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Research article

Restriction fragment length polymorphisms of mutans streptococci in forensic odontological analysis

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Humans bite each other with alarming frequency. Such injuries can result in forensic investigation and, ultimately, criminal prosecution. Bite mark analysis techniques include odontometric measurement, ABO serotyping and DNA profiling, none of which is infallible. Research into additional techniques would be advantageous. Biting involves, also, bacterial transfer from teeth to skin and vice versa. The principal species found on anterior teeth is *Streptococcus mutans*, which is universal among humans. The aim of this research was to establish, in the context of forensic odontology, whether chromosomal DNA profiles of this ubiquitous oral bacterium would vary significantly among a sample of Caucasian individuals ($P = 0.05$). Hence, if sufficient discriminatory power were present, the technique could be deemed useful to forensic investigation. Oral fluid was recovered from the lower incisors of 10 adults. Samples were cultured selectively using mitis salivarius bacitracin agar and microbiological tests carried out for the purpose of differential identification. These included visual assessment of colony morphology, Gram staining and microscopic analysis, followed by chemical testing for the enzyme catalase. Chromosomal DNA was extracted from subcultured cells, resolved by agarose gel electrophoresis and viewed using ultraviolet transillumination. The presence of DNA was confirmed. Subsequently, the 16S ribosomal RNA gene was amplified by polymerase chain reaction using specific 27 forward and 1492 reverse primers. Amplicons were resolved and viewed as previously. Amplified products were digested by incubation with restriction endonuclease *Hae*III, resolved by polyacrylamide gel electrophoresis and viewed. Linear regression analysis of gel profiles was used to calculate restriction fragment lengths. A Kruskal–Wallis (analysis of variance) test was performed on ranked data ($H = 8.161$, $df = 9$, $P = 0.518$). Consequently, the null hypothesis (no inter-subject variation) was accepted. However, all but two profiles were proved to be unique. Proof of principle was provided regarding the application of oral bacterial genotyping to forensic bite mark cases.

Key words: *Streptococcus mutans*, restriction fragment length polymorphism, polymerase chain reaction, mitis salivarius bacitracin agar, restriction endonuclease *Hae*III, 16S ribosomal RNA gene.

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Introduction

Background and theory

Darwin described biting as a form of assault exhibited by all the Carnivora.[1] In primates, this occurs in self-defence[2] or as an act of either physical or sexual aggression.[3] Its prevalence in human conflicts, ranging from playground fights to rape and child abuse, bears witness to its evolutionary origins.[4] One per cent of all visits to American medical emergency departments are bite-related.[5] Many more cases doubtless go unrecorded. In the UK, bites from humans are even more common than those from either dogs or cats. Police officers, health workers and other institutional staff, who constitute a significant proportion of the population, are at particular risk. Males are bitten most frequently on the upper limbs, whereas females are more likely to suffer bites

on the legs, breasts and genitalia.[6] Evidence shows that perpetrators are significantly more likely to be male than female,[7] and the superior force exerted by male jaws increases the potential severity of the trauma.[8, 9]

Bite injuries can precipitate many clinical complications, including damage to nerves, vessels and tendons.[10] Transmission of infections is more serious: saliva contains up to 100 million microorganisms per millilitre, and it has been estimated that 10–18% of bites become infected.[10] Microbial pathogens include hepatitis B and C, Herpes simplex, tuberculosis, tetanus and syphilis.[11] Clearly, therefore, high incidence of such injuries warrants effective forensic analysis.

Odontological evidence is routinely presented in courts of law. Several aspects of bite mark analysis are currently used, but legal contention is well documented and has valid foundation.[12] Odontometry, or tooth measurement, is predicated on two assumptions: first, that each individual's dentition is unique and, secondly, that this uniqueness is identifiable from a bite mark.[13] Supporting evidence is inconclusive,[14] and laboratory protocols are unreliable.[15] Unusual dental characteristics include congenital malformations, incisal-edge fractures and longitudinal rotations. Although helpful in differential identification, the majority of individuals do not have them, which restricts their usefulness to the forensic odontologist.[16]

The matching of a bite mark to an individual requires experienced, and sometimes subjective, judgement.[17] Herein lies the controversy: the technique is flawed if it is not based on the scientific method.[18] There are, as yet, no established critical methodologies, databases, nor probabilistic models, and error rates remain undetermined.[19]

Skin is, by virtue of its curves, irregularities and elasticity, a poor impression material; oedema, lividity and blood-clotting can induce post-traumatic distortion.[4] Moreover, marks deteriorate on healing,[20] which introduces a time constraint. These drawbacks are compounded by innate limits to interpersonal dental anatomical variation. Even sex differentiation can prove difficult. Iscan and Kedici[21] reported specificity of <77%. More fundamentally, false-positive diagnoses have been published. An impression made by a bottle top was once erroneously identified as a bite mark.[22]

Since the first bite mark conviction, in 1975,[23] referrals to Courts of Appeal, and subsequent acquittals, have occurred.[24] Exonerations have been based on hair and fingerprint evidence,[25] DNA profiling[26] and irreconcilable disagreement among forensic odontologists.[27] Most infamously, a death sentence was overturned.[28] Drawbacks of such enormity imply that unless techniques are either improved or superseded, then forensic odontology could become devalued.

