1   **Title:**

2   Multiplex Touchdown PCR for Rapid Typing of the Opportunistic Pathogen

3   *Propionibacterium acnes*

4

5   **Running head:**

6   Multiplex PCR typing of *P. acnes*

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21 ABSTRACT

22 The opportunistic human pathogen *Propionibacterium acnes* is comprised of a number of

23 distinct phylogroups, designated types IA_1, IA_2, IB, IC, II and III, that vary in their

24 production of putative virulence factors, inflammatory potential, as well as biochemical,

25 aggregative and morphological characteristics. Although Multilocus Sequence Typing

26 (MLST) currently represents the gold standard for unambiguous phylogroup classification,

27 and individual strain identification, it is a labour and time-consuming technique. As a

28 consequence, we have developed a multiplex touchdown PCR assay that will, in a single

29 reaction, confirm species identity and phylogeny of an isolate based on its pattern of reaction

30 with six primer sets that target the 16S rRNA (all isolates), ATPase (type IA_1, IA_2, IC), *sodA*

31 (type IA_2, IB), *atpD* (type II) and *recA* (type III) housekeeping genes, as well as a Fic family

32 toxin gene (type IC). When applied to 312 *P. acnes* isolates previously characterised by

33 MLST, and representing type IA_1 (n=145), IA_2 (n=20), IB (n=65), IC (n=7), II (n=45) and III

34 (n=30), the multiplex displayed 100% sensitivity and 100% specificity for the detection of

35 isolates within each targeted phylogroup. No cross-reactivity with isolates from other

36 bacterial species was observed. The multiplex assay will provide researchers with a rapid,

37 high-throughput and technically undemanding typing method for epidemiological and

38 phylogenetic investigations. It will facilitate studies investigating the association of lineages

39 with various infections and clinical conditions, as well as a pre-screening tool to maximise

40 the number of genetically diverse isolates selected for downstream, higher resolution

41 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

92   recently, mAb typing with antibodies targeting types IA, IC and II, combined with *recA*

93   sequence analysis has obviated this problem, facilitating accurate differentiation of type IB

94   from all type IA strains (45). MALDI-TOF MS has also been described as a valuable and

95   powerful approach for rapid and high throughput phylogroup identification, but currently will

96   not differentiate type IA_1 from IA_2 (41); furthermore, the technology is not available within

97   all laboratories. At present, Multilocus Sequence Typing (MLST) of *P. acnes* (20, 21) still

98   represents the clear gold standard for unambiguous phylogroup identification, as well as

99   individual strain resolution (Table S1), and offers significant advantages over comparable

100   high resolution gel-based typing methodologies, including Random Amplification of

101   Polymorphic DNA (RAPD) (46) and Pulse Field Gel Electrophoresis (PFGE) (47, 48) which

102   have also been applied to this bacterium. The MLST method is, however, technically

103   demanding, time consuming and expensive, especially when analysing multiple isolates.

104   Consequently, and as a result of the growing interest surrounding the role of this microbe in

105   disease, there is a need for a rapid, less labour intensive and inexpensive method for typing

106   and stratification of *P. acnes* isolates, thus facilitating future molecular epidemiological and

107   phylogenetic studies. Against this background, we now describe the development and

108   validation of a multiplex touchdown PCR assay that can be used for quick, high-throughput

109   and accurate molecular confirmation of *P. acnes* isolates combined with parallel disclosure of

110   their phylogeny. This assay should prove valuable for researchers and, along with MLST,

111   form part of a 'molecular typing toolbox' that can be used for the analysis of *P. acnes*

112   isolates.

113

114 **MATERIALS AND METHODS**

115 **Bacterial strains and growth.** A total of 312 *P. acnes* isolates were used to validate the

116 multiplex PCR (145 type IA_1; 20 type IA_2, 65 type IB, 7 type IC, 45 type II and 30 type III).

117 These isolates were previously recovered from a wide range of clinical sources and healthy

118 skin, and their phylogroup status determined using an MLST scheme based on eight genes

119 (38). A representative sample of isolates from each group were also analysed by mAb typing

120 as previously described (18), and their reactivity was consistent with their phylogroup

121 designations based on MLST. Genomic DNA from a panel of 49 isolates representing 34

122 medically relevant bacterial species, and including other human *Propionibacterium* species,

123 was also used in the assessment of multiplex specificity (Table S2). All bacterial strains were

124 maintained at -80 °C in brain heart infusion (BHI) broth, containing 12% (v/v) glycerol.

125 Anaerobic isolates were cultured in BHI and on anaerobic horse blood agar (ABA) plates

126 (Oxoid Ltd, Hampshire, UK) at 37 °C in an anaerobic cabinet (Mark 3; Don Whitley

127 Scientific) under an atmosphere of 10% H_2, 10% CO_2, 80% N_2. Aerobic bacteria were

128 cultured on horse blood agar at 37 °C.

