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Comparison of the identification of *Acinetobacter* spp. with API20NE and 16S rRNA gene sequencing techniques

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Acinetobacter species are aerobic, encapsulated, oxidase-negative, non-motile, non-fermentative Gram-negative coccobacilli. Until recently, the genus *Acinetobacter* contained the single species *A. calcoaceticus*, which was subdivided into two subspecies or biovars (*A. calcoaceticus* subspecies *anitratus* and *A. calcoaceticus* subspecies *Iwoffii*). In 1986, the taxonomy of the genus *Acinetobacter* was altered extensively by Bouvet and Grimont,[1] who outlined 12 different species by DNA-DNA hybridisation, including the named species *A. baumannii, A. calcoaceticus, A. haemolyticus, A. johnsonii, A. junii,* and *A. Iwoffii*, and six unnamed genomic species. More recently, 16S rRNA gene sequence analysis has also revealed that *Acinetobacter* spp. represent a well-defined genus;[2] however, species delineation has been more problematic and although a total of 24 genomic species have so far been recognised, only nine have been provided with valid species names.[3] At present, there are 23 formally described species with standing in the literature, which include *A. baumannii, A. baylyi, A. beijerinckii, A. bereziniae, A. bouvetii, A. calcoaceticus, A. gerneri, A. guillouiae, A. grimontii, A. gyllenbergii, A. haemolyticus, A. johnsonii, A. junii, A. Iwoffii, A. parvus, A. radioresistens, A. schindleri, A. soli, A. tandoii, A. tjernbergiae, A. towneri, A. ursingii* and *A. venetianus* (www.bacterio.cict.fr/a/acinetobacter.html).

Acinetobacter species are opportunistic pathogens of low virulence. They are widely prevalent in nature, being found on both animate and inanimate objects.[4] Although generally regarded as commensals of the skin and the respiratory and genitourinary tracts,[5] they have been implicated as the cause of serious infectious diseases such as meningitis, pneumonia, tracheobronchitis, endocarditis, wound infection and septicaemia, mostly involving the immunocompromised host.[6] The contribution of *Acinetobacter* to nosocomial infection has been increasing over the past 30 years.[6,7] Several outbreaks of hospital infection have been described, some being due to contamination of hospital equipment and the hands of personnel. Treatment of serious *Acinetobacter* spp. infection is further complicated by the widespread multidrug resistance of the organism.[7]

There has been considerable difficulty in the identification of species within this genus.[8] Molecular methods may be able to assist with the correct identification over phenotypic methods, particularly for the correct naming of species causing clinically significant disease in this patient population. Hence, it is the aim of this study to identify retrospectively *Acinetobacter* organisms originating from blood culture from patients with haematological malignancy.

A total of 55 isolates belonging to the genus *Acinetobacter* were revived from storage at –80°C from the culture repository of the Northern Ireland Public Health Laboratory, Belfast City Hospital. These isolates were all originally from blood culture material from haematology/oncology patients at Belfast City Hospital during the period January 2005 to May 2008. All *Acinetobacter* isolates were cultured on Columbia blood agar (Oxoid, Basingstoke, UK) containing 5% (v/v) defibrinated horse blood (CBA+DHB).

All *Acinetobacter* isolates were examined using API20NE (bioMérieux, France). Identification of these isolates was performed in accordance with the manufacturer's instructions. Substrate assimilations were read after 24 and 48 h. Interpretation of the results was carried out after 48 h using the identification software version 6.0. Isolates were classified into one of the following three groups: (i) identification at species level, (ii) identification at genus level, (iii) no identification (i.e., low discrimination). According to the manufacturer's instructions, identification at the species level was divided into four subgroups: (i) excellent species identification (≥99.9% identification,

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$T \geq 0.75$); (ii) very good species identification (≥99.0% identification, $T \geq 0.5$); (iii) good species identification (≥90.0% identification, $T \geq 0.25$; and (iv) acceptable species identification (≥80.0% identification, $T \geq 0.0$).