Approximately 80% of humans are secretors.[29] ABO blood groups of 90% of these can be determined by serotyping cell-surface antigens present in saliva.[30] Hence, more than one-quarter of potential aggressors are unidentifiable. This, combined with relatively low discriminatory power, renders blood serotyping susceptible to eventual obsolescence. Presently, its legal application is limited to a small number of exoneration cases.[31]

The ultra-high discriminatory power of 'genetic fingerprinting' revolutionized forensic identification.[32] DNA profiles can be generated from epithelial cells deposited in bite marks. For example, Kobilinsky *et al.*[33] documented a criminal case in which a serial killer was convicted following analysis of salivary DNA recovered from a coffee cup. However, molecular degradation constitutes a major impediment to DNA extraction from bite mark residue.[34] This occurs when the phosphodiester bonds are hydrolysed by nucleases,[35] secreted by the parotid gland.[36] Thus, salivary DNA concentration decreases with time since deposition.[37]

Locard's Exchange Principle—'every contact leaves a trace'[38]—invites the question: what other biological entities, apart from blood-type antigens and DNA, could be transferred during biting? Perhaps the most obvious are microbes. Some species of oral bacteria adhere plentifully to enamel by means of glucans, whose synthesis is catalysed by glucosyltransferase (GTF).[39] For effective forensic application, bacterial isolates would require effective typeability. Phenotypic methods include biotyping and serotyping,[40] although discriminatory power is poor in comparison with genotypic alternatives, and antisera availability is limited to very few species.[41] Bacteriocin typing can provide an alternative but is expensive and technically demanding.[42]

Bacterial DNA profiling was considered for experimental study. The most prevalent genus found on anterior teeth is *Streptococcus*, [43] followed by *Actinomyces* and *Veillonella*.[44] Thermophysiological research has shown that oral streptococci thrive only in the oral cavity.[45] This implies no host contribution to the streptococcal flora of a bite mark on human skin. Recovered cells would be likely, therefore, to have originated solely from the perpetrator.

The 'mutans streptococci' (MS) (Fig. 1) are especially significant. *Streptococcus mutans* and *Streptococcus sobrinus* are commonly found; the others are extremely rare.[46] *Streptococcus mutans* is carried by all humans; *S. sobrinus* is found in only 8–35%, depending on geographical origin.[47] Thus, the ubiquity of *S. mutans*, combined with its low tendency to colonize extraorally, renders this species potentially useful in forensic identification.

As with salivary DNA degradation on human skin, the *S. mutans* cell count deteriorates with time, the rate of reduction of viable cells being ~50% per hour.[48] Nevertheless, Borgula *et al.*[49] were able to recover more than 1000 viable cells after 24 h. Loss of bacteria would appear less problematic than comparable host DNA degradation, as bacterial DNA can easily be replicated by further culture.

Characteristics of *S. mutans* are: coccobacilli; diameter 0.5–2.0 μm; paired or chained; non-motile and non-sporing;

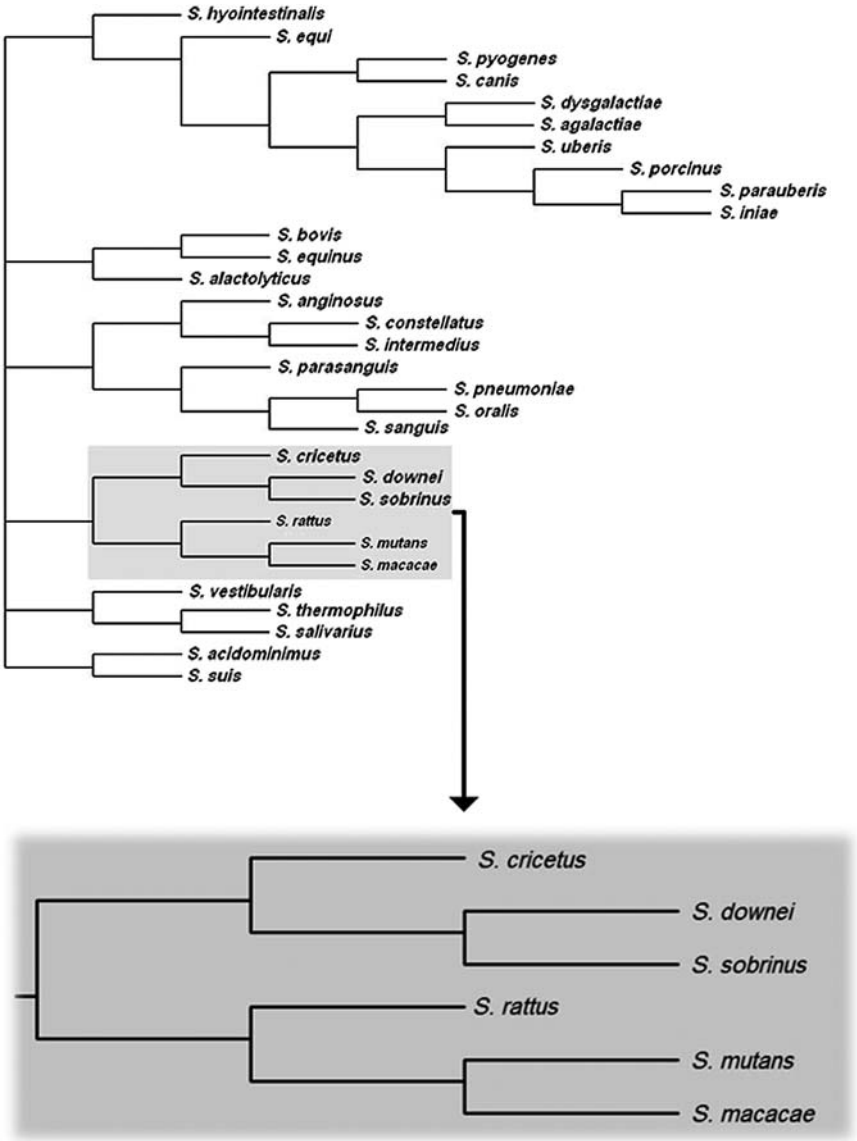

Figure 1. Dendrograms illustrating phylogenetic relationships of *Streptococcus* spp. (mutans group shaded).[81] Evolutionary interspecies distances are not represented quantitatively. Dendrograms constructed using GenoPro2007® Online Software.

