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Genotypic comparison of bacteria recovered from human bite marks and teeth using arbitrarily primed PCR

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ABSTRACT

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Aims: This study assessed, for forensic purposes, the feasibility of genotypically matching oral streptococci recovered from recent human bite marks with those from the teeth of the biter.

Methods and Results: Streptococci were isolated from the incisors of eight volunteers. Arbitrarily primed PCR (AP-PCR) distinguished 106 streptococcal genotypes among the participants, each harbouring at least eight distinct strains. In a crime simulation, a sample from an experimental bite mark was analysed by an experimenter unaware of its origin. The bacteria were unambiguously matched to the biter by comparing the amplicon profiles with those from the eight participants. In contrast, bacteria from an additional bite mark (not generated by one of the original participants) could not be matched to any of the eight participants. Between 20 and 78% of catalogued bacterial genotypes were recovered 12 months later from each participant. Throughout the study period, none of the bacterial genotypes were shared between participants.

Conclusions: Streptococci isolated from recent bite marks can be catalogued by AP-PCR and matched to the teeth responsible for the bite.

Significance and Impact of the Study: The study provides 'proof of concept' that genotypic analysis of streptococci from bite marks may provide valuable forensic evidence in situations where the perpetrator's DNA cannot be recovered.

Keywords: arbitrarily-primed PCR, bacterial genotyping, bite mark, forensic dentistry, *Streptococcus*

INTRODUCTION

Bite marks inflicted by human teeth on skin are difficult to analyse by morphometric methods, especially when lateral movement of the dentition had occurred during biting (Rothwell 1995). For many reasons, both known and unknown, the skin constitutes a poor impression material. Therefore, analysis of the bite mark often involves a subjective element of interpretation (Whittaker and MacDonald 1989). Consequently, conclusions drawn for forensic purposes are often challenged in a court of law (Whittaker and MacDonald 1989). Nevertheless, bite marks should always be considered as a potential source of evidence (Sweet and Hildebrand 1999). Recent advances in DNA technology offer alternative, objective approaches to many aspects of forensic analysis. However, DNA is rapidly digested by nucleases when exposed to human saliva (Smith *et al.* 1997; Mercer *et al.* 1999) and, until recently, an understanding that the perpetrator's DNA could not be recovered from bite marks was widely held (Blumenthal 1994). In some instances this problem may be surmounted by recovering intact oral epithelial cells from the bite mark; the DNA sequestered within intact cells is protected from the nucleases and can be subsequently analysed for

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discriminative sequences (Sweet *et al.* 1997). However, recovery of the biter's DNA is not always successful even under controlled laboratory conditions (Sweet *et al.* 1997) and alternative approaches should be explored.

We recently demonstrated that oral bacteria of the genus *Streptococcus* can be recovered in large numbers for up to 24 h from experimental bite marks inflicted on human skin (Borgula *et al.* 2003). When they do so, female victims of sexual assault seek medical assistance within 12 h (on average) of the incident (Peipert and Domagalski 1994) and thus there may be an opportunity to recover bacteria from evidentiary bite marks. Streptococci can be isolated from the mouths of most humans and are genotypically extremely diverse (Rudney *et al.* 1992; Alam *et al.* 1999; Wisplinghoff *et al.* 1999). Previously, we reported that the genomic 'fingerprints' of bacteria recovered from each of eight experimental bite marks could be matched exclusively to isolates recovered from the teeth of the biter (Borgula *et al.* 2003). Furthermore, each distinguishable bacterial genotype was harboured by not more than one participant in the study (Borgula *et al.* 2003). The findings suggest that a bacterial genotypic approach could provide a valuable aid to the forensic analysis of bite marks.

The present investigation aimed to extend this approach by applying an arbitrarily primed polymerase chain reaction (AP-PCR) technique to allow greater numbers of oral bacteria to be rapidly analysed. This method was used to identify the perpetrator of an experimental bite mark, to assess the natural distribution of oral streptococcal genotypes, and to examine the retention of identifiable genotypes after a 12-month period.

