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ORIGINAL ARTICLE

Using oral microbial DNA analysis to identify expirated bloodspatter

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Abstract Distinguishing expirated bloodstains (blood forced by airflow out of the nose, mouth or a chest wound) from impact spatter (blood from gunshots, explosives, blunt force trauma and/or machinery accidents) is an important challenge in forensic science. Streptococcal bacteria are only found in the human mouth and saliva. This study developed a polymerase chain reaction (PCR) method that detects DNA from these bacteria 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 very 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. In a blind trial, the PCR method was able to distinguish three mock forensic samples that contained expirated blood from four that did not. Our data show that bacteria present in the oral cavity can be detected in bloodstains that contain saliva and therefore can potentially be used as a marker in forensic work to distinguish mouth-expirated bloodstains from other types of bloodstains.

Keywords Expirated bloodstains · Saliva · DNA analysis · Glucosyltransferase genes · *Streptococcus*

Introduction

Bloodstain pattern analysis (BPA) is a forensic term used to describe the examination, identification and interpretation of bloodstain patterns in relation to the events that gave rise to them. One of the limitations of BPA is that some bloodstain patterns do not appear to have sufficient individual characteristics to enable analysts to reliably distinguish between the various causal mechanisms. Expirated bloodstains are bloodstain patterns resulting from blood forced by airflow out of the nose, mouth or a wound [1] and may result in the formation of very small bloodstains comparable to those found in impact bloodstain patterns from gunshots, blunt force trauma, explosives and machinery accidents [2]. In several high profile homicide trials in various jurisdictions, bloodstain pattern analysts have found themselves at the centre of arguments over the interpretation of the mechanisms that produce these small bloodstains [3, 4]. Of particular concern has been the differentiation between impact spatter patterns and expirated bloodstain patterns. Expirated bloodstains on an accused person's clothing could occur when assisting an injured person, a finding which would tend to exonerate

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that individual, whereas impact spatter stains on clothing tend to occur due to the proximity of the person to the bloodshedding event, consistent with guilt.

One method used to determine if bloodstains are expirated in origin involves the use of chemical tests detecting the salivary enzyme amylase, such as the Phadebas® test [5–7]. However, amounts of salivary amylase are affected by food and salivation [8] and can vary between individuals [9, 10]. In addition, current methods for the detection of salivary amylase lack sensitivity when they are used on stains less than 3 mm in diameter [11].

A potential alternative approach for the detection of saliva is the use of salivary oral microbes as a biomarker. Oral microbes are present in large numbers in saliva, and their presence in a bloodstain would indicate that the blood was expiratory in origin. The majority of the bacteria found in the oral cavity are unique to this habitat, with *Streptococcus* species comprising almost 50% of the total cultivable microflora from the oral cavity [12]. Identification of streptococcal bacteria requires the use of specialist growth media and expertise to distinguish them from other bacterial species. In addition, these bacteria lose viability quite rapidly outside the oral cavity [13].

An attractive alternative approach to determine if streptococci are, or have been, present in expirated bloodstains is to test for the presence of *Streptococcus*-specific DNA. Oral streptococci possess surface proteins that permit them to be primary colonisers of the oral cavity. One group of these proteins is produced from glucosyltransferase (*gtf*) genes, which encode enzymes that hydrolyse sucrose to synthesise extracellular polysaccharides. These polysaccharides (and hence the *gtf* genes) are unique to oral streptococci and contribute to the structural integrity of dental plaque and adhesion of the bacteria on tooth surfaces [12]. Each *Streptococcus* species possesses at least one glucosyltransferase enzyme. Glucosyltransferases are large enzymes that consist of four domains, one of which is not conserved between species and is thought to be species specific [14, 15]. This has led to the development of polymerase chain reaction (PCR) primers targeting the non-conserved regions of *gtf* genes (gtf primers) that can be used to detect and differentiate between species of oral streptococci [15].

Recent research, using either gtf primers or primers for genes encoding ribosomal RNA, has shown that PCR can be used to detect streptococcal DNA in saliva and in blood–saliva mixtures [16, 17]. The purpose of this study was to investigate whether detection of the non-conserved region of the *gtf* genes could provide a sensitive and specific test for the presence of DNA from oral streptococci in saliva and consequently a potential tool to distinguish mouth-expirated bloodstains from other bloodstains.