Gram-positive; facultatively anaerobic; fermentative metabolism producing lactate without gas; catalase negative; and mesophilic.[50] *Streptococcus mutans* colonizes the oral cavity when the first (deciduous) teeth erupt.[51] Transmission occurs principally via maternal saliva,[52] and the first colonizing strain is purported to persist throughout life.[53] Therefore, if the *S. mutans* profile is not liable to change with time, as is true with the host DNA fingerprint, then its forensic application would, in principle, be further endorsed. Lindquist and Emilson[54] discovered that *S. mutans* colonization was equally high on all teeth, whereas that of *S. sobrinus* was relatively low on incisors.[55] Bite marks, generally, involve only incisors and canines,[56] where the frequency ratio of *S. mutans* to *S. sobrinus* happens to be greatest. Hence, differential transfer during biting would be maximized in favour of *S. mutans*. These data are highly convenient to forensic bacterial genotyping.

Justification of methodology

Experimental objectives were: extraction of *S. mutans* from human incisors; selective microbiological culture and subsequent identification; formulation of an effective protocol for DNA extraction from Gram-positive cells; and generation of individual genetic profiles using a specific polymorphic marker. Methods of tissue sampling, bacterial culture and DNA profiling were selected in the light of previous research.

Sampling techniques, compared by Wennerholm *et al*.,[57] showed that recovery of greatest bacterial yield was provided by wooden toothpicks, as opposed to metal instruments or dental floss. Alternative techniques, using plastic strips, precluded bacterial growth in a significant proportion of samples.[58]

Several agar media have been used to culture *S. mutans* selectively. These include mitis salivarius bacitracin (MSB),[59] tryptone yeast-extract cystine[60] and trypticase yeast-extract cystine sucrose bacitracin.[61] The yeast-extract media produced greater recovery than MSB[60] but yielded numerous non-mutans bacteria also.[61] Given the greater selectivity of MSB agar, in which bacitracin is utilized for its long-proven antibiotic capability against extraneous Gram-positive species,[62] it is used most frequently for *S. mutans* isolation.[63, 64]

Species identification methods include the use of monoclonal antibodies raised against MS strains, enzymes or cell markers.[41] High specificity has been observed using two different types of immunoassay.[65] Biochemical tests are a common alternative,[66] and frequently involve carbohydrate fermentation.[67, 68] Colony morphology provides dependable provisional differentiation.[69–71] Gram staining and light microscopy enhances diagnostic power, which can be increased further by catalase-testing.[50]

Several markers have been genotyped successfully. Hamada and Slade[69] analysed extrachromosomal DNA but noted that plasmid occurrence in human *S. mutans* was only 5%. Analysed chromosomal genes include those coding for dextranase,[72] GTF,[73, 74] 16S ribosomal RNA (rRNA)[75] and the 16S–23S rRNA intergenic spacer.[76] Matsuyama *et al*.[77] compared the dextranase, GTF and 16S rRNA genes on the basis of detection frequency using their respective primers and polymerase chain reaction (PCR) amplification. It was concluded that the 16S rRNA gene offered greatest sensitivity for MS detection. Consequently, it is widely used to identify MS and many other bacterial pathogens.[78]

The *S. mutans* (UA159) genome has been sequenced fully.[79] There are 2 030 936 bp,[80] and the 16S rRNA gene is 1552 bp long.[79] Bentley *et al*.[81] analysed a 1334-nucleotide region and calculated that it exhibited 94.5% sequence homology with *S. sobrinus*. In fact, up until the mid-1980s, the two human MS species (*S. mutans* and *S. sobrinus*) were indistinguishable.[82]

Restriction fragment length polymorphism (RFLP) analysis, the first forensic DNA technique,[83] has been applied frequently following DNA extraction from Gram-positive bacteria.[84] 16S rRNA genes of other oral genera, *Veillonella*[85] and *Actinomyces*, [86] have been similarly typed. The *Hae*III enzyme, isolated from *Haemophilus aegyptius*, [87] recognizes the palindromic nucleotide sequence GGCC and cleaves both DNA strands at the same location, producing blunt-ended fragments.[88] It has been used to digest the 16S rRNA gene of *S. mutans*, [89] yielding RFLP patterns in accordance with Genbank.[75] The gene contains 13 potential *Hae*III restriction sites (Table 1), and the number of possible fragment lengths comprising an RFLP

Table 1. *Streptococcus mutans* UA159 16S rRNA complete nucleotide sequence (1552 bp) (NCBI, 2008, Sequence Viewer 2.0.β, Accession NC_004350.1, nucleotides 185 749–187 300 inclusive)