129

130 **Development of phylogroup-specific primers**. Housekeeping gene sequences representing

131 *aroE* (424bp), *atpD* (453bp), *gmk* (400bp), *guaA* (493bp), *lepA* (452bp) and *sodA* (450bp)

132 were retrieved from the *P. acnes* MLST database (http://pubmlst.org/pacnes/). Sequences for

133 each gene were then aligned using MEGA v5.1 software and inspected for phylogroup-

134 specific polymorphisms. Phylogroup-specific genomic regions were also investigated using

135 the progressiveMauve algorithm (v2.3.1) and the Artemis Comparison Tool (ACT;

136 http://www.sanger.ac.uk/Software/ACT/) using whole genome sequences (WGS) currently

137 available as part of the HMP, as well as other sequencing projects

138 (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)

164 **RESULTS**

165 **Primer design.** Polymorphisms in multiple aligned sequences of the *sodA* gene specific for

166 types IA_2 and IB, and *atpD* and *recA* genes specific to types II and III, respectively, were

167 identified as candidate regions for primer development (Table 1). Primers were also

168 developed against the ATP-binding component (ATPase; GenBank accession no.

169 ABB20821.1) of a previously described ABC-type peptide uptake operon (DQ208967)

170 present in the closely related type IA_1, IA_2 and IC groups, but absent in type IB, II and III

171 strains (Table 1) (43). This operon also includes genes encoding permeases (ABB20819.1;

172 ABB20820.1) and a solute binding protein (ABB20823.1), alongside genes for a glycoside

173 hydrolase (ABB20818.1) and chitinase (ABB20824). For type IC strains, we developed a

174 primer set targeting a Fic family-toxin gene located on an approximately 7.3 kb genomic

175 fragment present in the draft genome sequences of the type IC strains PRP-38

176 (TICEST70_07737) and HL097PA1 (HMPREF9344_02057), but not other phylogroups

177 (Table 1). This genomic fragment also contained restriction enzyme-associated genes and a

178 gene encoding a DEAD/DEAH box helicase (HMPREF9344_02061). Our previously

179 described *P. acnes*-specific 16S rDNA-based primers were also included in the assay to

180 confirm species identity (Table 1) (11, 50). Primer sets incorporated phylogroup-specific

181 mismatches at the 3' end, and elsewhere in the sequence when available, and were designed

182 to have identical annealing temperatures where possible, and to generate amplicons with

183 characteristic size differences that would facilitate easy visual identification on a gel after

184 multiplexing.

185

186 **Multiplex PCR development and validation.** Each individual phylogroup-specific primer

187 set was initially examined against a small panel of strains (n=40) representing types IA_1, IA_2,

188 IB, IC, II and III. Amplicons of predicted size were correctly generated from the targeted

189 phylogroup, and no products were unexpectedly observed in divisions outside those targeted

190 by the primers (data not shown). The identity of each PCR product was confirmed by direct

191 nucleotide sequence analysis (data not shown). Each primer set was then combined into a

192 single multiplex touchdown PCR reaction, which was optimised for final primer and $MgCl_2$

193 concentrations, as well as amplification cycles, as outlined in the methods section. As the

194 *sodA* primers PAMp-3/ PAMp-4 had a lower annealing temperature (62°C) than all the other

195 primer sets (66°C), a touchdown PCR approach was adopted to ensure satisfactory, highly

196 specific amplification of all gene targets within the assay. Using this approach, it proved

197 possible to reliably determine the phylogeny of an isolate based on the combination of

198 different phylogroup-specific amplification products as illustrated in Fig 1.

199   To assess the sensitivity and specificity of the multiplex assay, especially in relation

200 to primers targeting genomic regions that were presumptively present/absent between

201 phylogenetic divisions based on *in silico* analysis of WGS data, we screened a large panel of

202 312 *P. acnes* isolates previously characterised by MLST, and representing a total of 97

203 unique sequence types (ST) covering all the phylogroups. Based on this current sample

204 cohort, the multiplex PCR displayed 100% specificity and 100% sensitivity for the detection

205 of isolates within each targeted phylogroup (Table 2); no cross-reactivity with isolates from a

206 wide range of other medically relevant bacterial species was observed, including other

207 cutaneous *Propionibacterium* and *Staphylococcus* species (Table 2 and Table S2).