All isolates were also identified using the 16S rRNA gene. All DNA isolation procedures were carried out in a Class II biological safety cabinet (MicroFlow) in a room physically separated from that used to set up nucleic acid amplification reaction mixes and also from the 'post-polymerase chain reaction (PCR)' room, in accordance with the Good Molecular Diagnostic Procedures (GMDP) guidelines of Millar *et al.*,[9] in order to minimise contamination and hence the possibility of false-positive results. Genomic DNA was extracted from a single colony using the Roche High Purity PCR template kit (Roche Diagnostics), in accordance with the manufacturer's instructions. All reaction mixes were set up in a PCR hood in a room separate from that used to extract DNA and from the amplification and post-PCR room to minimise contamination. The PCR reaction mixes (50 µL) contained: 10 mmol/L Tris-HCl (pH 8.3), 50 mmol/L KCl, 2.5 mmol/L $MgCl_2$, 200 µmol/L (each) dATP, dCTP, dGTP and dTTP, 1.25 units *Thermus aquaticus* (*Taq*) DNA polymerase (Amplitaq, Perkin Elmer), 0.2 µmol/L (each) of the 16S rRNA primers 8FPL and PSR, and 4 µL DNA template containing approximately 50 ng of DNA per mL of extract. Following a 'hot start' (to avoid non-specific priming prior to initial extension), the reaction mixtures were subjected to the following thermal cycling conditions in a Perkin Elmer 2400 thermocycler: 96°C for 3 min followed by 40 cycles of 96°C for 1 min, 55°C for 1 min, 72°C for 1 min, followed by a final extension at 72°C for 10 min. During each run, molecular grade water (Biowhittaker) instead of DNA was included randomly as a negative control and *Staphylococcus aureus* DNA was included as a positive control. After amplification, portions (15 µL) were removed, electrophoresed (80 V, 45 min) in agarose gels (Gibco, 2% w/v) in TAE buffer (40 mmol/L Tris, 20 mmol/L acetic acid, 1 mmol/L EDTA [pH 8.3]) and stained with ethidium bromide (5 µg/100 mL). Gels were visualised under ultraviolet (UV) illumination with a gel image analysis system (UVP Products) and all images were archived as digital (*.bmp) graphic files.

