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**Title:**

# Multiplex Touchdown PCR for Rapid Typing of the Opportunistic Pathogen *Propionibacterium acnes*

**Running head:**

Multiplex PCR typing of *P. acnes*

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ABSTRACT

The opportunistic human pathogen *Propionibacterium acnes* is comprised of a number of distinct phylogroups, designated types $IA_1$, $IA_2$, IB, IC, II and III, that vary in their production of putative virulence factors, inflammatory potential, as well as biochemical, aggregative and morphological characteristics. Although Multilocus Sequence Typing (MLST) currently represents the gold standard for unambiguous phylogroup classification, and individual strain identification, it is a labour and time-consuming technique. As a consequence, we have developed a multiplex touchdown PCR assay that will, in a single reaction, confirm species identity and phylogeny of an isolate based on its pattern of reaction with six primer sets that target the 16S rRNA (all isolates), ATPase (type $IA_1$, $IA_2$, IC), *sodA* (type $IA_2$, IB), *atpD* (type II) and *recA* (type III) housekeeping genes, as well as a Fic family toxin gene (type IC). When applied to 312 *P. acnes* isolates previously characterised by MLST, and representing type $IA_1$ (n=145), $IA_2$ (n=20), IB (n=65), IC (n=7), II (n=45) and III (n=30), the multiplex displayed 100% sensitivity and 100% specificity for the detection of isolates within each targeted phylogroup. No cross-reactivity with isolates from other bacterial species was observed. The multiplex assay will provide researchers with a rapid, high-throughput and technically undemanding typing method for epidemiological and phylogenetic investigations. It will facilitate studies investigating the association of lineages with various infections and clinical conditions, as well as a pre-screening tool to maximise the number of genetically diverse isolates selected for downstream, higher resolution sequence-based analyses.

## INTRODUCTION

*Propionibacterium acnes* is an anaerobic-to-aerotolerant Gram-positive bacterium which exists in nature as a human commensal and opportunistic pathogen. It is a major component of the human skin microbiota, but can also be recovered from the oral cavity, gastrointestinal and genitourinary tracts (1). Although *P. acnes* is the main cause of opportunistic human infections within the 'cutaneous' group of propionibacteria, and is well known for its association with the inflammatory skin condition acne vulgaris (2, 3), its role in other human infections and clinical conditions is likely to have been significantly underestimated (4-6). Despite this, we now see a growing recognition that the bacterium is an important cause of human disease, especially in relation to indwelling medical device-related infections (7-12), and may also play a role in chronic conditions that cause significant morbidity and mortality, including low back pain associated with modic type I changes (13), sarcoidosis (14, 15) and prostate cancer (16, 17).

Within the last ten years, phylogenetic studies based on single and multilocus gene sequencing (18-21), as well as whole genome analyses of isolates from the Human Microbiome Project (HMP) and other studies (22-28), have provided valuable insights into the genetic population structure of *P. acnes*, particularly in the context of health and disease. The bacterium has an overall clonal structure and isolates can be classified into a number of statistically significant clades or phylogroups, designated types $IA_1$, $IA_2$, IB, IC, II and III, which appear to display differences in their association with specific types of infections (20-21), and also vary in their production of putative virulence determinants (19, 20, 29-32), inflammatory potential (33-36), antibiotic resistance (21, 37), aggregative properties (16) and morphological characteristics (19). In particular, a number of independent epidemiological studies have shown a strong association between clonal complexes from the type $IA_1$ phylogroup and moderate-to-severe acne, while lineages from all other divisions appear more

67 frequently isolated from medical device and soft tissue infections, or associated with health as
68 true commensals (20, 21, 31, 38). Despite these associations, much uncertainty still exists
69 regarding their exact clinical relevance, particularly in the context of acne where skin
70 sampling methods may not be optimal or appropriate (39), as well as the wider issue of
71 whether isolates recovered from different clinical samples are truly representative of infection
72 in all contexts, or are simply skin contaminants/ passive bystanders within a sample; such
73 issues are common when dealing with an opportunistic pathogen that is also part of the
74 normal microbiota, and untangling clinically relevant isolates from background contaminants
75 can be a challenge. Future studies aimed at addressing such issues will undoubtedly provide a
76 more solid platform on which we can make definite conclusions regarding the association of
77 specific *P. acnes* phylogroups with human disease.

