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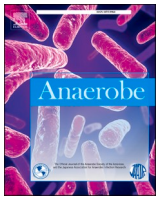

Anaerobes in human infections (dental/oral infections)

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MALDI-TOF mass spectrometry misidentification of *Cutibacterium namnetense* and *Cutibacterium modestum*: Implications for multiplex PCR phylotyping of *Cutibacterium acnes*

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ABSTRACT

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) can misidentify *Cutibacterium namnetense* and *Cutibacterium modestum* as *Cutibacterium acnes*. We now describe how such MALDI-TOF MS misidentification explains previous reports of *C. acnes* isolates that could not be characterised using a multiplex PCR phylotyping assay.

Cutibacterium acnes is a gram-positive anaerobe that plays an important role in human skin health and disease, as well as diseases beyond the skin. In 2015, a multiplex PCR method was described that enabled isolates of *C. acnes* to be assigned to phylotypes IA_1, IA_2, IB and IC within *C. acnes* subsp. *acnes*, as well as phylotypes II (*C. acnes* subsp. *defendens*) and III (*C. acnes* subsp. *elongatum)* [1]. This simple, high-throughput and relatively inexpensive typing approach has now been used by many different research groups to help characterise the genetic diversity of *C. acnes* isolates [2–6]. Despite the success of the multiplex PCR assay, two studies have described a small number of isolates that cannot be genotyped using this method, including PCR-negative samples and those with banding patterns that do not correspond to known phylotypes of the bacterium [4,5]. In the latter case, isolates that react with the 16S rRNA gene primers PArA-1 and PArA-2 specific for *C. acnes*, but not primers targeting protein-encoding loci critical for phylotype assignment, have been observed [4]. To better understand the phylogenetic nature of strains with these particularly unusual results, we conducted a detailed molecular study on five clinical isolates available from the previous study of Burnham et al. [4]. that were either multiplex PCR-negative (n = 1) or positive with PArA-1 and PArA-2 only (n = 4); these isolates had been identified as *C. acnes* using the VITEK® MALDI-TOF MS microbial identification system (v 2.0) (bioMérieux) at Washington University School of Medicine in St. Louis, MO, USA [4].

All isolates were routinely cultured on brain heart infusion agar (Sigma-Aldrich; UK), supplemented with 0.5% (w/v) glucose, at 37 °C in an AnaeroBox™ (ThermoFisher Scientific, UK). Genomic DNA was extracted using a MasterPure™ gram-positive DNA purification kit (Lucigen, Cambio Ltd, UK). All PCR assays were performed as previously reported (see below) with amplicons resolved on 1 % (w/v) agarose gels containing 1 x Tris-acetate-EDTA buffer (ThermoFisher Scientific) and 1 x GelRed® nucleic acid gel stain (Cambridge Bioscience, UK). As expected based on the previous report [4], one isolate (7646) was negative on multiplex PCR while four isolates (7106, 7215, 7361, 10628) were only positive with the *C. acnes* 16S rRNA gene primers PArA-1 and PArA-2 (designated pattern G) (Table 1). Further analysis revealed that all pattern G strains were also positive with the previously described primers PAR-1 and PAR-2 that target the *C. acnes recA* housekeeping gene (1201 bp) [7], but showed no reaction with primers used for PCR amplification of the SLST locus of *C. acnes* (612 bp) [8] (Table 1). In contrast, isolate 7646 was *recA* PCR-negative, but SLST PCR-positive (Table 1). For unambiguous identification of these strains, Sanger sequencing was performed on the *recA* and SLST amplicons, along with 16S rRNA gene amplicons generated using the broad-range primers

Abbreviations: MALDI-TOF MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; SLST, single locus sequence typing; $BLAST_n$®, nucleotide basic local alignment search tool.

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UFPL and URPL [9]. Amplified products were purified using a QIAquick® PCR purification kit (QIAGEN, UK) according to the manufacturer's instructions. Sequencing reactions were performed using BigDye Terminator sequencing chemistry (ThermoFisher Scientific) with the relevant forward and reverse PCR primers. Samples were then run on an ABI Prism® 3100 capillary electrophoresis system (ThermoFisher Scientific). Phylogenetic analysis was carried using Molecular Evolutionary Genetics Analysis (MEGA) software v 11.0 [10], with sequence alignments generated using the Multiple Sequence Comparison by Log-Expectation (MUSCLE) algorithm.