Materials and methods

Samples and bacterial strains

All bacterial strains used in this study are listed in Table 1. Strains of *Streptococcus* species were obtained from Professor John Tagg (Department of Microbiology and Immunology, University of Otago). Other species were from laboratory stocks. Streptococci were grown on tryptic soy agar with 5% sheep's blood (Fort Richard Laboratories, New Zealand) overnight for 8–12 h at 37°C, in a 5% CO_2 incubator. For liquid cultures, a single colony was inoculated into 10-ml Todd Hewitt broth and incubated overnight at 37°C in a 5% CO_2 incubator. The other microbes were grown on Luria Broth agar plates (Gibco BRL) overnight at 37°C or in Luria Broth (Gibco BRL) overnight at 37°C in a shaking incubator.

Human DNA from white blood cells was kindly provided by Marilyn Merriman (Department of Biochemistry, University of Otago). Microbial DNA extracted from soil was provided by the Institute of Environmental Science and Research (ESR) Ltd in Porirua [18]. DNA was extracted from samples of soil using a fast prep® soil kit (BIO 101).

Non-stimulated human saliva was collected from 100 healthy adult volunteers, aged between 18 and 65 years, with 58 participants being female and 42 being male. The volunteers expectorated approximately 50 μl of saliva into a plastic 1.5-ml microcentrifuge tube. The saliva samples were processed for microbial DNA extraction with Instagene matrix within 12 h of saliva collection. All saliva samples were stored at 4°C until processing. Ethics approval was obtained from the University of Otago Human Ethics Committee, and informed consent was obtained from all participants, before samples were collected.

Bloodstain production

Human blood was collected from one individual by a trained phlebotomist. Bloodstains were produced in two ways. The blood was placed into a plastic spray bottle, and the blood was then sprayed on the target surface from approximately 150 mm. Alternatively, 1 ml of blood was pipetted directly from approximately 300 mm onto the target surface. To prepare expirated bloodstains, a volunteer transferred 1 ml of their own blood into their mouth and then coughed it out approximately 300 mm onto the target surface. Alternatively, 5 ml of blood was mixed with human saliva in a spray bottle (ratios from 1:1 to 1:1,000 blood:saliva), and the mixed blood/saliva was sprayed from approximately 150 mm onto the target. The target surfaces were clean non-sterile cloth fabrics, carpet and white glossy cardboard. The cardboard samples were dried, and the

Table 1 Bacterial strains used in this study

Bacterial species	Strain number	Reference
Enterococcus faecalis	JH2	[35]
Escherichia coli	DH5α	[36]
Pseudomonas aeruginosa	PAO1	[37]
Streptococcus gordonii (ATCC type strain)	Challis DL1	[38]
S. gordonii	D105	J. Tagg
Streptococcus mutans (NCTC type strain)	10449	[39]
S. mutans	MT8148	[40]
S. mutans	NY266 (formerly T2)	[41]
S. mutans	OMZ175	[42]
S. mutans	UA159	J. Novak, University of Alabama [43]
Streptococcus pyogenes	FF22	[44]
Streptococcus salivarius	DC156A	J. Tagg
S. salivarius	G32	J. Tagg
S. salivarius (ATCC type strain)	JIM8777	J. Tagg
S. salivarius	K12	[45]
S. salivarius	MIN5	[13]
S. salivarius	MP5	[46]
S. salivarius	PIRIESt^{R}	J. Tagg
S. salivarius	2OP3	[46]
S. salivarius	#5	[46]
S. salivarius	#9	[46]
Streptococcus sanguinis (ATCC type strain)	10556 aka SK59	[47]
S. sanguinis	H15	[48]
S. sanguinis	H18	[48]
S. sanguinis	H25	[48]
S. sanguinis	K4	[48]
S. sanguinis	K11	[48]
S. sanguinis	K16	[48]
S. sanguinis	K18	[48]
S. sanguinis	K23	[48]
S. sanguinis	K28A	[48]

ATCC American Tissue Cell Culture, *NCTC* National Collection of Type Cultures

bloodstains were swabbed with sterile cotton swabs (NZ Medical & Scientific LTD) that had been moistened with sterile milliQ water. The cloth samples were dried, and 1× 1 cm square fabric swatches were excised. These samples were kept in sealed plastic bags at room temperature throughout the duration of the study. Stains on denim fabric were also washed (warm machine wash) using a biological laundry detergent. The washed stains were then air dried on a clothes line for 3 h before cutting out the stain swatch and testing.

Bloodstains for blind trial testing were generated on cardboard. These were provided for analysis (three as spots on excised pieces of cardboard and four swabbed onto cotton) with no information regarding the nature of each sample.