78 To date, a number of phenotypic and/ or molecular approaches have been investigated
79 as methods for phylogroup identification, ranging from very simple biochemical tests based
80 on haemolysis (19) or fermentation profiles (40), to matrix assisted laser
81 desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) (41), monoclonal
82 antibody (mAb) typing (18) and DNA-based analysis; the latter includes ribotyping (23),
83 DiversiLab analysis (42), direct PCR assays (43) and protein-encoding gene sequencing (18,
84 20, 21, 44). Unfortunately, many of these approaches suffer from specificity or sensitivity
85 limitations, which constrain their diagnostic value. For example, previously described direct
86 PCR assays do not differentiate $IA_1$ from type $IA_2$ or IC, or target type III strains (43); they
87 may also give ambiguous results (44). Single locus nucleotide sequencing, which to date has
88 been primarily based on the *recA* housekeeping and *tly* methyltransferase/ haemolysin genes,
89 is robust for identification of types I, II and III, but displays reduced specificity for
90 differentiation of type IB strains from type $IA_2$ and some strains within the type $IA_1$ clade as
91 they contain identical alleles due to horizontal gene transfer (HGT) (20, 21, 23, 38). More

recently, mAb typing with antibodies targeting types IA, IC and II, combined with *recA* sequence analysis has obviated this problem, facilitating accurate differentiation of type IB from all type IA strains (45). MALDI-TOF MS has also been described as a valuable and powerful approach for rapid and high throughput phylogroup identification, but currently will not differentiate type $IA_1$ from $IA_2$ (41); furthermore, the technology is not available within all laboratories. At present, Multilocus Sequence Typing (MLST) of *P. acnes* (20, 21) still represents the clear gold standard for unambiguous phylogroup identification, as well as individual strain resolution (Table S1), and offers significant advantages over comparable high resolution gel-based typing methodologies, including Random Amplification of Polymorphic DNA (RAPD) (46) and Pulse Field Gel Electrophoresis (PFGE) (47, 48) which have also been applied to this bacterium. The MLST method is, however, technically demanding, time consuming and expensive, especially when analysing multiple isolates. Consequently, and as a result of the growing interest surrounding the role of this microbe in disease, there is a need for a rapid, less labour intensive and inexpensive method for typing and stratification of *P. acnes* isolates, thus facilitating future molecular epidemiological and phylogenetic studies. Against this background, we now describe the development and validation of a multiplex touchdown PCR assay that can be used for quick, high-throughput and accurate molecular confirmation of *P. acnes* isolates combined with parallel disclosure of their phylogeny. This assay should prove valuable for researchers and, along with MLST, form part of a 'molecular typing toolbox' that can be used for the analysis of *P. acnes* isolates.





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## MATERIALS AND METHODS

**Bacterial strains and growth.** A total of 312 *P. acnes* isolates were used to validate the multiplex PCR (145 type $IA_1$; 20 type $IA_2$, 65 type IB, 7 type IC, 45 type II and 30 type III). These isolates were previously recovered from a wide range of clinical sources and healthy skin, and their phylogroup status determined using an MLST scheme based on eight genes (38). A representative sample of isolates from each group were also analysed by mAb typing as previously described (18), and their reactivity was consistent with their phylogroup designations based on MLST. Genomic DNA from a panel of 49 isolates representing 34 medically relevant bacterial species, and including other human *Propionibacterium* species, was also used in the assessment of multiplex specificity (Table S2). All bacterial strains were maintained at -80 °C in brain heart infusion (BHI) broth, containing 12% (v/v) glycerol. Anaerobic isolates were cultured in BHI and on anaerobic horse blood agar (ABA) plates (Oxoid Ltd, Hampshire, UK) at 37 °C in an anaerobic cabinet (Mark 3; Don Whitley Scientific) under an atmosphere of 10% $H_2$, 10% $CO_2$, 80% $N_2$. Aerobic bacteria were cultured on horse blood agar at 37 °C.