BLASTn® analysis of the resulting 16S rRNA and trimmed *recA* amplicon sequences for isolates 7106, 7215, 7361 and 10628 revealed the organisms to belong to the sp. *Cutibacterium namnetense* (Fig. 1), the sister taxon of *C. acnes* based on a whole genome phylogeny [11]. Identification of these isolates as *C. namnetense* and not *C. acnes* further extends the clinical source of these organisms to brain wounds and ocular fluid [4]. In contrast, SLST database analysis of the trimmed SLST amplicon sequence from 7646 demonstrated that the isolate was in fact *Cutibacterium modestum* (Fig. 2); cross-reaction of the SLST primers with *C. modestum* is known, and SLST alleles (prefix M) for this organism are also deposited in the *C. acnes* SLST database [8]. The negative reaction of *C. namnetense* with SLST primers reflects the absence of the SLST locus in this bacterium. Furthermore, in the original study of Barnard et al. [1], no cross-reaction between the multiplex PCR primers and *C. modestum* was observed in line with the results described here.

Our work adds to previous reports regarding the failure of commercially used bioMérieux VITEK® and Bruker Daltonics MALDI-TOF MS microbial identification systems to identify *C. namnetense* and *C. modestum* [11–14]. This can be explained by the absence of these organisms in the reference databases; currently, the VITEK® MS PRIME only has *C. acnes*, *C. avidum* and *C. granulosum* as claimed organisms, while the Bruker MALDI Biotyper® CA library only has *C. acnes* as a listed cutibacteria. As a result, *C. namnetense* and *C. modestum* strains may be returned as *C. acnes* due to the biological relatedness of the spp., or alternatively they can generate inconclusive results. The incorporation of distinct, validated spectra for *C. namnetense* and *C. modestum* into commercially available MALDI-TOF MS reference databases is therefore needed for correct identification of these organisms in clinical settings. However, while MALDI-TOF MS spectra characteristic for *C. modestum* has previously been described, albeit based on a relatively small number of isolates [11,14,15], to date this has not been the case for *C. namnetense*.

Interestingly, in the study of Nguyen et al. [5], where a small number of *C. acnes* isolates from periprosthetic shoulder infections also generated negative multiplex PCR results (n = 4) or untypable profiles (n = 4), MALDI-TOF MS was similarly applied for identification. While the nature of the non-typable profiles (e.g., pattern G), or the database used, were not described, it is likely these isolates were also incorrectly classified as *C. acnes* and, therefore, represented other cutibacteria; however, we are not able to confirm this.

While our study explains the atypical multiplex PCR phylotype results previously obtained [4], we wanted to understand why *C. namnetense* isolates were also positive with primers PArA-1 and PArA-2 originally designed for *C. acnes* [16]. Sequence alignment of 16S rDNA sequences from *C. namnetense* isolates 7106, 7215, 7361 and 10628 with those from the *C. acnes* type strain ATCC6919 (phylotype IA_1) revealed very high sequence identity (~99% overall). More importantly, identical sequences between *C. namnetense* and *C. acnes* at the annealing sites for PArA-1 and PArA-2 were observed (data not shown); this explains why a correctly sized 16S rRNA gene amplicon is generated from *C. namnetense* on multiplex PCR analysis. In contrast, *C. modestum* was found to have a base insertion and a number of other mismatches at the annealing site for the forward primer PArA-1; however, the annealing site sequence for PArA-2 in *C. modestum* was identical to that in *C. acnes* (data not shown). In the former case, the sequence differences in *C. modestum* versus *C. acnes* for PArA-1 binding were sufficient to ensure a negative reaction under the multiplex PCR conditions used. As the *C. acnes* 16S rRNA gene primers PArA-1 and PArA-2 used in the assay were originally described in 2009 [16], *C. namnetense* sequences would not have been included in the original primer design process as the organism was only reported in 2016 after isolation from a human bone infection [12]. The absence of any cross-reaction between *C. namnetense/C. modestum* versus the other primers used in the *C. acnes* multiplex phylotyping assay likely reflects greater levels of neutral sequence divergence within the protein-encoding loci these primers target; protein-encoding genes have faster rates of sequence divergence compared to the 16S rRNA gene [17]. Despite this, we did see cross reaction between *C. namnetense* and the previously described *C. acnes recA* housekeeping gene primers PAR-1 and PAR-2; these primers flank the entire *recA* open reading frame and have been used for sequence-based typing of the bacterium [7,18]. Inspection of PAR-1 and PAR-2 primer annealing regions in *C. acnes*

Table 1
Results for isolates that could not be phylotyped by multiplex PCR.