Nucleotides	Base sequence
1–40	AGAGTTTGATCCTGGCTCAGGACGAACGCTGGCGGCGTGC
41–80	CTAATACATGCAAGTGGGACGCAAGAGGACACACTGTGCT
81–120	TGCACACCGTGTTTTCTTGAGTCGCGAACGGGTGAGTAAC
121–160	GCGTAGGTAACCTGCCTATTAGCGGGGGATAACTATTGGA
161–200	AACGATAGCTAATACCGCATAATATTAATTATTGCATGAT
201–240	AATTGATTGAAAGATGCAAGCGCATCACTAGTAGATGGAC
241–280	CTGCGTTGTATTAGCTAGTTGGTAAGGTAAGAGCTTACCA
281–320	AGGCGACGATACATAGCCGACCTGAGAGGGTGATC**GGCC**A
321–360	CACTGGGACTGAGACAC**GGCC**CAGACTCCTACGGGAGGCA
361–400	GCAGTAGGGAATCTTCGGCAATGGACGAAAGTCTGACCGA
401–440	GCAACGCCGCGTGAGTGAAGAAGGTTTTCGGATCGTAAAG
441–480	CTCTGTTGTAAGTCAAGAACGTGTGTGAGAGTGGAAAGTT
481–520	CACACAGTGACGGTAGCTTACCAGAAAGGGACGGCTAACT
521–560	ACGTGCCAGCAGCCGCGGTAATACGTAGGTCCCGAGCGTT
561–600	GT**CCGG**ATTTATTGGGCGTAAAGGGAGCGCAGGCGGTCAG
601–640	GAAAGTCTGGAGTAAAAGGCTATGGCTCAACCATAGTGTG
641–680	CTCTGGAAACTGTCTGACTTGAGTGCAGAAGGGGAGAGTG
681–720	GAATTCCATGTGTAGCGGTGAAATGCGTAGATATATGGAG
721–760	GAACACCAGTGGCGAAAGCGGCTCTCTGGTCTGTCACTGA
761–800	CGCTGAGGCTCGAAAGCGTGGGTAGCGAACAGGATTAGAT
801–840	ACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAGGTGT
841–880	TA**GGCC**CTTT**CCGG**GGCTTAGTG**CCGG**AGCTAACGCAATA
881–920	AGCACTCCGCCTGGGGAGTACGACCGCAAGGTTGAAACTC
921–960	AAAGGAATTGACGGG**GGCC**CGCACAAGCGGTGGAGCATGT
961–1000	GGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTT
1001–1040	GACATCCCGATGCTATTCTTAGAGATAGGAAGTTACTTCG
1041–1080	GTACATCGGAGACAGGTGGTGCATGGTTGTCGTCAGCTCG
1081–1120	TGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAAC
1121–1160	CCTTATTGTTAGTTGCCATCATTAAGTTGGGCACTCTAGC
1161–1200	GAGACTG**CCGG**TAATAAA**CCGG**AGGAAGGTGGGGATGACG
1201–1240	TCAAATCATCATGCCCCTTATGACCTGGGCTACACACGTG
1241–1280	CTACAATGGTCGGTACAACGAGTTGCGAG**CCGG**TGACGGC
1281–1320	AAGCTAATCTCTGAAAGCCGATCTCAGTTCGGATTGGAGG
1321–1360	CTGCAACTCGCCTCCATGAAGTCGGAATCGCTAGTAATCG
1361–1400	CGGATCAGCACGCCGCGGTGAATACGTTC**CCGGGGCC**TTGT
1401–1440	ACACACCGCCCGTCACACCACGAGAGTTTGTAACACCCGA
1441–1480	AGTCGGTGAGGTAACCTTTTAGG**GGCC**AGCCGCCTAAGGT
1481–1520	GGGATGGATGATTGGGGTGAAGTCGTAACAAGGTAGCCGT
1521–1552	ATCGGAAGGTGCGGCTGGATCACCTCCTTTCT

*Hae*III restriction sites are given in bold type and underlined.

profile is given by the expression: $1/2\ (x^2 + 3x + 2)$, where x represents the number of restriction sites. (Note: This algebraic expression is original and, therefore, requires no citation.)

Each subject would, therefore, yield between 1 and 14 restriction fragments from a combination of 105 possible lengths, assuming that all sizes were different. This could provide adequate informativeness. In the 1980s, RFLP analysis was often impractical, partly because of the relatively large amount of DNA required. This has been alleviated by the advent of PCR.[90]

Selected specific primers were 27f (forward) and 1492r (reverse) (Fig. 2).[77, 91] Given that some DNA fragments were predicted to be shorter than 500 bp (Table 2), electrophoresis using polyacrylamide gel was preferred, for quantitative analysis, to agarose gel by virtue of its higher resolution capability.[92] However, for qualitative purposes—that is, to confirm the presence (or absence) of DNA—agarose was deemed adequate.

The hypothesis to be tested was that individual DNA profiles of *S. mutans* would exhibit inter-subject variation (at a confidence level of 5%).

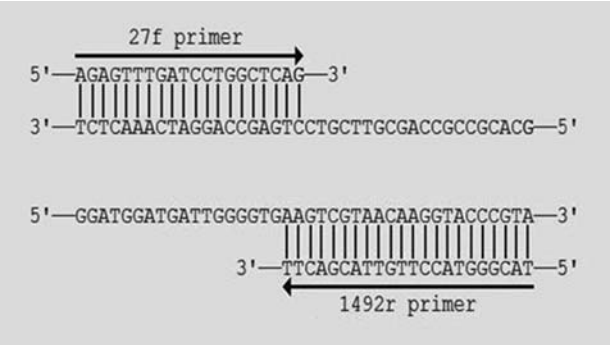

Figure 2. Schematic representation of PCR amplification of *S. mutans* 16S rRNA gene, using 27 (forward) and 1492 (reverse) primers.

Materials and methods

Microbiological culture and identification

About 660 mg bacitracin powder (Fluka®, Chemie AG, Buchs, Switzerland) was dissolved in 250 ml dH_2O. A 20 ml aliquot was extracted by a sterile syringe and injected through a sterile, surfactant-free cellulose acetate syringe filter, diameter 26 mm, pore size 0.2 μm (Corning®, Corning, NY, USA), and collected in a sterile 30 ml bottle. (Fresh solutions were prepared for each subsequent culture.)

Twenty-seven grams of mitis salivarius agar powder (Fluka®) were dissolved in 300 ml dH_2O, autoclaved for 15 min at 121°C before being cooled in a water bath to ~55°C. Three hundred microlitres of sterile 1% potassium tellurite solution (Fluka®) and 375 μl bacitracin solution were added, in turn, and agitated gently for 10 s. Approximately 20 ml was dispensed into each of 12 sterile Petri plates, covered, allowed to cool to 20°C and refrigerated until required.