**Development of phylogroup-specific primers**. Housekeeping gene sequences representing *aroE* (424bp), *atpD* (453bp), *gmk* (400bp), *guaA* (493bp), *lepA* (452bp) and *sodA* (450bp) were retrieved from the *P. acnes* MLST database (http://pubmlst.org/pacnes/). Sequences for each gene were then aligned using MEGA v5.1 software and inspected for phylogroup-specific polymorphisms. Phylogroup-specific genomic regions were also investigated using the progressiveMauve algorithm (v2.3.1) and the Artemis Comparison Tool (ACT; http://www.sanger.ac.uk/Software/ACT/) using whole genome sequences (WGS) currently available as part of the HMP, as well as other sequencing projects (http://www.ncbi.nlm.nih.gov/genome/genomes/1140) (Table S1). Based on these analyses,

139 phylogroup-specific primer sets were developed as listed in Table 1.

140

141 **Multiplex PCR analysis.** Bacterial genomic DNA was prepared using an AquaGenomic™
142 kit (Multi Target Pharmaceuticals). PCR amplification was carried out using a MultiGene
143 thermocycler (Labnet International Inc, UK). Samples contained 1 x PCR buffer, 200 µM of
144 each deoxynucleoside triphosphate (Invitrogen Life Technologies, UK), six primer sets
145 targeting each phylogroup at concentrations described in Table 1, 1.5 mM $MgCl_2$, 1x
146 *Redi*Load™ (Invitrogen Life Technologies), 1.25 U *Taq* DNA polymerase (Invitrogen Life
147 Technologies) and 1µl of pure genomic DNA preparation in a total volume of 10 µl. Samples
148 were initially heated at 94°C for 1 min, followed by 14 cycles consisting of 94°C for 30 s,
149 66°C (decreasing incrementally 0.3°C per cycle) for 30 s, and 72°C for 1 min, followed by 11
150 cycles at 94°C for 30 s, 62°C for 30 s and 72°C for 1 min, culminating with a final cycle at
151 72°C for 10 min. A negative control (PCR water) and six positive control samples
152 representing all phylogroups were included in all experiments. PCR products were analysed
153 by electrophoresis on 1.5% (w/v) agarose gels containing 1x Tris-acetate-EDTA buffer.
154 Molecular size markers were run in parallel on all gels. Resolved DNA products were stained
155 with 1x GelRed™ Nucleic Acid Gel stain (Cambridge Biosciences, UK).

156

157 **Nucleotide sequencing**. Sequencing reactions were performed using BigDye® reaction
158 terminator cycle sequencing kits (version 1.1) (Life Technologies, UK) according to the
159 manufacturer's instructions. Samples were then analysed on an ABI PRISM 3100 genetic
160 analyser capillary electrophoresis system (Life Technologies).

161

162 **Split decomposition analysis.** Split decomposition analysis was performed using SplitsTree4
163 version 4.13.1 (49)