Isolate	Phylotype	Source	Multiplex PCR primer sets[a]						Multiplex PCR pattern	*recA*-PCR[b]	SLST-PCR[c]
			PArA-1/ PArA-2	PAMp-1/ PAMp-2	PAMp-3/ PAMp-4	PAMp-5/ PAMp-6	PAMp-7/ PAMp-8	PAMp-9/ PAMp-10			
Controls											
ATCC6919	IA_1	Acne	+	+	–	–	–	–	A	+	+
P. acn17	IA_2	Corneal scraping	+	+	+	–	–	–	B	+	+
W1392	IB	Dental	+	–	+	–	–	–	C	+	+
Prp-38	IC	Acne	+	+	–	+	–	–	D	+	+
NCTC10390	II	NK	+	–	–	–	+	–	E	+	+
Asn12	III	Disc tissue	+	–	–	–	–	+	F	+	+
Untypable											
7106	–	Brain wound	+	–	–	–	–	–	G	+	–
7215	–	Brain wound	+	–	–	–	–	–	G	+	–
7361	–	Bone	+	–	–	–	–	–	G	+	–
7646	–	Joint fluid	–	–	–	–	–	–	–	–	+
10628	–	Ocular fluid	+	–	–	–	–	–	G	+	–

NK = not known.

[a] PArA-1/PArA-2 targets 16S rRNA; PAMp-1/PAMp-2 targets ATPase gene; PAMp-3/PAMp-4 targets *sodA* gene; PAMp-5/PAMp-6 targets Fic family toxin gene; PAMp-7/PAMp-8 targets *atpD* gene; PAMp-9/PAMp-10 targets *recA* gene [1].

[b] *recA*-PCR following the method of McDowell et al. [7] with primers PAR-1 and PAR-2 (1201 bp).

[c] SLST-PCR following the method of Scholz et al. [8] with primers SLST-1 and SLST-2 (612 bp).

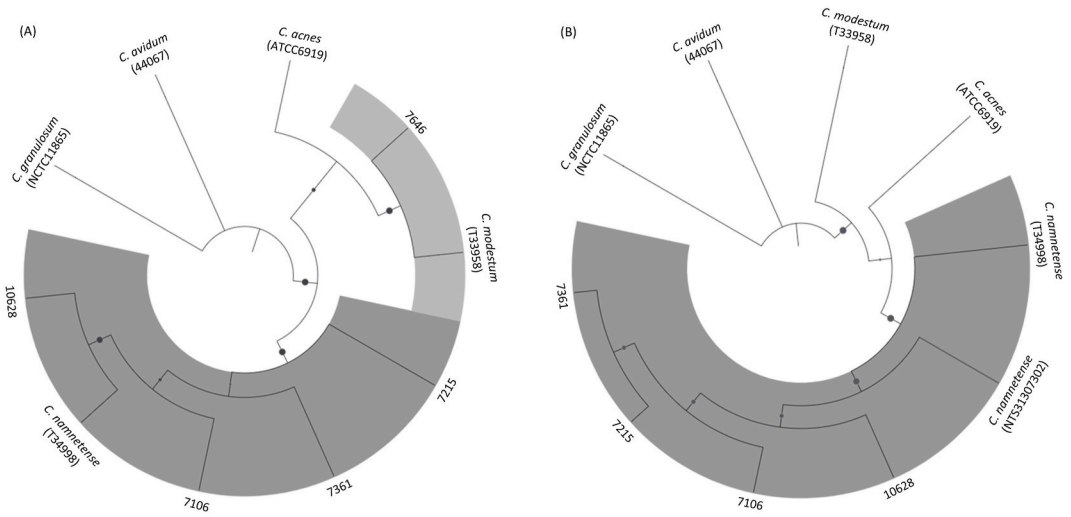

Fig. 1. Molecular evolution tree with the Tajima-Nei method for (A) 16S rRNA gene sequences (1365–1368 bp) and (B) *recA* gene sequences (1046–1048 bp) from strains with multiplex PCR pattern G (7106, 7215, 7361, 10628) or a negative-reaction (7646; 16S rRNA only). Sequences from known cutibacteria spp. (*C. acnes, C. avidum, C. granulosum, C. namnetense* and *C. modestum*) are included for comparison; these sequences were obtained from whole genomes available at National Centre for Biotechnology Information. Sequence input order was randomized, and bootstrapping resampling statistics were performed using 500 datasets. The trees were generated using MEGA and iTOL v 6.0 software (https://itol.embl.dr/). Bootstraps ≥70% are shown (circles). The different shades of grey represent the *C. namnetense* or *C. modestum* strains analysed.

