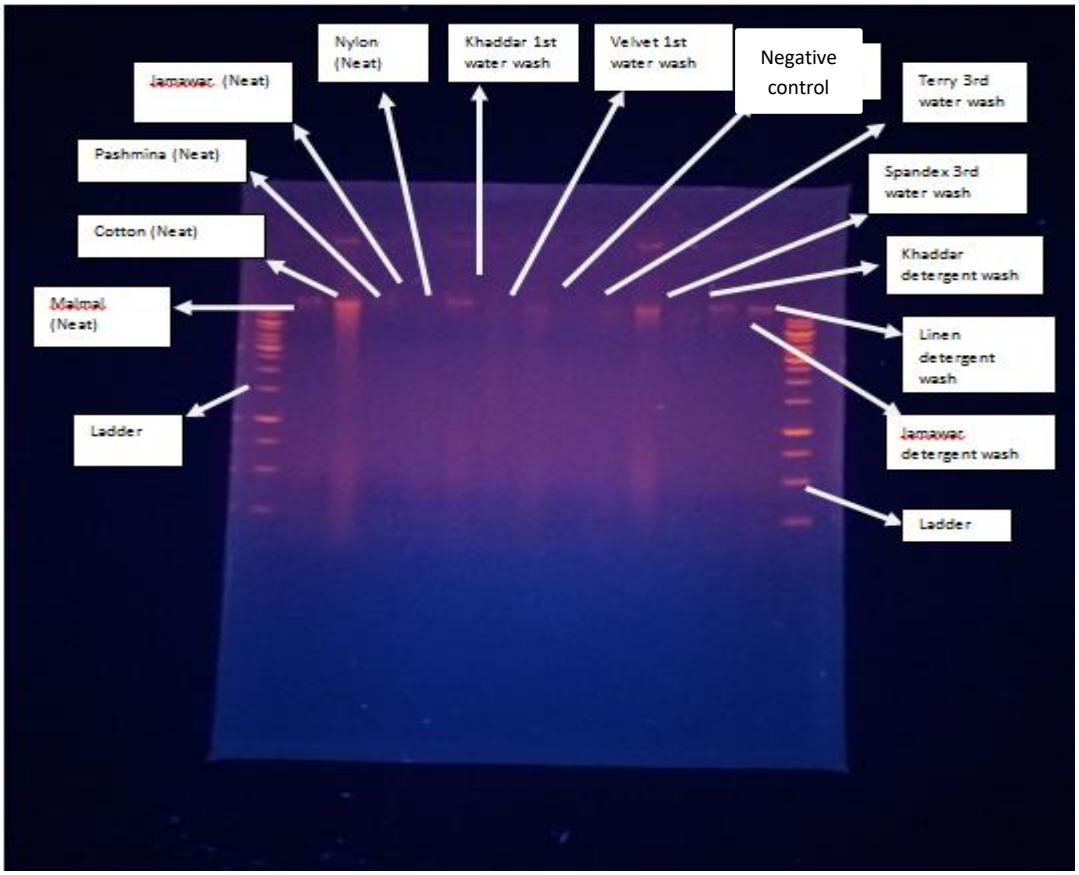
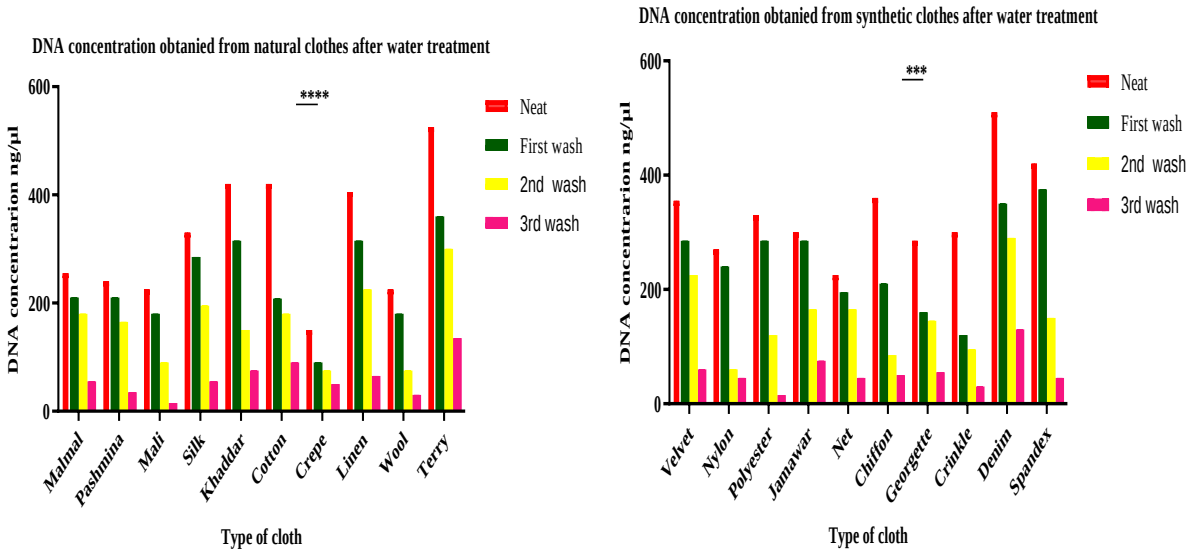