## RESULTS

**Primer design.** Polymorphisms in multiple aligned sequences of the *sodA* gene specific for types $IA_2$ and IB, and *atpD* and *recA* genes specific to types II and III, respectively, were identified as candidate regions for primer development (Table 1). Primers were also developed against the ATP-binding component (ATPase; GenBank accession no. ABB20821.1) of a previously described ABC-type peptide uptake operon (DQ208967) present in the closely related type $IA_1$, $IA_2$ and IC groups, but absent in type IB, II and III strains (Table 1) (43). This operon also includes genes encoding permeases (ABB20819.1; ABB20820.1) and a solute binding protein (ABB20823.1), alongside genes for a glycoside hydrolase (ABB20818.1) and chitinase (ABB20824). For type IC strains, we developed a primer set targeting a Fic family-toxin gene located on an approximately 7.3 kb genomic fragment present in the draft genome sequences of the type IC strains PRP-38 (TICEST70_07737) and HL097PA1 (HMPREF9344_02057), but not other phylogroups (Table 1). This genomic fragment also contained restriction enzyme-associated genes and a gene encoding a DEAD/DEAH box helicase (HMPREF9344_02061). Our previously described *P. acnes*-specific 16S rDNA-based primers were also included in the assay to confirm species identity (Table 1) (11, 50). Primer sets incorporated phylogroup-specific mismatches at the 3' end, and elsewhere in the sequence when available, and were designed to have identical annealing temperatures where possible, and to generate amplicons with characteristic size differences that would facilitate easy visual identification on a gel after multiplexing.

**Multiplex PCR development and validation.** Each individual phylogroup-specific primer set was initially examined against a small panel of strains (n=40) representing types $IA_1$, $IA_2$, IB, IC, II and III. Amplicons of predicted size were correctly generated from the targeted

phylogroup, and no products were unexpectedly observed in divisions outside those targeted by the primers (data not shown). The identity of each PCR product was confirmed by direct nucleotide sequence analysis (data not shown). Each primer set was then combined into a single multiplex touchdown PCR reaction, which was optimised for final primer and $MgCl_2$ concentrations, as well as amplification cycles, as outlined in the methods section. As the *sodA* primers PAMp-3/ PAMp-4 had a lower annealing temperature (62°C) than all the other primer sets (66°C), a touchdown PCR approach was adopted to ensure satisfactory, highly specific amplification of all gene targets within the assay. Using this approach, it proved possible to reliably determine the phylogeny of an isolate based on the combination of different phylogroup-specific amplification products as illustrated in Fig 1.

To assess the sensitivity and specificity of the multiplex assay, especially in relation to primers targeting genomic regions that were presumptively present/absent between phylogenetic divisions based on *in silico* analysis of WGS data, we screened a large panel of 312 *P. acnes* isolates previously characterised by MLST, and representing a total of 97 unique sequence types (ST) covering all the phylogroups. Based on this current sample cohort, the multiplex PCR displayed 100% specificity and 100% sensitivity for the detection of isolates within each targeted phylogroup (Table 2); no cross-reactivity with isolates from a wide range of other medically relevant bacterial species was observed, including other cutaneous *Propionibacterium* and *Staphylococcus* species (Table 2 and Table S2).

**DISCUSSION:**

Since the 16S rRNA gene of *P. acnes* has very high intraspecific sequence identity (18, 19), it afforded little opportunity for the design of phylogroup-specific primers on which we could base our multiplex assay. As an alternative, we examined various protein-encoding housekeeping loci, and interrogated available whole genome sequences representing all





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known phylogroups, for unique genetic regions that could act as platforms for assay development. By adopting this approach, we were able to design primers based on *atpD* (PAMp-7/ PAMp-8) and *recA* (PAMp-9/ PAMp-10) housekeeping loci that specifically identified phylogroup II and III strains, respectively. Type IC strains were identified by their reaction with the primers PAMp-5/ PAMp-6 that targeted a Fic family toxin gene present on a genomic region only found in type IC strains; such toxins form part of toxin-antitoxin (TA) systems which are believed to be important in bacterial persistence in response to specific environmental stresses, as well as pathogenicity (51). While types $IA_1$, $IA_2$ and IC all reacted with primers targeting an ATPase gene (PAMp-1/ PAMp-2) that was part of an ABC-type peptide uptake operon, differentiation of type $IA_2$ and IC strains from $IA_1$, and each other, was achieved due to their separate reaction with the primers PAMp-3/ PAMp-4 and PAMp-5/ PAMp-6, respectively. The restriction of an ABC-type peptide uptake operon containing chitinase to type IA and IC divisions, but not other phylogroups, is of particular interest and may be potentially advantageous for the cleavage of chitin from the cell wall of the fungus *Malassezia* and/or *Demodex* mites, which also colonise human skin (20). Since type $IA_2$ isolates contain alleles of the *sodA* locus that are identical (allele 4), or very closely related (allele 5), to those present in all type IB strains (21, 38), both phylogenetic groups displayed reaction with the *sodA* primer pair PAMp-3/ PAMp-4. Their identity was, however, easily determined based on differential reaction with the ATPase primers PAMp-1/PAMp-2; type IB isolates show no product with the latter primer set. Interestingly, as the type $IA_2$ clade shares *recA* and *tly* alleles with type IB isolates it provides evidence for HGT of large genomic fragments in the natural history of the bacterium (20, 21, 38).