Extraction of DNA

Each sample was added to 1 ml of sterile water in a 1.5-ml mini-centrifuge tube, mixed by vortexing for 10 s at maximum and allowed to stand at room temperature for 30 min. Each tube was vortexed again for 10 s and then the fabric swatch or swab tip was removed. The sample was then processed using Instagene matrix (BioRad) following the manufacturer's instructions (whole blood protocol). The DNA present in the supernatant resin of the Instagene matrix was stored at −20°C until required. Genomic DNA from laboratory-grown bacteria was extracted using a Microbial Genomic DNA isolation Kit (MoBio), following the protocol provided by the manufacturer. The DNA was stored at −20°C until required.

Polymerase chain reaction

PCR primers have been described previously [15, 19] or were developed in this study and are listed in Table 2. PCR was carried out in 20-µl reaction mixtures with HotStarTaq Master Mix polymerase (Qiagen Corporation, USA). Amplification was performed with a BioRad thermocycler using the following protocol: 95°C (15 min); 94°C (5 min); 30 cycles of 94°C (10 s), 56°C (15 s) and 72°C (1 min); 72°C (5 min). PCR products were analysed via electrophoresis in 0.85% sodium borate agarose gels containing ethidium bromide to a final concentration of 0.001% [20] with 1 Kb plus DNA marker (Invitrogen) included on all gels. The DNA was visualised using UV light and a BioRad Gel Doc illuminator.

QPCR was carried out in 10-µl reaction mixtures on a 96-well plate (Applied Biosystems, Australia and New Zealand) with LC480 2× Master Mix (Roche Applied Science, Germany). Amplifications were performed with a Light Cycler® 480 thermo cycler (Roche Applied Science, Germany). The QPCR program was performed using the following protocol: 95°C (10 min); 45 cycles of 95°C (10 s), 60°C (10 s) and 72°C (20 s); 40°C (10 s). Melting curve analysis was carried out using the software provided with the Light Cycler to confirm that the intended products had been amplified. QPCR results were analysed using the Absolute Quantification software program with the 480 thermo cycler (Roche Applied Science, Germany). Absolute quantification involves comparing the amplification of target nucleic acids in an unknown sample against a standard curve with known concentrations of the same target.

Phadebas blue paper test

The Phadebas blue paper press test (Phadebas®) was used to detect the presence of amylase in stains [7, 21, 22]. The blue paper consists of starch polymer chains that are chemically attached to a water-soluble blue dye. In the presence of amylase, the starch is hydrolysed releasing the dye. The intensity of the blue colour is indicative of the amylase activity in the stain. Phadebas® press test sheets were placed onto each object of interest and moistened with water. Test sheets were held down with weights and left at room temperature for 30–60 min. After this time, the sheets were removed and examined for hydrolysis of the dye.

Results

Specificity of gtf primers

The gtf primers were used in PCR reactions with purified DNA from multiple strains of the four target *Streptococcus* species as well as DNA from four other microbes (*Escherichia coli*, *Pseudomonas aeruginosa*, *Enterococcus faecalis* and *Streptococcus pyogenes*), DNA extracted from soil and human DNA. Each of the four primer sets produced the expected PCR product from multiple strains within its target species, although not all products were made in equal amounts. Data for *Streptococcus sanguinis* and *Streptococcus salivarius* are shown in Supplementary Fig. S1. DNA sequencing of PCR products confirmed that they had been amplified from the targeted *gtf* genes. None of the primer sets gave PCR product with DNA from any of the other *Streptococcus* species or any of the other microbes, with microbial DNA extracted from soil samples or with human DNA (data not shown) demonstrating high specificity for the oral streptococcal target species. The *Streptococcus gordonii* gtf primers resulted in a small amount of a larger PCR product using *S. pyogenes* template DNA, but this product was easily distinguished from the correctly sized *S. gordonii* product.

The gtf primer sets were also compared to sequenced microbial genes that were present in Genbank (www.ncbi.nlm.nih.gov) [23]. This comparison showed that amongst sequenced microbes, the primers are very specific for the target streptococcal *gtf* genes.