Figure 4: DNA extracted from different type of clothes, containing neat saliva and those treated with water and surfactant wash, loaded and run through agarose gel electrophoresis.

UV absorbance analysis

Diluted samples of DNA were analyzed under spectrophotometer at wavelength 260 and 280nm to determine the purity and concentration of the extracted DNA from samples. Like amylase activity of clothes in radial gel diffusion test, neat natural clothes and those treated with water wash gave the higher amounts of DNA concentration in comparison to synthetic clothes treated with water as well as other natural and synthetic clothes treated with surfactant wash. Below **Figure 5** exhibits that after water washing of natural clothes terry cloth gave the higher ranges of DNA concentration throughout neat, 1st, 2nd and 3rd water wash while remaining clothes in comparison to each other demonstrate the DNA concentration with some differences. Crepe cloth overall gave the lower amounts for neat, 1st and 2nd wash while mali cloth distributed the lowest amount for 3rd wash relatively to all other DNA samples extracted from clothes.After water washing of synthetic clothes, denim cloth gave the higher values of DNA concentration throughout neat, 1st and 3rd water wash while spandex cloth overall exhibited the higher value for 2nd water wash and other clothes show variance in comparison to each other. Overall, net cloth gave the minimum concentration for DNA extracted from neat cloth, crinkle cloth gave minimum value for 1st wash, nylon cloth exhibited the lower range for 2nd water wash and polyester cloth gave lower range for 3rd water wash. **Figure 5** also demonstrates that in comparison to all other natural clothes, terry gave the higher values of DNA concentration for surfactant and 3rd wash while linen cloth displayed the higher values of concentration for 1st and 2nd wash. However, overall, crepe and wool cloth exhibited minimum ranges of concentration for surfactant, 1st, 2nd and 3rd wash.After surfactant washing of synthetic clothes, denim cloth overall exhibited higher range for surfactant wash. Overall, nylon and denim both displayed the higher and equal amounts of concentration for 1st wash. Denim and spandex both demonstrated the equal ranges of concentration for 2nd wash. Denim and jamawar gave almost same values for DNA concentration after 3rd wash.

Overall, chiffon cloth gave the minimum quantities for surfactant wash, while all remaining clothes displayed quantities with some differences after 1st, 2nd and 3rd wash. Below **Table 5** and s7 demonstrate the individual quantities of absorbance of each cloth after water and surfactant wash; however, comparatively these quantities were gradually decreasing after each wash.