By combining these different primer sets, along with the 16S rDNA primers PArA-1 and PArA-2, into a single multiplex assay, we have been able to provide researchers with a robust method for rapid, high-throughput molecular identification of presumptive *P. acnes*

isolates, combined with valuable phylogenetic typing information. This assay should prove a useful tool for epidemiological studies, as well as stratification of isolates for various downstream analyses. It offers enhanced discrimination and specificity over comparable molecular (PCR, single gene sequencing) and mAb typing approaches which have been previously described in the literature, and is also less time consuming, especially over methods that require multiple, separate analyses on each isolate. The multiplex PCR will facilitate future retrospective and prospective studies aimed at investigating the association of specific phylogenetic lineages with different human infections, clinical conditions and antibiotic resistance, and will now also provide a technically undemanding way to rapidly map multiple isolates from the same clinical sample so that the presence and pattern of mixed population types can be determined, especially at different body sites/ niches. The method should also provide a useful complement to the more detailed, and technically complicated, study of *P. acnes* populations in complex microbiotas based on metagenomic analysis (23). Although in this study we utilized purified genomic DNA as our template for multiplex PCR, future optimization of the method for direct analysis of bacterial colonies (colony PCR) would further enhance the rapid nature of the assay. Furthermore, of the *P. acnes* isolates tested, numbers representing types $IA_2$ and IC were lower than those from other phylogroups, especially types IC which are infrequently recovered. As a consequence, further analysis of additional isolates from these clusters will be important to confirm the multiplex PCR specificity and sensitivity results for these types.

To date, our understanding of *P. acnes* population structure within and between different body habitats of individuals is poor. These sites not only include various areas of, and regions within, the skin, but also the oral cavity and genitourinary tract. Such data may prove especially valuable in our attempts to better understand the potential origin of different lineages associated with clinical samples, particularly in relation to blood culture, and





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whether the pattern of isolates recovered from primary surgical samples matches those on the overlying/ surrounding skin, thus indicating potential contamination. In the surgical setting, current methods used for pre-operative skin antisepsis do not always prevent microbial contamination of surgical wounds with viable bacteria (52, 53). Although acute surgical site infections may not ensue due to effective i.v. prophylactic antibiotic administration, these bacteria may still cause downstream chronic biofilm-associated implant infection. It seems reasonable to assume that contamination from the skin would result in a mixture of different phylogenetic groups within a sample, while significant counts of a monotype may be more indicative of infection. Under such circumstances, the multiplex could prove a valuable and simple molecular screening tool to highlight such a scenario, and thus aid the diagnosis of biofilm-associated implant infections and bacteremia etc within a clinical setting (54). Furthermore, the detection of phylogroups with a potentially greater propensity to cause infection within a clinical sample, such as $IA_1$, may also be more indicative of infection when compared to phylogroups, such as types II and III, believed to be associated with a more commensal existence (21).