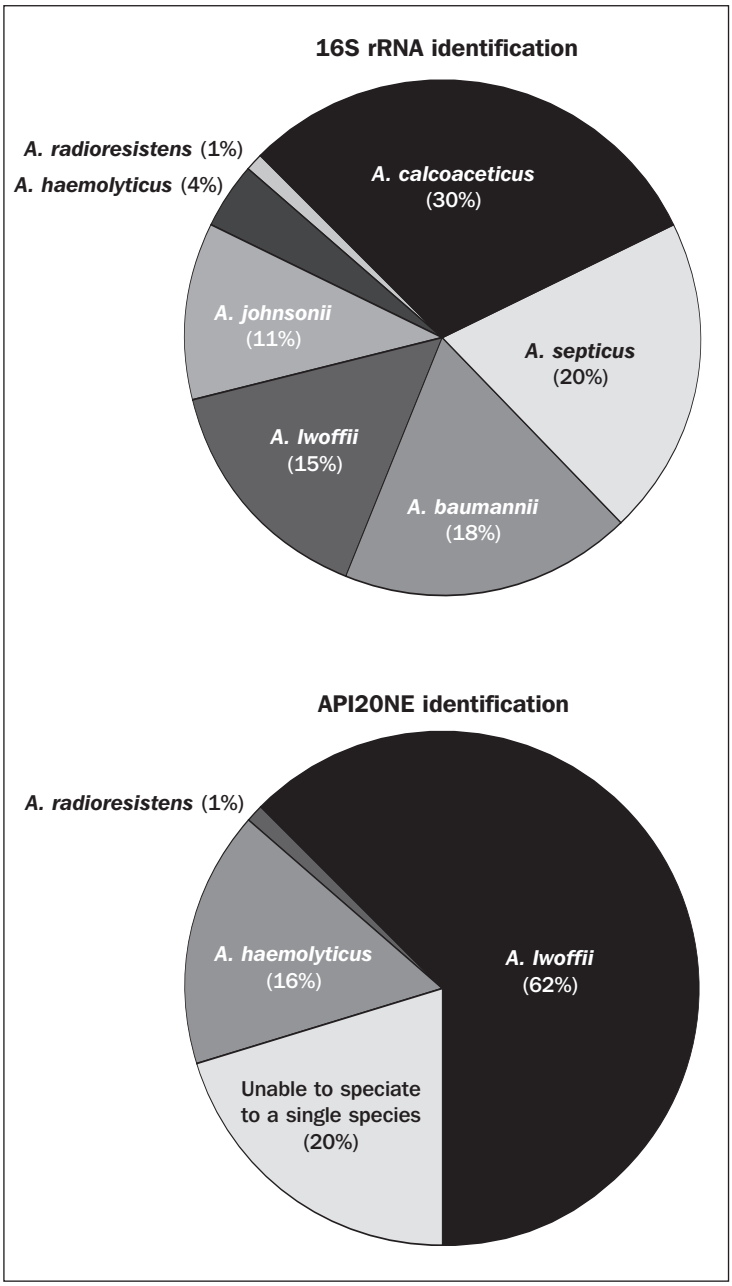

Fig. 1. Comparison of species identified when using 16S rRNA gene sequencing methods and API20NE phenotyping methods.

Amplicons for sequencing were purified with the QIAquick PCR purification kit (Qiagen) following the manufacturer's instructions and these were then eluted in Tris-HCl (10 mmol/L, pH 8.5) before sequencing. Sequences of the partial regions of all five gene loci were determined using the BigDye Terminator cycle sequencing kit and ABI 3100 genetic analyser. The sequences obtained were compared with those stored in the GenBank data system with BLAST alignment software (www.blast.genome.ad.jp/).

The 16S rRNA gene sequencing was able to assign a species designation to each of the 55 isolates examined, from which seven species were identified (Table 1, Fig. 1). Partial 16S rRNA gene sequences have now been deposited in GenBank for all species identified, with accession numbers FJ263916–FJ263931. API identification was able to assign a species in 44 isolates, where 11 isolates gave two species possibilities (Table 1). In those species that were identified phenotypically, three species were noted (Fig. 1). There was a marked difference between the species identified by molecular and phenotypic methods, where only 12 out of the 55 isolate (22%) results concurred between molecular and API methods.

Previous findings indicate that *Acinetobacter* is a relatively uncommon causal agent of bacteraemia in patients with a haematological malignancy or in oncology patients. Jugo *et al.*,[10] in a previous study at the same hospital in which the current study was performed, showed that the rate of positive blood cultures due to *Acinetobacter* was 3.3% and 2.3% for haematology and oncology patients, respectively.

When using the API20NE scheme, the present results showed that an incorrect result was reported on 78% of isolates examined, when considering the 16S rRNA gene sequencing method as the gold standard. This may be due to

Table 1. Comparison of 16S rRNA gene sequencing versus API20NE identification of *Acinetobacter* spp.