Table 2 Polymerase chain reaction primers used in this study

Target	Primer sequence (5′–3′)	Fragment size (bp)	Reference
S. mutans gtfD for	ggcaccacaacattgggaagctcagtt	433	[15]
S. mutans gtfD rev	ggaatggccgctaagtcaacaggat		[15]
S. sanguinis gtfP for	gatgttaagcaggtggcagttcaag	505	This study
S. sanguinis gtfP rev	catcatgctcagtattaacaggcg		This study
S. gordonii gtfG for	ctatgcggatgatgcttaatcaagtg	440	[15]
S. gordonii gtfG rev	ggagtcgctataatcttgtcagaaa		[15]
S. salivarius gtfK for	gtgttgccacatcttcactcgcttcgg	548	[15]
S. salivarius gtfK rev	atgaatacttgatgtgcttgaaaggg		This study
16Sr for	ggaggttgatcatggctcag	219	[19]
16Sr rev	acaacgcaggtccatct		[19]

These findings indicate that the gtf primers are very specific, giving gtf PCR product with multiple strains of the target streptococcal species and no product with any other DNA tested.

Sensitivity of the gtf primers

To determine the limits of detection, PCR was carried out with serial dilutions of genomic DNA. The detection limits for *S. salivarius* and *S. sanguinis* were 3.8 pg (corresponding to approximately 1,500 bacterial genomes) and 60 fg (corresponding to approximately 24 bacterial genomes), respectively (Supplementary Fig. S2). The detection limits for *Streptococcus mutans* and *S. gordonii* were 1 ng (corresponding to 3.98×10^5 genomes) and 0.5 ng (corresponding to 1.98×10^5 genomes), respectively (data not shown). QPCR was also carried out with different amounts of *S. sanguinis* genomic DNA to determine the sensitivity of the assay in this system. The QPCR was quantitative between 6 ng and 3 fg of *S. sanguinis* DNA (Supplementary Fig. S3 and Supplementary Table S1). Collectively, these results show that the PCR assay provides a very sensitive test for the detection of streptococcal DNA.

PCR of streptococcal DNA in saliva

PCR was carried out to test whether the gtf primers could detect streptococcal DNA in saliva. The *S. sanguinis* and *S. salivarius* primer sets gave PCR product in 97/100 and 89/100 saliva samples, respectively (Fig. 1), and 99/100 saliva samples gave a PCR product with at least one of these primer sets. The *S. gordonii* and *S. mutans* also gave product though with fewer numbers of samples. Subsequent experiments were conducted with only the *S. sanguinis* and *S. salivarius* primer sets as these gave PCR product with the highest number of saliva samples and were the most sensitive primer sets.

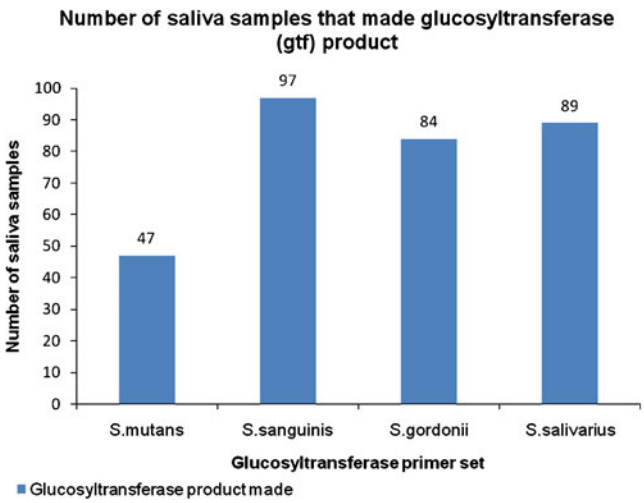

Fig. 1 The number of samples that produced gtf polymerase chain reaction (PCR) product with each primer set. Samples of saliva from 100 different people underwent PCR with each of the four gtf primer sets. The number of samples that gave PCR product with each gtf primer set is shown

QPCR was also carried out with *S. sanguinis* gtf primers using as template DNA extracted from saliva. The crossing points (Supplementary Table S1) corresponded to amounts of between 4.1 and 142 pg of template DNA. This equates to between 1.1×10^7 and 3.9×10^8 *S. sanguinis* genomes per millilitre of saliva.

Effects of blood in PCR amplification

Blood can inhibit PCR reactions [24, 25]. Mixtures of saliva and blood were prepared to investigate the effect of the presence of blood on the detection of streptococcal DNA. The *S. salivarius* gtf primers gave product in saliva: blood ratios ranging from 1:1 to 1:200, and *S. sanguinis* primers made PCR product for all ratios tested (Supplementary Fig. S4). Both *S. salivarius* and *S. sanguinis* produced gtf product for all saliva:water ratios (data not shown), which suggests that blood slightly inhibited PCR with the *S. salivarius* primer set.