MATERIALS AND METHODS

Isolation of bacteria

Swabbed samples were taken from the biting edges of the lower incisors of eight volunteers, none of whom were using or had used antiseptic mouthwashes or had received antibiotic treatment in the previous 3 months. Swabs were vigorously agitated in 5 ml of Bacto^{TM} tryptic soy broth (TSB; Becton Dickinson and Co., Sparks, MD, USA) and the TSB serially diluted by 10-fold steps. Aliquots of each dilution (to 10^{-4}) were plated onto Difco Mitis-Salivarius agar (Becton Dickinson) and the agar plates incubated for 3 days at 37°C under anaerobic conditions, essentially following the previously described method (Borgula *et al.* 2003). Fifty bacterial colonies (≤2 mm diameter) were randomly selected from each sample, patched onto TSB agar and re-incubated. Small amounts of bacterial growth from each patch were recovered with a sterile toothpick and suspended in 50 μl of molecular biology-grade nuclease-free deionised water (Eppendorf AG, Hamburg, Germany). Template DNA was released by lysis of bacteria during the initial denaturation step of the PCR protocol. This is in contrast to the method of Li and Caufield (1998) in which DNA was extracted by enzymatic and chemical treatment of cells. The involvement of human participants was approved by the University of Otago Ethics Committee.

Amplification and comparison of bacterial DNA

Several oligonucleotide primers were initially tested and compared under a variety of PCR conditions using a collection of laboratory strains of *Streptococcus gordonii*, *Streptococcus oralis* and *Streptococcus mitis*. The following protocol, using the primer 5′-TGCCGAGCTG-3′ (OPA-02; Invitrogen Life Technologies, Auckland, New Zealand), based on the method of Li and Caufield (1998), gave the most consistent and informative results and was adopted for this study. Except for the primer and deoxynucleotide (dNTP: 10 mmol l^{-1} each of dATP, dTTP, dCTP and dGTP) mixture (Roche Diagnostics New Zealand Ltd., Auckland, New Zealand), the PCR reagents were from the same commercial source (MasterTaq Kit, Eppendorf). Reaction preparations (25 μl) contained bacterial suspension (2·5 μl), $MgCl_2$ (4 mmol l^{-1}), dNTP mixture (200 μmol l^{-1}), Taq polymerase (1·25 units), and primer (4 μmol l^{-1}). Each run of AP-PCRs included a tube containing *S. mitis* strain I18 (Hartley *et al.* 1984) as an internal control. In addition, one tube containing all reagents except the bacterial suspension provided the negative control for each PCR run. Preparations were initially denatured by heating at 94°C for 2 min followed by 35 amplification cycles of denaturation at 94°C for 30 s, annealing at 36°C for 1 min, extension at 72°C for 2 min, followed by a final polymerization step at 72°C for 10 min. The resulting amplicons were resolved by agarose (1·0%) electrophoresis, stained with ethidium bromide and photographed with UV transillumination (Sambrook and Russell 2001). Relative migration distances of DNA bands were normalized against DNA molecular weight markers (1 kb Plus DNA Ladder, Invitrogen Corp., Carlsbad, CA, USA) included in each gel. A 'binning' method (Krawczak and Schmidtke 1998), which obviates the need to standardize the size of the gel images in order to align the calibration bands, was used to compare amplicon profiles. Each amplicon profile was assigned a column in a spreadsheet and the most prominent bands from each profile were registered as an 'X' in the appropriate cells or 'bins', which were ascribed molecular size ranges of 0·2 kb. This facilitated the search for similar amplicon profiles derived from different individuals appearing on separate gels. AP-PCR profiles that were initially observed on different gels, but appeared to match, were electrophoresed in adjacent lanes on a second gel to allow a direct comparison.