Oral fluid was sampled from the incisal/labial enamel surfaces of the each subject's anterior teeth, using a sterile wooden toothpick, and streaked directly across its corresponding plate.[58] A positive control plate was prepared

Table 2. Potential *Hae*III-digested restriction fragment lengths (bp)

		Primer	Restriction site no. (3′ end)													Primer
		27f	1	2	3	4	5	6	7	8	9	10	11	12	13	1492r
Primer	27f	–	297	319	544	824	832	845	917	1149	1160	1251	1371	1374	1445	1465
Restriction site no. (5′ end)	1		–	22	247	527	535	548	620	852	863	954	1074	1077	1148	1168
	2			–	225	505	513	526	598	830	841	932	1052	1055	1126	1146
	3				–	280	288	301	373	605	616	707	827	830	901	921
	4					–	8	21	93	325	336	427	547	550	621	641
	5						–	13	85	317	328	419	539	542	613	633
	6							–	72	304	315	406	526	529	600	620
	7								–	232	243	334	454	457	528	548
	8									–	11	102	222	225	296	316
	9										–	91	211	214	285	305
	10											–	120	123	194	214
	11												–	3	74	94
	12													–	71	91
	13														–	20
Primer	1492r															–

with precultured *S. mutans*, and a negative with *Escherichia coli*.[93] The plates were placed in an anaerobic system (BBL™ GasPak™, Becton, Dickinson & Company, Oxford, UK) and incubated at 37°C for 72 h.[94]

All plates were assessed for bacterial growth, characteristics being: number, colour, size, form, elevation, margin, consistency and odour.[95] A small drop of sterile dH_2O was placed onto each of a series of clean microscope slides. A single, well-isolated colony was removed from each plate using a sterile metal loop and emulsified on its corresponding slide. Gram staining was carried out on each slide in turn. Oil immersion microscopy was used to assess cell morphology and aggregation.

A single colony was recovered from each plate and streaked across a corresponding nutrient agar (NA) (Lab M™ Ltd, Bury, UK) plate.[96] These were incubated anaerobically, as previously,[94] and exposed to air for 30 min.[97] One drop of 30% (w/v) hydrogen peroxide (Fisher Scientific, Loughborough, UK) was placed onto a clean microscope slide. A single, well-isolated colony was recovered, using a sterile wooden toothpick, from each NA plate, emulsified in fresh solution and any gaseous production observed.[98] *Staphylococcus aureus* was employed as a positive control,[97] *Lactococcus lactis* as a negative control.[50]

From each MSB plate, a single, well-isolated colony was streaked across a corresponding fresh MSB plate and incubated anaerobically.[94] Following incubation, the procedure was repeated using the resultant (first) subcultures, and subsequent (second) subcultures were grown.

Liquid culture of *Streptococcus mutans*

Yeast glucose buffered broth was prepared by dissolving the following ingredients in 500 ml dH_2O: 6.5 g Nutrient Broth No. 2 (Lab M™, Bury, UK); 0.5 g D-glucose (anhydrous) (Fisons Scientific Equipment, Loughborough, UK); 1.84 g potassium dihydrogen orthophosphate (VWR International Ltd, Poole, UK); and 0.66 g di-potassium hydrogen orthophosphate (anhydrous) (Fisher Chemicals, Loughborough, UK).[99] pH was adjusted to 7.2 by adding 1.0 M aqueous sodium hydroxide, as required. The buffered solution was autoclaved at 121°C for 15 min. A single colony from each second MSB subculture was suspended in a corresponding sterile glass universal bottle containing 4 ml broth and incubated at 37°C for 72 h.[94]

DNA extraction and isolation

DNA was extracted from each broth sample using the Wizard® Genomic Purification Kit A1120 (Promega Corporation, Madison, WI, USA). In accordance with recommended proportions, 100 mg lysozyme preparation (Sigma®, St Louis, MO, USA) was dissolved in 10 ml dH_2O. One millilitre broth culture was transferred to a 1.5 ml microcentrifuge tube and centrifuged for 2 min at 15 000 rpm. The supernatant was removed and the cells were suspended in 480 µl 50 mM ethylenediaminetetraacetic acid (EDTA) (pH 8.0). One hundred and twenty microlitres of lysozyme solution were added and the mixture was incubated at 37°C for 45 min. Centrifugation was repeated, for 2 min at 15 000 rpm, and the supernatant discarded. Six hundred microlitres of Nuclei Lysis Solution (Promega) were added and agitated gently. This was incubated at 80°C for 5 min and cooled to room temperature (20°C). Three microlitres of RNase solution (Promega) were added, mixed, incubated at 37°C for 60 min, and then cooled to room temperature. Two hundred microlitres of Protein Precipitation Solution (Promega) were added, vortexed for 10 s and incubated on ice for 5 min. The mixture was centrifuged for 3 min at 15 000 rpm. The supernatant was transferred to a clean 1.5 ml tube containing 600 µl (room temperature) isopropanol and mixed gently by repeated inversion. This was centrifuged for 2 min at 15 000 rpm and the supernatant decanted. Six hundred microlitres of (room temperature) 70% ethanol were added, mixed and centrifuged for 2 min at 15 000 rpm. The ethanol was aspirated, and the pellet air-dried for 15 min and rehydrated in 100 µl Rehydration Solution (Promega) for 1 h at 65°C. The isolated DNA was refrigerated at 2°C until required.

PCR amplification and restriction fragment resolution

Two grams of agarose (Fisher Scientific) were added to 100 ml dH_2O and heated until dissolution was complete. After cooling to ~60°C, 2 ml 50× Tris-Acetate-EDTA (TAE) buffer and 10 µl GelRed™ fluorescent nucleic acid gel stain (Biotium Inc., Cambridge, UK) were added. The mixture was stirred to homogenize and poured into a casting tray. Fifteen microlitres of each (unamplified, whole genomic) DNA sample were transferred to a corresponding, fresh 0.5 ml tube. Ten microlitres of a 1 kb marker were added to an additional tube. Three microlitres of 6× blue/orange Loading Dye (Promega) were added to each sample and the marker. After solidification, 500 ml 1× TAE running buffer was poured into the tray until the gel was submerged. Twelve microlitres of each sample were introduced into its corresponding well. Electrophoresis was carried out at 100 V for 80 min. The gel was removed from the tray, placed in a Gel Doc 2000 ultraviolet (UV) transilluminator (BioRad, Hemel Hempstead, UK) and DNA products were visualized.