208

209 **DISCUSSION:**

210 Since the 16S rRNA gene of *P. acnes* has very high intraspecific sequence identity (18, 19), it

211 afforded little opportunity for the design of phylogroup-specific primers on which we could

212 base our multiplex assay. As an alternative, we examined various protein-encoding

213 housekeeping loci, and interrogated available whole genome sequences representing all

214   known phylogroups, for unique genetic regions that could act as platforms for assay

215   development. By adopting this approach, we were able to design primers based on *atpD*

216   (PAMp-7/ PAMp-8) and *recA* (PAMp-9/ PAMp-10) housekeeping loci that specifically

217   identified phylogroup II and III strains, respectively. Type IC strains were identified by their

218   reaction with the primers PAMp-5/ PAMp-6 that targeted a Fic family toxin gene present on

219   a genomic region only found in type IC strains; such toxins form part of toxin-antitoxin (TA)

220   systems which are believed to be important in bacterial persistence in response to specific

221   environmental stresses, as well as pathogenicity (51). While types IA$_1$, IA$_2$ and IC all reacted

222   with primers targeting an ATPase gene (PAMp-1/ PAMp-2) that was part of an ABC-type

223   peptide uptake operon, differentiation of type IA$_2$ and IC strains from IA$_1$, and each other,

224   was achieved due to their separate reaction with the primers PAMp-3/ PAMp-4 and PAMp-5/

225   PAMp-6, respectively. The restriction of an ABC-type peptide uptake operon containing

226   chitinase to type IA and IC divisions, but not other phylogroups, is of particular interest and

227   may be potentially advantageous for the cleavage of chitin from the cell wall of the fungus

228   *Malassezia* and/or *Demodex* mites, which also colonise human skin (20). Since type IA$_2$

229   isolates contain alleles of the *sodA* locus that are identical (allele 4), or very closely related

230   (allele 5), to those present in all type IB strains (21, 38), both phylogenetic groups displayed

231   reaction with the *sodA* primer pair PAMp-3/ PAMp-4. Their identity was, however, easily

232   determined based on differential reaction with the ATPase primers PAMp-1/PAMp-2; type

233   IB isolates show no product with the latter primer set. Interestingly, as the type IA$_2$ clade

234   shares *recA* and *tly* alleles with type IB isolates it provides evidence for HGT of large

235   genomic fragments in the natural history of the bacterium (20, 21, 38).

236   By combining these different primer sets, along with the 16S rDNA primers PArA-1

237   and PArA-2, into a single multiplex assay, we have been able to provide researchers with a

238   robust method for rapid, high-throughput molecular identification of presumptive *P. acnes*

239   isolates, combined with valuable phylogenetic typing information. This assay should prove a

240   useful tool for epidemiological studies, as well as stratification of isolates for various

241   downstream analyses. It offers enhanced discrimination and specificity over comparable

242   molecular (PCR, single gene sequencing) and mAb typing approaches which have been

243   previously described in the literature, and is also less time consuming, especially over

244   methods that require multiple, separate analyses on each isolate. The multiplex PCR will

245   facilitate future retrospective and prospective studies aimed at investigating the association of

246   specific phylogenetic lineages with different human infections, clinical conditions and

247   antibiotic resistance, and will now also provide a technically undemanding way to rapidly

248   map multiple isolates from the same clinical sample so that the presence and pattern of mixed

249   population types can be determined, especially at different body sites/ niches. The method

250   should also provide a useful complement to the more detailed, and technically complicated,

251   study of *P. acnes* populations in complex microbiotas based on metagenomic analysis (23).

252   Although in this study we utilized purified genomic DNA as our template for multiplex PCR,

253   future optimization of the method for direct analysis of bacterial colonies (colony PCR)

254   would further enhance the rapid nature of the assay. Furthermore, of the *P. acnes* isolates

255   tested, numbers representing types IA_2 and IC were lower than those from other phylogroups,

256   especially types IC which are infrequently recovered. As a consequence, further analysis of

257   additional isolates from these clusters will be important to confirm the multiplex PCR

258   specificity and sensitivity results for these types.

259   To date, our understanding of *P. acnes* population structure within and between

260   different body habitats of individuals is poor. These sites not only include various areas of,

261   and regions within, the skin, but also the oral cavity and genitourinary tract. Such data may

262   prove especially valuable in our attempts to better understand the potential origin of different

263   lineages associated with clinical samples, particularly in relation to blood culture, and

11

264　　whether the pattern of isolates recovered from primary surgical samples matches those on the

265　　overlying/ surrounding skin, thus indicating potential contamination. In the surgical setting,

266　　current methods used for pre-operative skin antisepsis do not always prevent microbial

267　　contamination of surgical wounds with viable bacteria (52, 53). Although acute surgical site

268　　infections may not ensue due to effective i.v. prophylactic antibiotic administration, these

269　　bacteria may still cause downstream chronic biofilm-associated implant infection. It seems

270　　reasonable to assume that contamination from the skin would result in a mixture of different

271　　phylogenetic groups within a sample, while significant counts of a monotype may be more

272　　indicative of infection. Under such circumstances, the multiplex could prove a valuable and

273　　simple molecular screening tool to highlight such a scenario, and thus aid the diagnosis of

274　　biofilm-associated implant infections and bacteremia etc within a clinical setting (54).