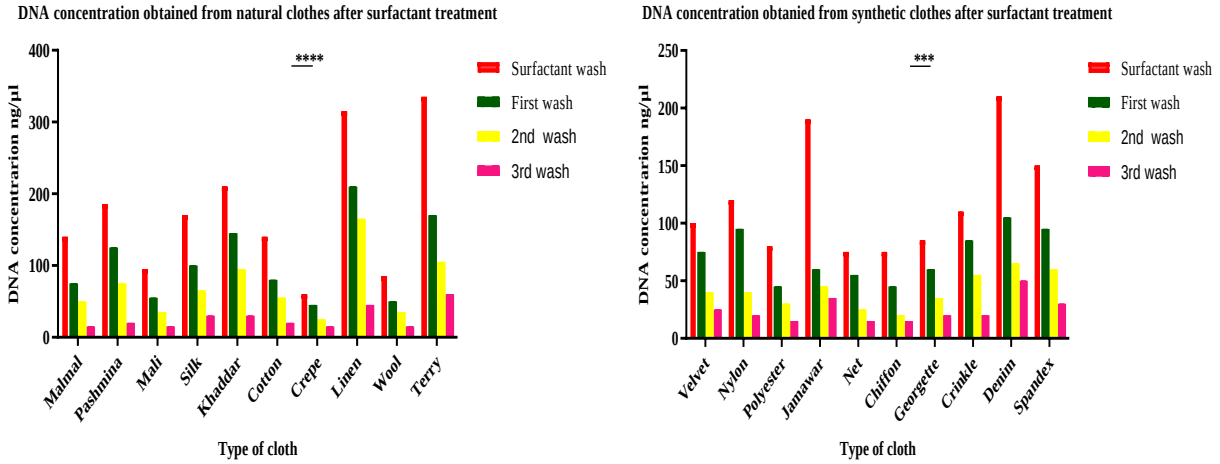


Figure 5: Absorbance analysis of extracted DNA to determine DNA concentration of natural and synthetic clothes after treatment with water and surfactant wash

Table 5: DNA concentration of natural and synthetic clothes containing neat saliva and those treated with water wash

Natural water wash DNA concentration					
No of Samples	**Cloth Type**	**Neat**	**1st wash**	**2nd wash**	**3rd wash**
1	Malmal	255	210	180	55
2	Pashmina	240	210	165	35
3	Mali	225	180	90	15
4	Silk	330	285	195	55
5	Khaddar	420	315	150	75
6	Cotton	420	208	180	90
7	Crepe	150	90	75	50
8	Linen	405	315	225	65
9	Wool	225	180	75	30
10	Terry	525	360	300	135
Synthetic water wash DNA concentration					
No of Samples	**Cloth Type**	**Neat**	**1st wash**	**2nd wash**	**3rd wash**
11	Velvet	355	285	225	60
12	Nylon	270	240	60	45
13	Polyester	330	285	120	15
14	Jamawar	300	285	165	75
15	Net	225	195	165	45
16	Chiffon	360	210	85	50
17	Georgette	285	160	145	55
18	Crinkle	300	120	95	30
19	Denim	510	350	290	130
20	Spandex	420	375	150	45

DISCUSSION

In this study saliva containing clothes were analyzed under alternate light source (ALS) at 455nm wavelength, it was noted that after washing the ability of saliva detection under ALS immensely decreases. Moreover, the color, design, background and texture of cloth influence the detection. These results correlate with findings of Kulstein and Wiegand that revealed the saliva detection on clothes (synthetic and cotton clothes) under ALS at wavelength 430-470 nm, prior to washing, while the luminescence vanished after laundering at 40℃ (Kulstein and Wiegand 2018). Similarly, another study demonstrated that washing, even at low temperature made the biological fluids undetectable when viewed under UV light sources (Fiedler 2008). However, these findings demonstrated that type of clothes does not influence the fluorescent signals, while dark color clothes absorb light and decrease the chances of detection of biological fluids. This research contradicts with findings of Fielder, according to which pure black clothes does not allow the detection of stains at any wavelength on the other hand; this study showed the clear fluorescence of saliva on black denim and spandex clothes under alternate light source (ALS). Previous research of Wang exhibited that saliva stains can be visually detected from bath towel at wavelength 447nm with a blue laser light (Wang 2017), in contrast to this study in which saliva stain was undetectable on terry cloth when visualized under ALS. Another research (Vandenberg and van Oorschot 2006) concluded that type and color of clothes, background color and their absorbency effects the detection of saliva under ALS. Similarly, excitation/emission conditions, contrast and wavelength of the equipment influences the detection.