Analysis of bacteria recovered from a bite mark

One of the eight volunteers, whose identity was withheld from the laboratory investigator, and one extraneous individual, firmly bit their own upper arms with sufficient force to produce indentations that lasted for at least 10 min. The bite marks were covered with loose clothing for 6 h and the area impacted by the mandibular incisors was swabbed with a moistened cotton-tipped applicator (Borgula *et al.* 2003) by an associate. The two samples (ostensibly from two of the eight original volunteers) were given to the laboratory investigator and processed as for the incisor samples. The bacterial genotypes recovered from the bite marks were then compared with those from the eight incisor samples.

Temporal stability of oral streptococcal genotypes

Twelve months following the original isolation and AP-PCR analysis of oral streptococci, an additional sample was recovered from the incisors of each of the eight participants and the streptococci cultured and analysed as above. Amplicon profiles from the two samples (taken 12 months apart) were compared.

RESULTS

Distribution of streptococcal genotypes

From the teeth of eight unrelated individuals, approximately 400 colonies were analysed to yield a total of 106 genotypically distinguishable streptococcal strains (Table 1). The number of genotypes recovered from each participant ranged between eight and 23. The two most dominant genotypes from each individual composed more than 35% of all isolates from that site (Table 1). None of the genotypic profiles harboured by an individual were identified among the isolates from any other individual. Examples of AP-PCR amplicon profiles are shown in Fig. 1. Not more than three per cent of isolates failed to produce visible amplicons by the method described.

Analysis of bacteria recovered from a bite mark

Comparison of genotypes of bacteria isolated from a self-inflicted bite mark with those from the teeth of each of the eight volunteers revealed several genotypes identical to those isolated only from the teeth of participant No. 4 (Fig. 1), who was indeed the originator of the bite mark sample. In contrast, the bite mark sample derived from a ninth participant revealed no genotype profiles that appeared in anyway similar to those from any of the original eight participants (data not shown).

Table 1 Occurrence (%) of bacterial genotypes recovered from the incisors of each of eight participants

	Participant no.							
Genotype*	1	2	3	4	5	6	7	8
A	82	84	36	66	40	38	42	20
B	4	4	24	10	34	10	12	16
C	4	2	20	4	4	8	6	12
D	2	2	6	4	4	8	6	6
E	2	2	4	4	2	6	4	4
F	2	2	4	2	2	6	2	4
G	2	2	2	2	2	6	2	4
H	2	2	2	2	2	4	2	4
I			2	2	2	4	2	2
J				2	2	2	2	2
K				2	2	2	2	2
L					2	2	2	2
M					2	2	2	2
N						2	2	2
O							2	2
P							2	2
Q							2	2
R							2	2
S							2	2
T							2	2
U								2
V								2
W								2
Total	8	8	9	11	13	14	20	23

*The genotype designations A, B, C, …etc. categorize genotypes from each individual in descending order of prevalence. Genotypes with the same letter designation but from different individuals were not the same (i.e. they produced distinct amplicon profiles). Thus, genotype A1 (harboured by participant 1) was distinct from genotype A2 (harboured by participant 2). There was no evidence that genotypes were shared between individuals in this study.

Temporal stability of oral streptococcal genotypes

From each of the eight participants, between 20 and 78% of bacterial isolates recovered at the start of the study were genotypically matched with isolates recovered 12 months later (Table 2, Fig. 2). Furthermore, over the entire study period, no indistinguishable bacterial genotypes were shared between any two participants in the study.

DISCUSSION

In a previous study (Borgula *et al.* 2003), we demonstrated the feasibility of recovering oral streptococci from bite marks and matching their genomic 'fingerprints' to isolates exclusively from the teeth of the biter. The 'fingerprinting' method involved extraction, purification and restriction