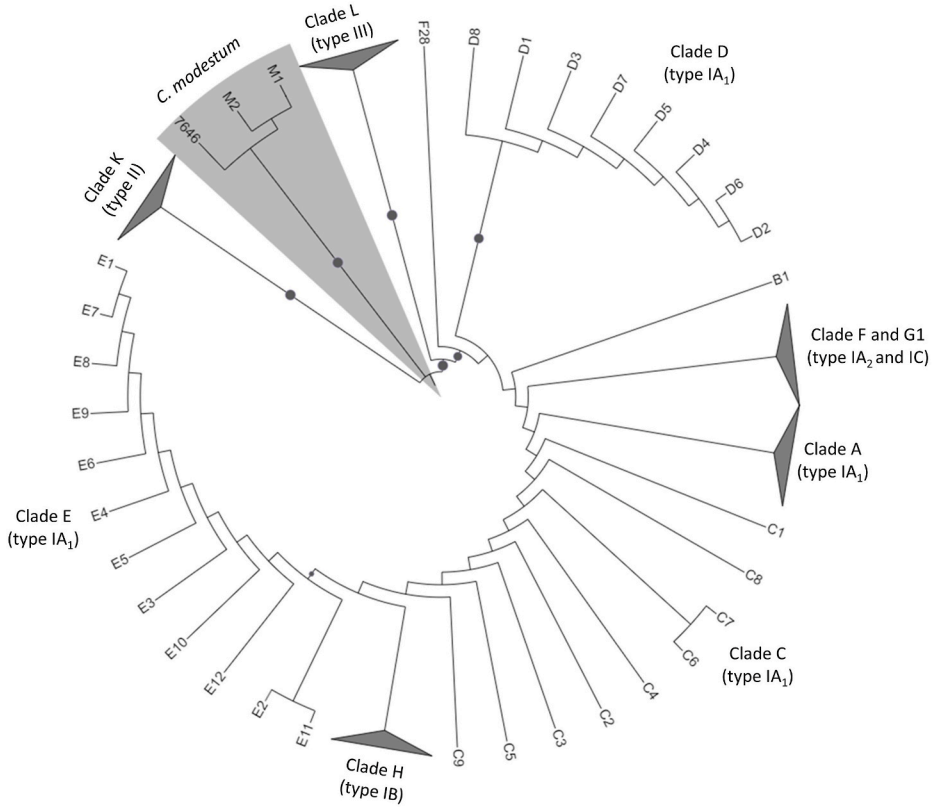

Fig. 2. Molecular evolution tree with the Tajima-Nei method for *C. acnes* and *C. modestum* SLST allele sequences obtained from the SLST database, as well as the SLST allele sequence for strain 7646 (489 bp). Sequence input order was randomized, and bootstrapping resampling statistics were performed using 500 datasets. The tree was generated using MEGA and iTOL v 6.0 software (https://itol.embl.dr/). Bootstraps ≥70% are shown (circles). *C. modestum* alleles from the database and the 7646 sequence are highlighted in grey. Clade K═*C. acnes* subsp. *defendens* (type II); Clade L = *C. acnes* subsp. *elongatum* (type III); all other clades are *C. acnes* subsp. *acnes* (type I).

compared to *C. namnetense* did identify a base deletion at the PAR-1 binding site in *C. namnetense* (near the 5' end), along with other base mismatches at both primer-binding locations in the bacterium; however, the 3' end sequences in PAR-1 and PAR-2 generally matched their complementary annealing sites in *C. namnetense*. While these differences did not inhibit *recA* amplicon formation from *C. namnetense* at the

published annealing temperature of 55 °C used, at increased temperatures product formation could be compromised.

Overall, this work has shown that assumed *C. acnes* isolates that generate negative or pattern G amplification results on multiplex PCR phylotyping likely reflect other misidentified cutibacteria, especially if analysed by MALDI-TOF MS systems that have incomplete reference databases for the genus. For definitive identification of such isolates we would recommend sequencing of the 16S rRNA gene or other loci such as *recA* [7], *gyrB* [13] or SLST [8]. From experience, it is unusual to encounter genuine *C. acnes* isolates that cannot be typed with the multiplex PCR assay, especially given the bacterium's clonal nature. Occasionally, other aberrant amplification patterns with the multiplex PCR primers that target protein-encoding genes have been observed, thus suggesting potentially new lineages. However, on further investigations these have proved to be mixed phylotypes (e.g., type IA_1 and II) that likely failed to separate on culture; this explains the unusual amplicon combinations detected. Interestingly, *C. acnes* has a tendency to aggregate, especially organisms from the type IA and IC phylotypes [19], and clumping of *C. acnes* cells grown from a clinical sample could generate these results due to a mixed phylotype colony or set of colonies. Other possibilities that could explain mixed phylotype patterns are cross-contamination between different phylotype DNA samples, or PCR reagent contamination [20]; however, the latter should be easily identified with appropriate controls.

CRediT authorship contribution statement

Joseph McLaughlin: Writing – original draft, Investigation, Formal analysis. **Carey-Ann D. Burnham:** Writing – review & editing, Resources. **Andrew McDowell:** Writing – original draft, Visualization, Validation, Supervision, Resources, Project administration, Methodology, Funding acquisition, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare that there are no conflicts of interest.

Data availability

Data will be made available on request.

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