To date, two MLST schemes based on eight ($MLST_8$) and nine ($MLST_9$) different protein-encoding genes have been described for *P. acnes* (20, 21); the methods are essentially concordant in respect to the clustering of strains into different clonal complexes (CCs), although more subtle differences in the resolution of particular lineages within these CCs exist (21, 38). While MLST provides high resolution typing of *P. acnes*, and generates not only phylogroup information, but also ST data that is highly amenable to phylogenetic and evolutionary analyses, the method is laborious and time consuming when investigating multiple isolates. The development of new approaches to help streamline the MLST workflow are very attractive, and recently we described how cross-referencing a refined four gene MLST allelic profile to the full eight gene versions available in the MLST database at

289 http://pubmlst.org/pacnes/ could be used to correctly predict and assign phylogroup, clonal
290 complex and, in the vast majority of cases, ST for a *P. acnes* isolate (38). In this context,
291 rapid pre-screening of isolates by multiplex PCR could also prove an extremely valuable way
292 to maximise the number of genetically diverse isolates selected for downstream MLST
293 analyses, thus reducing sequencing costs. Furthermore, MLST and whole genome analyses
294 have shown that types $IA_2$, IB, IC and III represent tight phylogenetic clusters, especially
295 when compared to types $IA_1$ and II (21, 22, 38) (Table S1); this is reflected in a more
296 restricted number of STs, some of which are highly dominant and widely disseminated. As a
297 consequence, high resolution MLST typing after multiplex PCR provides less phylogenetic
298 information for types $IA_2$, IB, IC and III isolates when compared to types $IA_1$ and II which
299 are genetically more heterogeneous and contain deeper levels of phylogenetic structure. In
300 keeping with the desirability of a simpler approach to high resolution typing of *P. acnes*, a
301 single locus typing scheme (SLST) for the bacterium based on nucleotide sequencing of an
302 amplified target region (484 bp) immediately upstream of the *camp1* gene (identified by a
303 genome mining approach) was described during the preparation of this manuscript (55).
304 While the $MLST_8$ and $MLST_9$ schemes resolve a greater number of genotypes than SLST
305 (Table S1), the latter method does correctly cluster isolates into phylogenetic groupings that
306 are congruent with a core genome reference tree (55). In addition, there is little evidence of
307 recombination within the locus based on a network tree analysis (phi test p=0.976) (Fig S1).
308 The SLST method is, therefore, a valuable complement, and technically simpler approach, to
309 current MLST methods of typing *P. acnes*.

310 Rapid screening of isolates by multiplex PCR will also aid the discovery of novel taxa
311 via atypical PCR reactions. For example, sole reaction with the 16S rDNA primer set PArA-
312 1/ PArA-2 may indicate a new closely related species of *Propionibacterium* with high 16S
313 rDNA identity to *P. acnes*, or a novel *P. acnes* phylogroup or ST that contains base



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mismatches at primer binding sites within the protein encoding genes of the assay. Indeed, as a direct result of multiplex PCR screening of skin derived isolates, we have recently come across such a scenario and identified a new *Propionibacterium* species that has very high 16S rDNA identity to *P. acnes* (and reacts with PArA-1/ PArA-2) but is quite distinct from the latter, and other cutaneous propionibacteria, based on whole genome analysis (currently unpublished).

In conclusion, the multiplex PCR described here facilitates rapid molecular confirmation of presumptive *P. acnes* isolates along with parallel phylogenetic typing. It should provide researchers with a flexible typing tool that can be used in isolation, or as an adjunct to more detailed sequence-based analysis depending on the epidemiological questions being asked and resolution required. It is also a technically simple methodology for the rapid analysis of mixed *P. acnes* populations, and should therefore help improve our understanding of the role of different *P. acnes* lineages in clinical conditions.