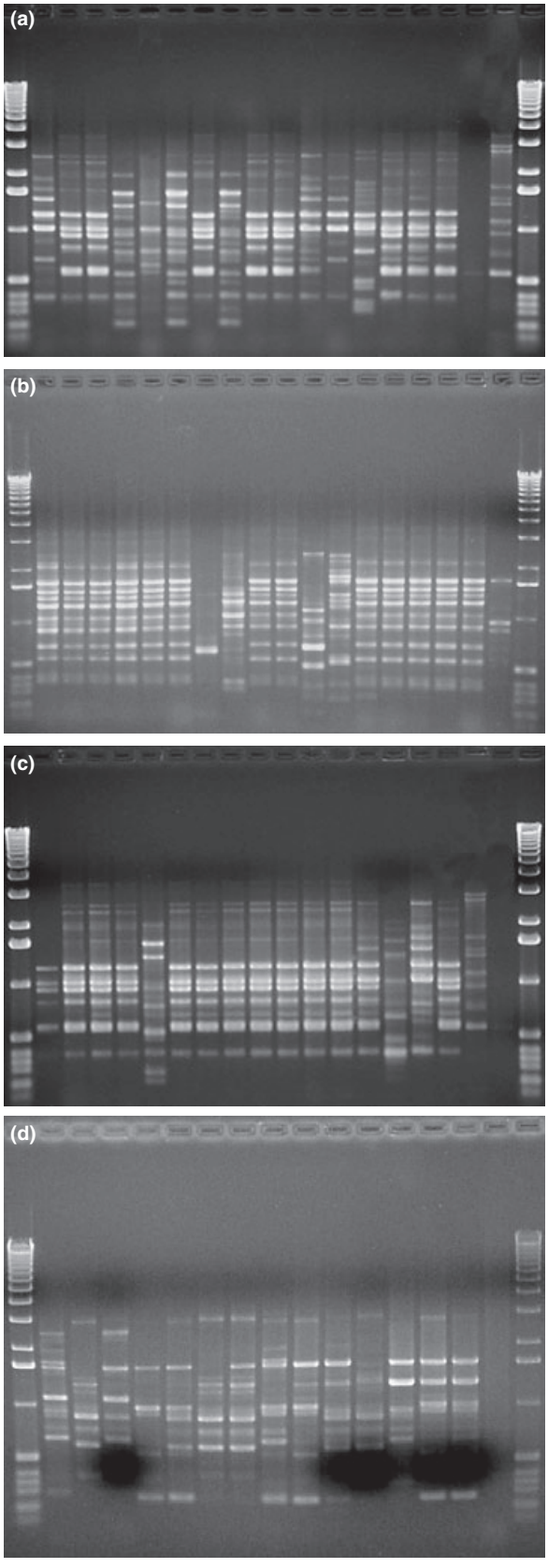

Table 2 The occurrence (%) of streptococcal genotypes from the eight participants at the start of the study compared with those recovered 12 months later

	Participant no.							
Genotype*	1	2	3	4	5	6	7	8
$A_{t=0}$	82	84	36	66	40	38	42	20
$A_{t=12}$	52	30	28	4	52	18	36	22
$B_{t=0}$	4	4	24	10	34	10	12	16
$B_{t=12}$	8	0	10	26	26	2	6	8
$C_{t=0}$	4	2	20	4	4	8	6	12
$C_{t=12}$	4	0	4	0	0	0	0	4
$D_{t=0}$	2	2	6	4	4	8	6	6
$D_{t=12}$	0	0	0	0	0	0	0	2
%match†	64	30	42	30	78	20	42	36

*Genotypes with the same letter designation but from different individuals produced distinct amplicon patterns (see footnote to Fig. 1). $A_{t=0}$ indicates the percentage of isolates producing the same amplicon pattern recovered at the start of the trial; $A_{t=12}$ indicates the percentage of isolates recovered at 12 months that generated amplicon patterns indistinguishable from those designated $A_{t=0}$.
†Isolates (% of total) recovered after 12 months that were genotypically indistinguishable from strains recovered at the beginning of the experiment.