275　　Furthermore, the detection of phylogroups with a potentially greater propensity to cause

276　　infection within a clinical sample, such as IA_1, may also be more indicative of infection when

277　　compared to phylogroups, such as types II and III, believed to be associated with a more

278　　commensal existence (21).

279　　　　　　To date, two MLST schemes based on eight ($MLST_8$) and nine ($MLST_9$) different

280　　protein-encoding genes have been described for *P. acnes* (20, 21); the methods are essentially

281　　concordant in respect to the clustering of strains into different clonal complexes (CCs),

282　　although more subtle differences in the resolution of particular lineages within these CCs

283　　exist (21, 38). While MLST provides high resolution typing of *P. acnes*, and generates not

284　　only phylogroup information, but also ST data that is highly amenable to phylogenetic and

285　　evolutionary analyses, the method is laborious and time consuming when investigating

286　　multiple isolates. The development of new approaches to help streamline the MLST

287　　workflow are very attractive, and recently we described how cross-referencing a refined four

288　　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

314   mismatches at primer binding sites within the protein encoding genes of the assay. Indeed, as

315   a direct result of multiplex PCR screening of skin derived isolates, we have recently come

316   across such a scenario and identified a new *Propionibacterium* species that has very high 16S

317   rDNA identity to *P. acnes* (and reacts with PArA-1/ PArA-2) but is quite distinct from the

318   latter, and other cutaneous propionibacteria, based on whole genome analysis (currently

319   unpublished).

320   In conclusion, the multiplex PCR described here facilitates rapid molecular

321   confirmation of presumptive *P. acnes* isolates along with parallel phylogenetic typing. It

322   should provide researchers with a flexible typing tool that can be used in isolation, or as an

323   adjunct to more detailed sequence-based analysis depending on the epidemiological questions

324   being asked and resolution required. It is also a technically simple methodology for the rapid

325   analysis of mixed *P. acnes* populations, and should therefore help improve our understanding

326   of the role of different *P. acnes* lineages in clinical conditions.

ACKNOWLEDGMENTS

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523   **FIGURE LEGEND.**

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525   FIG 1. Multiplex PCR analysis of *P. acnes* strains representing different phylogroups and

526   STs. Bottom lanes (left to right): 1, hdn-1 (ST1; type IA$_1$); 2, PRP-60 (ST20; type IA$_1$); 3,

527   76793 (ST101; type IA$_1$); 4, Pacn33 (ST2, type IA$_2$); 5, P.acn17 (ST2, type IA$_2$); 6, P. acn31

528   (ST2, type IA$_2$); 7, 6609 (ST5, type IB); 8, VA3/4 (ST78, type IB); 9, 74874 (ST43, type IB);

529   10, PRP-38 (ST70, type IC); 11, PV66 (ST85, type IC); 12, 5/1/3 (ST107, type IC); 13,

530   ATCC11828 (ST27, type II); 14, VA2/9N (ST28, type II); 15, 6187 (ST30, type II); 16, 12S

531   (ST32, type III); 17, Asn12 (ST33, type III); 18, Asn10 (ST81, type III); 19, *P. avidum*

532   44067; Ma molecular size markers. ST is based on the eight-gene MLST scheme of

533   McDowell et al. (21) and database at http://pubmlst.org/pacnes/. Gene amplicons (left to

534   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 PArA-2	*P. acnes*	16S rRNA	AAGCGTGAGTGACGGTAATGGGTA CCACCATAACGTGCTGGCAACAGT	442-465 1118-1095	0.2μM	66	677
PAMp-1 PAMp-2	$IA_1/IA_2/IC$	ATPase	GCGTTGACCAAGTCCGCCGA GCAAATTCGCACCGCGGAGC	451-470 944-925	0.25μM	66	494
PAMp-3 PAMp-4	IA_2/IB	*sodA*	CGGAACCATCAACAAACTCGAA GAAGAACTCGTCAATCGCAGCA	168-189 312-291	0.6μM	62	145
PAMp-5 PAMp-6	IC	Toxin, Fic family	AGGGCGAGGTCCTCTTCTACCAGCG ACCCTCCAACTGCAACTCTCCGCCT	17-41 321-297	0.1μM	66	305
PAMp-7 PAMp-8	II	*atpD*	TCCATCTGGCCGAATACCAGG TCTTAACGCCGATCCCTCCAT	339-360 689-669	0.15μM	66	351
PAMp-9 PAMp-10	III	*recA*	GCGCCCTCAAGTTCTACTCA CGGATTTGGTGATAATGCCA	641-660 865-846	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)

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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

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Multiplex Amplification Profiles