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**FIGURE LEGEND.**

FIG 1. Multiplex PCR analysis of *P. acnes* strains representing different phylogroups and STs. Bottom lanes (left to right): 1, hdn-1 (ST1; type $IA_1$); 2, PRP-60 (ST20; type $IA_1$); 3, 76793 (ST101; type $IA_1$); 4, Pacn33 (ST2, type $IA_2$); 5, P.acn17 (ST2, type $IA_2$); 6, P. acn31 (ST2, type $IA_2$); 7, 6609 (ST5, type IB); 8, VA3/4 (ST78, type IB); 9, 74874 (ST43, type IB); 10, PRP-38 (ST70, type IC); 11, PV66 (ST85, type IC); 12, 5/1/3 (ST107, type IC); 13, ATCC11828 (ST27, type II); 14, VA2/9N (ST28, type II); 15, 6187 (ST30, type II); 16, 12S (ST32, type III); 17, Asn12 (ST33, type III); 18, Asn10 (ST81, type III); 19, *P. avidum* 44067; Ma molecular size markers. ST is based on the eight-gene MLST scheme of McDowell et al. (21) and database at http://pubmlst.org/pacnes/. Gene amplicons (left to right): a, 16S rRNA; b, ATPase; c, *sodA*; d, toxin; e, *atpD*; f, *recA*.

535 **TABLE 1. Multiplex PCR primer characteristics**

| Primers[a] | Specificity | Gene targeted | Sequence (5'-3') | Position | Concentration | Annealing Temp (°C) | Amplicon size (bp) |
|---|---|---|---|---|---|---|---|
| PArA-1<br>PArA-2 | *P. acnes* | 16S rRNA | AAGCGTGAGTGACGGTAATGGGTA<br>CCACCATAACGTGCTGGCAACAGT | 442-465<br>1118-1095 | 0.2µM | 66 | 677 |
| PAMp-1<br>PAMp-2 | $IA_1/IA_2/IC$ | ATPase | GCGTTGACCAAGTCCGCCGA<br>GCAAATTCGCACCGCGGAGC | 451-470<br>944-925 | 0.25µM | 66 | 494 |
| PAMp-3<br>PAMp-4 | $IA_2/IB$ | *sodA* | CGGAACCATCAACAAACTCGAA<br>GAAGAACTCGTCAATCGCAGCA | 168-189<br>312-291 | 0.6µM | 62 | 145 |
| PAMp-5<br>PAMp-6 | IC | Toxin, Fic family | AGGGCGAGGTCCTCTTCTACCAGCG<br>ACCCTCCAACTGCAACTCTCCGCCT | 17-41<br>321-297 | 0.1µM | 66 | 305 |
| PAMp-7<br>PAMp-8 | II | *atpD* | TCCATCTGGCCGAATACCAGG<br>TCTTAACGCCGATCCCTCCAT | 339-360<br>689-669 | 0.15µM | 66 | 351 |
| PAMp-9<br>PAMp-10 | III | *recA* | GCGCCCTCAAGTTCTACTCA<br>CGGATTTGGTGATAATGCCA | 641-660<br>865-846 | 0.25µM | 66 | 225 |

536 [a]For protein-encoding housekeeping genes, primers relate to positions within the open reading frame. For the 16S rRNA gene, primers relate to positions
537 within the sequence for NCTC737 (Genbank accession no. AB042288)

**TABLE 2. Multiplex PCR assay accuracy**

| Phylogroup[a] | Number of isolates[b] | | Sensitivity (%) | Specificity (%) |
|---|---|---|---|---|
| | Positive | Negative | | |
| $IA_1$ | 145/145 | 0/145 | 100 | 100 |
| all others | 0/216 | 216/216 | | |
| $IA_2$ | 20/20 | 0/20 | 100 | 100 |
| all others | 0/341 | 341/341 | | |
| IB | 65/65 | 0/65 | 100 | 100 |
| all others | 0/296 | 296/296 | | |
| IC | 7/7 | 0/7 | 100 | 100 |
| all others | 0/354 | 354/354 | | |
| II | 45/45 | 0/45 | 100 | 100 |
| all others | 0/316 | 316/316 | | |
| III | 30/30 | 0/30 | 100 | 100 |
| all others | 0/331 | 331/331 | | |

[a]All others relates to *P. acnes* isolates outside the target phylogroup, plus 49 isolates from other medically relevant species

[b]Positive relates to detection of the expected amplification pattern under consideration, while negative indicates that one of the alternate phylogroup profiles was detected, or no reaction was observed in the case of other spp