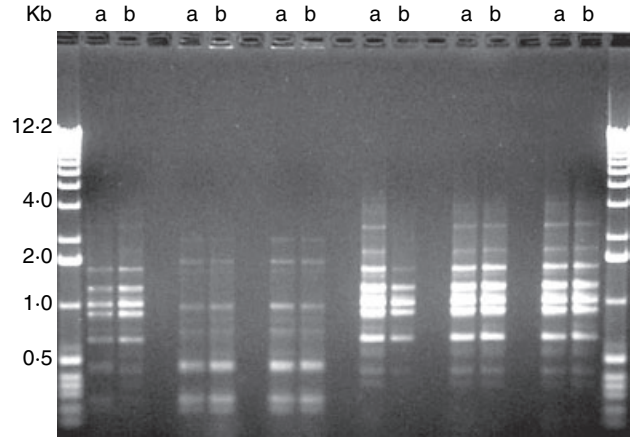

Fig. 2 Confirmation of identity of apparently matching AP-PCR amplicon profiles of streptococci isolated 12 months apart from the lower incisors of participant No. 7, by resolution on the same gel. (a) Strains isolated at 0 months. (b) Strains isolated at 12 months. Molecular size markers are provided in the extreme lanes of the gel

Fig. 1 Comparison of arbitrarily primed PCR amplicon profiles of bacteria isolated from an experimental bite mark (panel A), with those from the teeth of three participants (No. 3 [panel B], No. 4 [panel C], and No. 5 [panel D]). Note similarity between profiles of the bite-mark samples and that of participant No. 4. DNA size markers are provided in the extreme lanes on each side of each gel

endonuclease digestion of genomic DNA, which is labour-intensive and therefore restrictive with respect to the number of isolates that can be conveniently examined. The AP-PCR method described in the current study, facilitates faster analysis of larger numbers of bacteria with no evident loss of resolution. Thus, from eight participants, each contributing approximately 50 isolates, there was no evidence of genotype sharing between individuals, suggesting that the method may be discriminatory to a degree that could afford forensic application. Furthermore, the AP-PCR method would facilitate a large survey of streptococcal isolates from which to determine the frequency of genotype sharing among the general population. Note that as Mitis-Salivarius agar is probably not exclusive for streptococci, other oral organisms may occasionally have been included in the analysis. Further steps, such as growth on blood-containing agar media under micro-aerophilic conditions to select only non-pigmented alpha-haemolytic colonies, could be conveniently incorporated to reduce the likelihood of involving other genera.

Application of the AP-PCR approach unambiguously identified the perpetrator of an experimental bite mark from a group of eight participants, whereas none of the participants could be implicated in the generation of a further bite mark for which none of them was responsible. The bite marks were analysed some weeks following recovery of the samples from the participants' teeth. In a crime investigation, the bite mark would be examined and sampled as soon as possible after infliction but there may be a considerable elapse of time before a suspect is apprehended and his/her teeth sampled for comparison. Our findings indicate that the perpetrators are likely to retain some of the predominant streptococcal genotypes on their teeth for a prolonged period (in the absence of antimicrobial treatment). Therefore this approach to bite mark analysis could be applied in situations when a suspect is not identified for months following an assault, provided the bite mark is sampled and the recovered bacteria are stored appropriately. In four of the eight individuals, fewer than 40% of the isolates recovered at 12 months were matched to isolates from the initial sample. These four individuals comprised two men and two women and therefore gender-related factors, such as hormone cycling, are unlikely to have been responsible for the comparatively lower match rates. During the intervening 12 months we were unable to establish the extent of either antibiotic or antiseptic exposure of the participants, but as such, the group may constitute a realistic sample.

This approach to bite-mark analysis could have immediate application in the identification of an individual from a limited number of implicated persons. However, the frequency at which specific streptococcal genotypes are harboured among either the general or a particular population is not yet known and therefore application of this method could currently only provide evidence 'consistent with' the involvement of a specific individual. Future studies will be aimed at determining the probability of two unrelated individuals harbouring the same genotype.

In conclusion, the findings of this study indicate that the AP-PCR method described could provide valuable supportive information in the forensic analysis of bite marks in instances where human DNA is unrecoverable. Clearly it would be preferable to recover the biter's own DNA but as this will not always be successful then recovery of bacteria derived from the perpetrator's teeth may provide evidence linking a suspect to a crime.

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