PCR of expirated bloodstains

Expirated bloodstain patterns were prepared on cardboard and on cotton fabric. DNA was extracted from bloodspots (1–2 mm) and analysed by PCR. *S. sanguinis* gtf product was obtained from bloodspots for at least 92 days (cardboard) and 64 days (cotton fabric) after deposit and *S. salivarius* gtf product after 34 and 29 days. The age of stains in which DNA could be detected was similar to very recent data [17] using primers to rRNA genes to successfully detect streptococci on different fabrics for up to 62 days. No PCR product was obtained with bloodstain patterns that did not contain added saliva, with either the *S. salivarius* or *S. sanguinis* gtf primer sets.

PCR of different fabrics and washed fabrics

PCR was carried out for six different materials (cotton, wool, denim, carpet, polyester and elastane) that had been stained with expirated blood. All stained samples resulted in PCR product with *S. sanguinis* gtf primers (Fig. 2a). Some unstained samples also gave PCR product. PCR product was obtained with the remaining unstained samples if the PCR was extended from 30 to 35 cycles (data not shown). None of these samples gave PCR product with the *S. salivarius* gtf primers.

QPCR was carried out with the *S. sanguinis* gtf primer set to quantify the differences between stained and unstained samples (Supplementary Table S2). In all cases, fabrics stained with expirated blood gave rise to PCR product in amounts corresponding to at least fivefold more *S. sanguinis* genomes than the unstained fabrics. However, all of the unstained fabrics gave PCR product in the QPCR system.

Further testing was carried out with objects that were not known to be contaminated. Swabs were taken from 14 different surfaces, DNA was extracted from the swabs and PCR carried out with *S. sanguinis* gtf primers. *S. sanguinis* gtf product was detected in ten samples (two laboratory computer screens, one clean drinking cup, the outside surface of a fridge door, thermal curtains and the fronts of five worn shirts) when 35 amplification cycles were used but not for any of the samples when 30 cycles were used (data not shown).

The effect of washing on the detection of saliva and expirated bloodstains was also examined. The PCR method detected the presence of streptococcal DNA in both washed and unwashed stains for both saliva and expirated bloodstains (Fig. 2b, c).

The washed and unwashed samples used for this experiment were also tested for the presence of amylase using the Phadebas amylase test. The Phadebas test detected amylase in all unwashed stains that contained saliva or expirated blood but not in any stains that had been washed.

Blind trial test

Bloodstains for blind trial testing were prepared and analysed to test the accuracy and robustness of the PCR detection method. DNA was extracted from each sample and PCR carried out using *S. sanguinis* and *S. salivarius* gtf primer sets. PCR product was obtained with both primer sets for 3/7 samples, and 4/7 samples did not give PCR product with either primer set (Supplementary Table S3). These results indicated that three samples were likely to be expirated bloodstains and four samples were likely to be unmixed bloodstains. This conclusion matched exactly with the nature of the provided samples.

Discussion

Distinguishing expirated bloodstains from impact spatter is an important challenge in forensic science. The current study developed a PCR method to differentiate mouth-expirated bloodstains from other types of bloodspatter using DNA from human-specific oral microbes as a biomarker for the presence of saliva and hence oral expirated bloodstains. The PCR technique was specific and sensitive enough to identify the presence of oral streptococci in both saliva and expirated blood on a variety