Primer sequences were: 27F, 5′-AGAGTTTGATCCTGG CTCAG-3′; and 1492R, 5′-TACGGGTACCTTGTTACGA CTT-3′ (Eurofins MWG, London, UK).[77, 91] About 1608 µl sterile dH_2O was added to the 27F primer, and 1185 µl to the 1492R. About 50 µl of each was added to a 0.5 ml tube containing a further 100 µl sterile dH_2O, making a total volume of 200 µl. A 10 µl aliquot of primer mix was added to a fresh 0.5 ml tube containing 125 µl 2× Premix E reaction buffer (FailSafe™, Epicentre Biotechnologies, Madison, WI, USA). To this was added

5 µl (25 units) GoTaq™ DNA polymerase (Promega) and 60 µl sterile dH_2O, making a total volume of 200 µl. A 5 µl aliquot of each of the 10 DNA samples was transferred to a corresponding 0.5 ml PCR tube containing 20 µl master mix. The 10 samples were amplified using a TECHNE TC-312 thermal cycler, programmed as follows: 95°C for 15 min (initial heat activation); followed by 35 cycles of 94°C for 1 min (denaturation), 52°C for 1 min (annealing) and 72°C for 1.5 min (extension); and 72°C for 10 min (final extension).[91]

About 10 µl of each PCR product was transferred to a corresponding series of 10 fresh 0.5 ml tubes. Ten microlitres of DNA Step Ladder (1 kb) (Promega) were added to two further tubes. Two microlitres of loading dye were then added to all 12 tubes and samples were loaded into agarose wells. Amplicons were resolved by electrophoresis and profiles visualized.

A digestion master mix was prepared as follows: 195.6 µl sterile dH_2O was transferred to a fresh 0.5 ml tube; 24 µl 10× Multi-core™ buffer was added, followed by 2.4 µl acetylated bovine serum albumin (10 mg/ml), making a total volume of 222 µl, in accordance with a protocol issued by Promega.[100] About 18.5 µl master mix was transferred to a series of 12 fresh 0.5 ml tubes. One microlitre of PCR product was added, in turn, to 10 of these, leaving two to serve as controls (i.e. containing no DNA). About 0.5 µl (5 units) *Hae*III was added to all preparations apart from one of the controls (i.e. containing neither DNA nor restriction enzyme), making a total volume of 20 µl per reaction. Samples were incubated at 37°C for 2.5 h, before being frozen at −20°C until required.

A 10% Tris-Borate-EDTA (TBE) 15-well polyacrylamide gel (Invitrogen™, Paisley, UK) was loaded into a vertical electrophoresis kit. Approximately 1 litre 1× TBE buffer was added, ensuring complete coverage of wells. A 50 ml syringe was filled with buffer and any air bubbles above the gel were flushed out. The gel was pre-run at 300 V for 10 min, ensuring a surface temperature of 50°C. For each sample and two controls, 3 µl loading dye was added to a fresh 0.5 ml tube containing 10 µl restriction digest. The same amount of dye was added to 10 µl marker. The mixtures were spun in a microcentrifuge for 10 s. All wells were reflushed with buffer. A 10 ml aliquot of each sample was loaded into a corresponding well. The gel was run at 300 V for 60 min, removed from the cast and immersed in GelRed™ staining solution (Biotium Inc., Cambridge, UK). This was placed in a Stuart Orbital Incubator SI50 (Barloworld Scientific, Stone, UK) at 50 oscillations per minute for 30 min. The gel was transferred to a UV transilluminator and DNA products were visualized.

Statistical analysis

A Kruskal–Wallis (non-parametric) test for analysis of variance by ranks (Statistical Package for the Social Sciences, SPSS)[101] was conducted to compare PCR-RFLP profiles of the 10 test samples.

Results

Microbiological culture and identification

All colonies had a purple, metallic, frosted appearance. Colony diameter ranged from 0.5 to 1.5 mm. Elevation was variable, and margins were not well-defined, owing, at least partly, to coalescence. All plates were odour-free. The positive control plate, streaked with *S. mutans*, showed similar growth. The negative control plate, streaked with *E. coli*, was not growth-free but showed fewer than 10 colonies in total. A distinctive odour, characteristic of *E. coli*, was evident. Colonies recovered from test and positive control plates tested Gram-positive. Microscopy revealed ovoid coccobacilli ~0.5–1.0 µm in diameter. Cells were either isolated or aggregated in tightly packed chains 2–10 units in length. Colonies taken from the negative control plate tested Gram-negative; cells were exclusively bacilli and non-chained. Colonies recovered from NA test plates produced no gas on immersion in hydrogen peroxide solution. However, a single, isolated and markedly larger, colony taken from each of plates 4 and 9 effervesced vigorously. Gaseous production was apparent from colonies recovered from the positive control plate but not from the negative. Broth cultures of all test samples exhibited high turbidity and noticeable precipitation.

Electrophoresis and gel analysis

The presence of (unamplified) DNA was visualized in every lane except '2' (Fig. 3). All bands were of comparable length. The presence of DNA was visualized in every lane containing amplified 16S rRNA genes (Fig. 4). All amplicons appeared to be of the same length, although the signal in lane 10 was relatively weak. Amplicons measured between 1000 and 2000 bp, in accordance with the expected 1552 bp value.[79]

Multiple DNA restriction fragment bands were visualized in all 10 lanes containing digested samples. The maximum

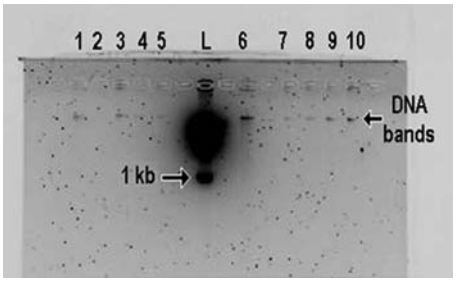

Figure 3. Electrophoregram of *S. mutans* whole genome, confirming presence of DNA.