Radial gel diffusion test demonstrated that neat saliva containing clothes distribute the maximum ranges of diameter in comparison to those treated with water and surfactant wash as diameter distribution decreased after washing. Furthermore, natural clothes distributed the higher ranges of mean diameter comparatively to synthetic clothes and gave positive results upto ten washings. However, these results align with earlier findings of Mussabekova in 2017, demonstrated that laundering conditions and surfactant usage degrades the biological fluids as hydrogen bonds in double helix of DNA tend to degrade through heat and surfactant effect thus make the saliva detection harder (Mussabekova 2017). Washing of clothes with a detergent depend on physical nature of cloth (cloth type, gauge, warp and weft), chemical nature of detergent (acidic/basic nature and ionic charge) and washing time provided to clothes (Simon et al., 2013; Bajpai and Tyagi 2007). Natural fibers like wool (animal source), cotton (plant source) and linen (flax plant source) have higher absorbency and retention for moisture. Wool has higher insulation properties even when wet but has low resistance while cotton and linen has high resistance for alkalis and cleaning agents, solvents and cold dilute acids and thus have low chances to lose of biological fluids. On the other hand, polyester is a synthetic fiber that has high resistance for dirt and decay of mold while low tendency for moisture absorbance and retention. At room temperature polyester shows resistance for bleaches and alkalis (Mushtaq et al; Crow et al, 1998). These findings correlate with the results of this study as natural clothes like cotton, terry (cotton and silk source), and linen and khaddar (cotton source) clothes gave the positive results even after surfactant wash till ten washings, however mean diameter decreased after each wash, while wool and polyester clothes minimum diameter range to negative results after water and surfactant wash.

This study relied on the agarose gel electrophoresis method for DNA extraction while UV absorbance of the samples was analyzed through spectrophotometer similar to an earlier research in 2012, manifested that DNA can be extracted from fresh saliva. Moreover, extraction seemed possible from dried old saliva stain as well but there was the considerable difference in the quantities of saliva extracted from fresh and dried saliva stains. The quantity of DNA concentration obtained from fresh saliva was 125-795µg/ml (Saroch and MP 2012). These results correlate with these findings, as in this study the recorded amount of DNA concentration from fresh saliva containing samples ranged from 15-525µg/ml. However, a different formula (A_{280}-A_{260} x 50 x dilution factor) was used in this study; furthermore, absorbance of each sample was noted at 260nm to determine the DNA concentration of the sample while at 280nm to determine the purity of

samples. Thus, impurities were eliminated and pure DNA concentration of the samples was determined.

The amount of saliva Furthermore, natural fibers have greater chances to hold back saliva stains for longer time than the synthetic fibers due to uneven structure and surface of natural clothes, whereas, synthetic clothes contain smooth surfaces and cannot persist saliva stain. Though, trace amount of saliva present on natural clothes can be detected more easily (Mussabekova 2017). This research somehow conflicts with this statement as many synthetic clothes gave effective results when analyzed through various tests; such as crinkle, chiffon, denim, georgette, spandex and nylon, all these synthetic clothes gave positive results under ALS. Similarly, denim and spandex cloth exhibited the sharp band for DNA till 3rd water wash when analyzed under UV as well as these two clothes gave efficient results for UV absorbance analysis. In another way, the results of this study align with the statement as for radial gel diffusion test natural clothes gave positive results till ten washings, similarly exhibited mean diameter and concentration range was comparatively higher for natural than synthetic clothes. Furthermore, these contradictions can be supported through Kulstein and Weigand's findings, revealed that saliva collected from two different individuals, results in the variance in amount of extracted DNA because of different composition and amount of cells. So, it can be hypothesized that DNA collected from different individual can give different results on same clothes. However, the increased washing cycle and delayed analysis can decrease the quantities of DNA on both cotton and synthetic clothes (Kulstein and Wiegand 2018). These earlier findings about DNA quantities also correlate with this study as low amounts of DNA were identified when visualized under UV similarly for UV absorbance analysis after washing of clothes with water and surfactant wash.

CONCLUSION

This study concluded that both natural clothes and synthetic can detect saliva even after water and surfactant wash in this way diameter decreases after each wash in comparison to neat clothes. Natural clothes like terry, cotton, linen and khaddar while synthetic clothes like denim and spandex gave efficient results overall while wool, polyester and nylon clothes gave poor results. Terry, linen, denim and spandex cloth gave the high amount of DNA under UV. Thus, a good DNA profile can be generated that helps to solve the criminal cases.

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