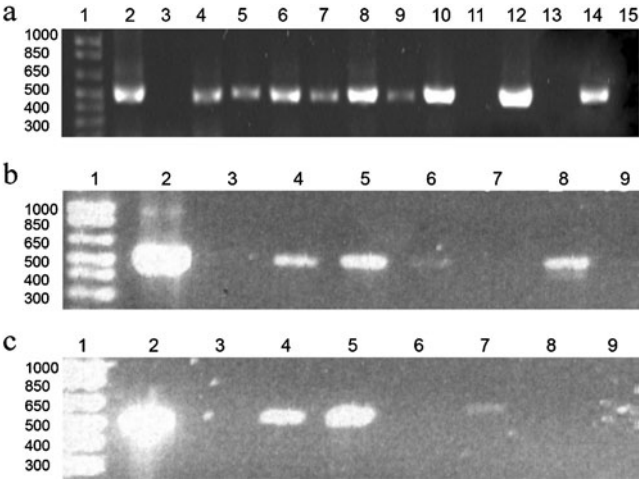

Fig. 2 Polymerase chain reaction (PCR) of saliva and expirated blood. PCR was carried out and the products analysed by agarose gel electrophoresis. **a** PCR with *Streptococcus sanguinis* gtf primers on expirated bloodstains on different fabrics. *Lane 1*: DNA ladder, *lane 2*: *S. sanguinis* 10556 genomic DNA, *lane 3*: no template, *lane 4*: stain on carpet, *lane 5*: unstained carpet, *lane 6*: stain on sweatshirt, *lane 7*: unstained sweatshirt, *lane 8*: stain on singlet, *lane 9*: unstained singlet, *lane 10*: stain on trousers, *lane 11*: unstained trousers, *lane 12*: stain on t-shirt, *lane 13*: unstained t-shirt, *lane 14*: stain on wool jersey, and *lane 15*: unstained wool jersey. **b** PCR with *S. sanguinis* gtf primers of washed and unwashed stains on denim. *Lane 1*: DNA ladder, *lane 2*: undiluted saliva, *lane 3*: no template, *lane 4*: unwashed expirated bloodstain on denim, *lane 5*: unwashed saliva stain on denim, *lane 6*: unwashed unstained denim, *lane 7*: washed expirated bloodstain on denim, *lane 8*: washed saliva stain on denim, and *lane 9*: washed unstained denim. **c** PCR with *S. salivarius* gtf primers of washed and unwashed stains on denim. *Lane 1*: DNA ladder, *lane 2*: undiluted saliva, *lane 3*: no template, *lane 4*: unwashed expirated bloodstain on denim, *lane 5*: unwashed saliva stain on denim, *lane 6*: unwashed unstained denim, *lane 7*: washed expirated bloodstain on denim, *lane 8*: washed saliva stain on denim, and *lane 9*: washed unstained denim

of surfaces for up to 92 days after staining. The specificity of this method was similar to data reported with *gtf* primers or primers to rRNA genes to detect oral streptococci in saliva [15–17]. Streptococcal DNA was detected in 99/100 saliva samples, and in another recent study, gtf primers gave PCR product with 20/20 saliva samples [16], indicating that this approach is not affected by differences in the oral microflora of different individuals. The method described here successfully distinguished mock expirated bloodstains from non-expirated bloodspatter in a blind trial, indicating its potential application in forensic applications.

Our data additionally showed that DNA could be detected on washed fabrics, although this would be difficult to establish and test for, as there was no obvious visible stain pattern. It may be possible to use alternative light sources to detect such stains [26, 27], but that was not investigated in this study. Haem from blood and indigo blue dye from denim can have an inhibitory effect on PCR [24, 28–30]. Little or no inhibition was observed in this study with streptococcal DNA being detected on denim even after

washing. This may be due to the use of Instagene matrix, a commercially prepared solution of Chelex®100, as the DNA extraction method that is able to remove PCR inhibitors [31, 32]. Our data also indicate that this method was efficient in recovering template DNA. The QPCR results (Supplementary Table S1) indicate that DNA equivalent to between 1.1×10^7 and 3.9×10^8 *S. sanguinis* genomes/ml was detected in undiluted saliva. This is similar to the reported salivary amounts of *S. sanguinis* (10^7 to 10^8 cfu/ml) [33], indicating near-quantitative recovery of bacterial template DNA from saliva.

The current study established that low but detectable amounts of streptococcal DNA were present on unstained fabrics and objects such as clothing, curtains, computer screens and drinking cups, even if the fabrics or objects were apparently clean. This finding was not completely unexpected because it has been shown that saliva can travel up to 184 cm from the mouth of a person who is talking, coughing or laughing [34]. Therefore, small numbers of oral streptococci are probably present on many surfaces that are associated with human use, although their short survival time outside the human mouth (2–6 days) [13, 17] means that they may not be viable. However, the amount of streptococcal DNA detected on unstained materials and objects was at least fivefold less than that detected on stained samples (Supplementary Table S2).

In conclusion, this study has demonstrated that bacteria present in the oral cavity can provide a potential biological marker for saliva and mouth-expirated blood. This marker could be used in forensic work as a way to distinguish mouth-expirated blood from other types of spatter stains including impact spatter. It cannot be used as a marker for all expirated blood as expirated blood can also come from the nose, and the current primers are specific to oral bacteria. This method requires further validation studies including testing expirated blood samples that have been exposed to various environmental conditions before being used in casework.

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