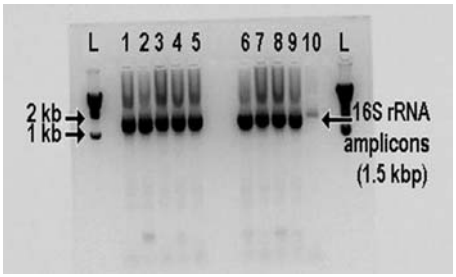

Figure 4. Electrophoregram of 16S rRNA gene. DNA amplicons 1–9 (inclusive) exhibited strong signals.

number was five (lanes 1, 3 and 9), minimum three (lanes 4 and 7). Profiles were dissimilar, with the exception of identical samples 5 and 6. No bands were apparent in the two control lanes (11 and 12) (Fig. 5).

Evidence of sample overload is apparent in each of the three gel images (Figs 4–6). This occurred, principally, as a result of a lack of prior knowledge with regard to the quantity of DNA extracted from the broth cultures.

Using Fig. 5, electrophoretic migration distances were measured (Table 3). Calibration data (Table 4) were used to generate a (log-lin) standard curve and its linear equation (SPSS) (Fig. 6). By interpolation, fragment lengths were calculated to range from 100 to 400 bp (Table 5).

A Kruskal–Wallis test revealed no significant difference between PCR-RFLP profiles of *S. mutans* samples at a confidence level of 5%, i.e. $H = 8.161$, $df = 9$, $P = 0.518$.

A graphical profile comparison is illustrated in Fig. 7.

Discussion

The extent of *S. mutans* genotypic variation was comparable to related research.[49, 94] Despite the Kruskal–Wallis *P*-value of 0.518, all but two of the 10 profiles, namely samples 5 and 6, were unique. For the purpose of positive forensic identification, individuality is key.[102] That is, profile variation is more crucial than the actual degree of variation. Numerical difference in DNA fragment length, providing that it exists and is discernible, has no effect on discriminatory power.

Microbiological methods were effective. MSB agar facilitated selective culture and standard concentrations of bacitracin and potassium tellurite[63] promoted sufficient MS growth. However, the presence of *E. coli* on the negative control plate demonstrated that false-positive results can occur. These have been documented previously.[103]

Confirmation that DNA had been extracted from the same species in each sample was provided by the whole-genome electrophoregram (Fig. 3). All bands migrated similar distances, indicating similar-sized genomes. For example, the *S. mutans* genome is 15% smaller than that of *Streptococcus sanguinis*[104] and less than half that of *E. coli*.[105] Such differences would have been detectable in the banding pattern. The band of the sample 10 amplicon (Fig. 4) was less intense than the others owing to random error. PCR-RFLP techniques were successful in generating legible profiles (Fig. 5), in accordance with Sato *et al.*[75]

Subject selection was, genetically, non-random. All subjects were Caucasian British, between 20 and 45 years old. It is not known whether *S. mutans* profiles vary significantly between groups of different racial or geographic origin, although *S. mutans* chromosomal genotypes have been shown to exhibit less variation among (unrelated) Caucasians than among Asians.[106]

Different restriction enzymes yield different numbers of fragment lengths per DNA sample. In this case, a mean average of only 4.1 restriction fragments per sample meant that discriminatory power was limited. This, and hence statistical power, could be increased by using additional enzymes in parallel with *Hae*III.[107]

Järvinen *et al.*[108] found that both *S. mutans* and *S. sobrinus* were susceptible to ampicillin, penicillin, cefuroxime and tetracycline. Consequently, recovery of *S. mutans* for genotyping could be diminished if a suspect had recently undergone antibiotic therapy. Furthermore,

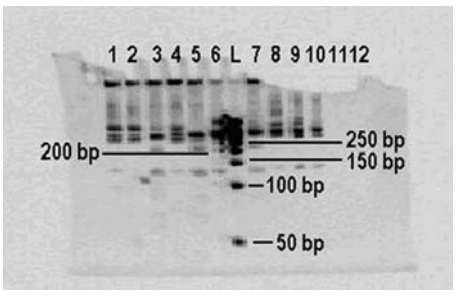

Figure 5. Electrophoregram of *Hae*III-restricted digests of 16S rRNA amplicons.

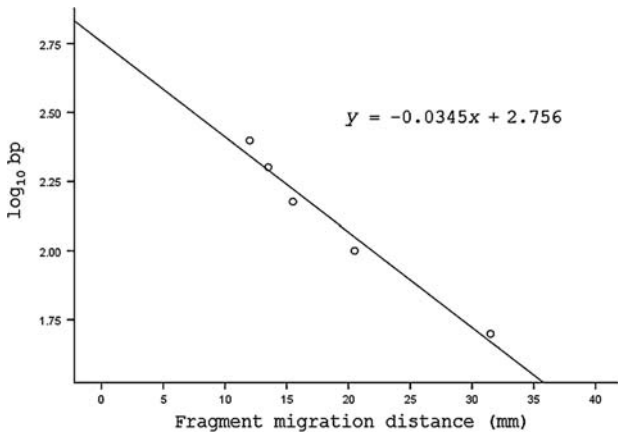

Figure 6. *Hae*III-restricted 16S rRNA gene standard curve (SPSS).

Table 3. Migration distances of *Hae*III-restricted *S. mutans* 16S rRNA gene

Sample									
1	2	3	4	5	6	7	8	9	10
Migration distances (mm)									
5.0	5.0								
							6.0		
								7.5	
			8.0						
8.5	8.5	8.5					8.5	8.5	8.5
						9.5	9.5	9.5	9.5
				10.0	10.0				
10.5	10.5	10.5	10.5					10.5	10.5
		11.0							
11.5	11.5		11.5	11.5	11.5				
		13.0		13.0	13.0	13.0			
							16.5	16.5	16.5
17.5		17.5		17.5	17.5	17.5			

Profiles 5 and 6 were seen to be identical.

sensitivity varies according to serotype.[93] Similar complications might result following the use of chlorhexidine, a commonly used agent in antiseptic mouthwashes.[109] Emilson *et al*.[70] isolated *S. mutans* from only 28% of incisors following intensive chlorhexidine therapy. The most widely used oral antibacterial, however, is fluoride.[110] Present almost universally in dentifrices, it denatures enolase and thus disrupts glycolysis.[110] Taking the form of sodium fluoride in commercial brands such as Crest®, [111] a synergistic effect has been observed when used with chlorhexidine.[112]