Sample	Molecular identification	Size (bp)	Homology (%)	API profile	API identification	% identification
AB1	*Acinetobacter baumannii*	1005	100	0004042	*Acinetobacter lwoffii*	99.6
AB2	*Acinetobacter johnsonii*	1336	99	0004042	*Acinetobacter lwoffii*	99.6
AB3	*Acinetobacter johnsonii*	1329	98	0004042	*Acinetobacter lwoffii*	99.6
AB4	*Acinetobacter calcoaceticus*	987	100	0004042	*Acinetobacter lwoffii*	99.6
AB5	*Acinetobacter baumannii*	1328	100	0004042	*Acinetobacter lwoffii*	99.6
AB6	*Acinetobacter lwoffii*	1002	100	0000010	*Acinetobacter lwoffii*	91.7
AB7	*Acinetobacter septicus*	991	99	0000071	*Acinetobacter junii/johnsonii*	63.4
AB8	*Acinetobacter lwoffii*	1004	100	0000010	*Acinetobacter lwoffii*	91.7
AB9	*Acinetobacter septicus*	1332	100	0004042	*Acinetobacter lwoffii*	99.6
AB10	*Acinetobacter septicus*	1331	99	0000071	*Acinetobacter junii/johnsonii*	63.4
AB11	*Acinetobacter calcoaceticus*	1000	99	0041073	*Acinetobacterbaumannii/calcoaceticus*	99.9
AB14	*Acinetobacter septicus*	1334	99	0010053	*Acinetobacter haemolyticus*	95.2
AB15	*Acinetobacter calcoaceticus*	1332	100	0004042	*Acinetobacter lwoffii*	99.6
AB16	*Acinetobacter johnsonii*	1333	99	1010053	*Acinetobacter haemolyticus*	92.2
AB17	*Acinetobacter calcoaceticus*	1301	99	0000010	*Acinetobacter haemolyticus*	91.7
AB18	*Acinetobacter haemolyticus*	1328	100	0004042	*Acinetobacter lwoffii*	99.6
AB21	*Acinetobacter calcoaceticus*	1333	99	0004042	*Acinetobacter lwoffii*	99.6
AB22	*Acinetobacter baumannii*	1328	99	0001073	*Acinetobacterbaumannii/calcoaceticus*	99.9
AB23	*Acinetobacter calcoaceticus*	1321	100	0004042	*Acinetobacter lwoffii*	99.6
AB24	*Acinetobacter baumannii*	1321	99	0000010	*Acinetobacter lwoffii*	91.7
AB25	*Acinetobacter calcoaceticus*	1332	100	0005042	*Acinetobacter lwoffii*	94.9
AB26	*Acinetobacter calcoaceticus*	1332	100	0004042	*Acinetobacter lwoffii*	99.6
AB27	*Acinetobacter calcoaceticus*	1328	99	0010051	*Acinetobacter haemolyticus*	99.9
AB28	*Acinetobacter johnsonii*	1332	99	0000010	*Acinetobacter lwoffii*	91.7
AB29	*Acinetobacter calcoaceticus*	1003	100	0004042	*Acinetobacter lwoffii*	99.6
AB30	*Acinetobacter calcoaceticus*	1321	100	0204042	*Acinetobacter lwoffii*	99.6
AB31	*Acinetobacter baumannii*	1330	100	0005042	*Acinetobacter lwoffii*	94.9
AB32	*Acinetobacter baumannii*	1320	100	0004042	*Acinetobacter lwoffii*	99.6
AB34	*Acinetobacter baumannii*	1320	99	0000010	*Acinetobacter lwoffii*	91.7
AB35	*Acinetobacter johnsonii*	1332	99	1010053	*Acinetobacter haemolyticus*	92.2
AB36	*Acinetobacter calcoaceticus*	1328	100	0004042	*Acinetobacter lwoffii*	99.6
AB37	*Acinetobacter johnsonii*	984	99	0000071	*Acinetobacter junii/johnsonii*	63.4
AB38	*Acinetobacter septicus*	1307	100	0000001	*Acinetobacter lwoffii*	47.3
AB39	*Acinetobacter calcoaceticus*	1327	99	0001073	*Acinetobacter baumannii/calcoaceticus*	99.9
AB40	*Acinetobacter lwoffii*	1320	99	0004042	*Acinetobacter lwoffii*	99.6
AB41	*Acinetobacter septicus*	1319	99	0000071	*Acinetobacter junii/johnsonii*	63.4
AB42	*Acinetobacter septicus*	1322	99	0000072	*Acinetobacter lwoffii*	55.4
AB43	*Acinetobacter septicus*	1004	99	0000031	*Acinetobacter junii/johnsonii*	49.1
AB44	*Acinetobacter septicus*	1006	99	0000040	*Acinetobacter lwoffii*	82.7
AB45	*Acinetobacter septicus*	1333	99	0040071	*Acinetobacter baumannii/calcoaceticus*	71.7
AB46	*Acinetobacter lwoffii*	1332	99	1000071	*Acinetobacter junii/johnsonii*	21.3
AB47	*Acinetobacter lwoffii*	1332	99	0000070	*Acinetobacter lwoffii*	78.4
AB48	*Acinetobacter septicus*	594	99	0000071	*Acinetobacter junii/johnsonii*	63.4