Forensic bacterial genotyping need not be confined to odontological cases, providing that fundamental conditions are met. These include: a bacterial species highly specific to the relevant area of the body; a means of reliable culture and DNA isolation; a genetic marker sufficiently polymorphic; and preservation of transferred cells for an adequate period. These criteria are satisfied by sex crimes where either assailant or victim has gonorrhoea, the causative agent, *Neisseria gonorrhoeae* (gonococcus), being a genital tract pathogen of both sexes.[113] In contrast to *S. mutans*, where interpersonal transmission takes place from a surface on which the species thrives (teeth) to one where lysis readily occurs (skin), the gonococcus has the advantage of exchanging one optimum environment for another. Thus, its survival, post-transmission, is more assured. The UK's first forensic analysis of this type was carried out in 2007.[114] Gonococcal isolates were recovered from a child and an adult male. Respective genotypes were

Table 4. Calibration data for *Hae*III-restricted 16S rRNA gene of *S. mutans*

Marker fragment size (bp)	Migration distance (mm)
50	31.5
100	20.5
150	15.5
200	13.5
250	12.0

Table 5. Calculated band lengths of *Hae*III-restricted *S. mutans* 16S rRNA gene

Sample									
1	2	3	4	5	6	7	8	9	10
Fragment lengths (bp)									
383*	383								
							354		
								314	
			302						
290	290	290					290	290	290
						268	268	268	268
				258	258				
248	248	248	248					248	248
		238							
229	229		229	229	229				
		203		203	203	203			
							154	154*	154
142		142		142	142	142			

*Fragments 1 and 37 in Fig. 7.

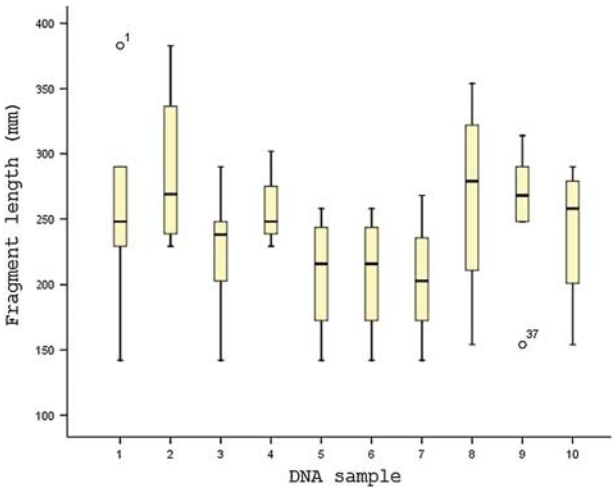

Figure 7. A boxplot of 16S rRNA gene PCR-RFLP profiles of *S. mutans* samples recovered from 10 subjects. Central bands represent median fragment lengths; whiskers represent highest and lowest values; and box lengths represent differences between respective 25th and 75th percentiles. Outliers are present in two samples: the 383 bp fragment in Sample 1 and the 154 bp fragment in Sample 9.

matched and a guilty verdict was secured. Recent figures suggest that a mere 6% of reported rape offences result in conviction, 40% of cases not reaching trial owing to insufficient evidence.[115] Further refinement of this technique could, therefore, benefit what is a complex, and at present unsatisfactory, area of the criminal justice system.

PCR-RFLP analysis of *S. mutans* could be extended to other polymorphic markers. Mattos-Graner *et al.*[116] digested GTF amplicons using enzymes *Bsr*I and *Ssp*I, which yielded a large total number of restriction sites. Total genomic bacterial DNA has also been analysed, using *Eco*RI and *Hin*dIII.[117]

Neither this nor the aforecited research involved sample recovery from human skin, which would mimic a forensic investigative scenario. Subsequent research might, therefore, involve swabbing self-inflicted bites and attempting to match genotypes with those generated from oral samples.[118] One approach would be to compare profiles of two sample groups—one set taken from teeth and the other from skin—in which only the 'perpetrator' had supplied both.

Skin might not be the only substrate from which *S. mutans* could be obtained. Others include pipe stems and writing utensils, although foodstuffs would be more likely.[119] These might create a different effect by virtue of nutrient content. *Streptococcus mutans* is aciduric and readily metabolizes sucrose.[120] Therefore, its capacity for survival might be influenced by foodstuff characteristics such as pH, and protein and carbohydrate content, as well as by environmental temperature.[50] Arakawa *et al.*[121] isolated *Streptococcus* spp. from spoilage microbiota found in milk biscuits and carried out 16S rRNA genotyping. It was concluded that milk-based substrates could prolong cell viability. Controlled experiments using different foodstuffs are indicated.

During the course of this study, Beecher-Monas[122] argued that court rulings are now regularly being overturned by DNA evidence because odontometric analysis has no empirical basis. Consequently, the number of exoneration cases could reach a critical threshold and precipitate a *Daubert* motion *in limine*, [123] causing forensic odontometric evidence to become inadmissible. If so, alternative techniques, such as bacterial genotyping, could be advocated for more intensive study.

Conclusion

Proof of principle has been provided to support the forensic application of PCR-RFLP analysis for genotypic comparison of *S. mutans* recovered from bite mark injuries. The emergence of bacterial genotyping could, therefore, extend the applicability of Locard's Exchange Principle:[38] an individual, when in any form of physical contact with another, stands to transfer not only his own genetic identity, but also those of the myriad other organisms with whom he inevitably co-exists.

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Paul Spradbery graduated with First Class Honours in Forensic Biology at the University of Chester. He also holds a degree in Dental Surgery and has been awarded the Freedom of the City of London. Having been recruited by a major international scientific research organization, his future aspirations involve a range of biological disciplines. These include odontology, genetics and further forensic work, in particular DNA profiling techniques.

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