Sample	Molecular identification	Size (bp)	Homology (%)	API profile	API identification	% identification
AB49	*Acinetobacter lwoffii*	1332	99	0000070	*Acinetobacter lwoffii*	78.4
AB50	*Acinetobacter radioresistens*	1332	99	0000032	*Acinetobacter radioresistens*	69.7
AB51	*Acinetobacter baumannii*	1332	100	0004042	*Acinetobacter lwoffii*	99.6
AB52	*Acinetobacter lwoffii*	1321	100	0004042	*Acinetobacter lwoffii*	99.6
AB53	*Acinetobacter baumannii*	1320	1320	0004042	*Acinetobacter lwoffii*	99.6
AB54	*Acinetobacter baumannii*	1321	99	0000010	*Acinetobacter lwoffii*	91.7
AB55	*Acinetobacter calcoaceticus*	1321	100	0004042	*Acinetobacter lwoffii*	99.6
AB56	*Acinetobacter haemolyticus*	1333	99	1010053	*Acinetobacter haemolyticus*	92.2
AB57	*Acinetobacter calcoaceticus*	1332	100	0004042	*Acinetobacter lwoffii*	99.6
AB58	*Acinetobacter lwoffii*	1333	99	1010053	*Acinetobacter haemolyticus*	92.2
AB59	*Acinetobacter calcoaceticus*	1301	99	0000010	*Acinetobacter haemolyticus*	91.7
AB60	*Acinetobacter calcoaceticus*	1334	99	0010053	*Acinetobacter haemolyticus*	95.2

a lack of phenotypic profiles in the databases which cover all 23 species of *Acinetobacter*, which is in contrast to the relative availability of 16S rRNA gene sequences freely available in GenBank for all 23 named species of *Acinetobacter*. Interestingly, the description of *A. septicus* as a the second most common species of *Acinetobacter* to be identified from blood culture from haematology patients is new, as this species has not been reported previously as a causal agent of bacteraemia in this patient population.

Full and partial 16S rRNA gene sequencing methods have now emerged as valuable tools for identifying phenotypically anomalous isolates. The findings of Bosshard *et al*.[11] have shown that 16S rRNA gene sequencing is a more accurate technique for the identification of Gram-negative non-fermenters than is the API20NE system. The results of the current study concur with these findings. Most laboratories will report the isolation of *Acinetobacter* as *A. calcoaceticus–A baumannii* complex (Abc), which is sufficient to direct clinical decision-making. However, specific species are implicated in disease and should not be considered contaminants, especially in the setting of repeatedly positive blood cultures.[4]

Correct and reliable identification, however, may be important with such organisms, particularly if these environmental organisms are responsible for serious infections associated with immunocompromised patients. As these organisms are likely to be clinically significant in such circumstances, we recommend that molecular identification methods be used where definitive identification is required. When the use of molecular identification methods is justified, employment of partial 16S rDNA PCR and sequencing provides a valuable and reliable method for the identification of *Acinetobacter* spp. □

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