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gyrB Multiplex PCR To Differentiate between *Acinetobacter calcoaceticus* and *Acinetobacter* Genomic Species 3▽

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A previously established multiplex PCR that identifies to the species level *Acinetobacter baumannii* and *Acinetobacter* genomic species 13TU (GS13TU) was expanded to include *Acinetobacter calcoaceticus* and *Acinetobacter* genomic species 3.

Identification of *Acinetobacter* isolates to the species level is often difficult, especially in routine diagnostic laboratories (1). The clinically relevant species *Acinetobacter baumannii* and *Acinetobacter* genomic species 3 and 13TU (GS3 and GS13TU) are often grouped together alongside the environmental *A. calcoaceticus* species as the *Acinetobacter calcoaceticus-A. baumannii* complex (Acb complex) because they are genetically closely related and phenotypically very difficult to differentiate from each other (7). However, there are considerable epidemiological and clinically relevant differences among these species. *A. calcoaceticus* is an environmental organism that, to our knowledge, has never been involved in serious human disease, and therefore it should not be misidentified as *A. baumannii*. The natural habitats of *A. baumannii* and GS13TU are unknown, as are the differences in their epidemic behaviors, resistance mechanisms, and pathogenicities. GS3 can be found regularly on human skin, as well as in aquatic environments (5). GS3 has also been implicated in nosocomial infections, but its tendency for epidemic spread and resistance development is far less pronounced than that of *A. baumannii* (5, 9). For epidemiological and clinical purposes, it is therefore highly desirable to differentiate among these species correctly.

Manual and semiautomated commercial identification systems, e.g., API 20NE, Vitek 2, Phoenix, and MicroScan WalkAway, do not differentiate among these species, resulting in misidentification of up to 25% of *Acinetobacter* isolates belonging to the Acb complex as *A. baumannii* (14). Additionally, there is no recognized biochemical method to distinguish reliably between *A. calcoaceticus* and GS3 (3, 7). Molecular methods for species identification, such as DNA-DNA hybridization and amplified rRNA gene restriction analysis (ARDRA), are labor-intensive, difficult to interpret, and rarely used routinely (2, 13), while tRNA spacer fingerprinting does not differentiate between *A. calcoaceticus* and GS3 and between *A. baumannii* and GS13TU (6). More recently, sequencing of the *rpoB* gene, its flanking spacer regions, and of the 16S-23S rRNA gene spacer region has been proposed for identification of *Acinetobacter* isolates to the species level (4, 10), but it is unlikely that these sequencing techniques will be used routinely, except in a few specialized reference laboratories. Multiplex PCR based on species-specific *gyrB* primers is a simple, specific, and rapid method to reliably identify *A. baumannii* and GS13TU (8). This study expanded the *gyrB* multiplex to enable the identification of *A. calcoaceticus* and GS3.

A total of 146 clinical, type, and reference strains, including 23 *A. calcoaceticus* strains, 36 GS3 strains, 21 *A. baumannii* strains, and 29 GS13TU strains obtained from our own clinical culture collection (12, 14), were used in this study. Clinical isolates of the species *A. beijerinckii* (1), *A. bereziniae* (1), *A. guillouiae* (2), *A. haemolyticus* (4), *A. johnsonii* (1), *A. junii* (3), *A. lwoffii* (2), *A. radioresistens* (2), *A. schindleri* (1), *A. ursingii* (3), *A. venetianus* (1), and the unnamed genomic species 6 (1), 14 (4), and 15TU (1) were also included (11). All clinical strains had been previously identified to the species level by

TABLE 1. Primers used in this study

Primer	Sequence (5′–3′)	Species	Reference
gyrB-2	CTTACGACGCGTCATTTCAC	*A. calcoaceticus* and GS3 reverse sequencing primer	This study
D14	GACAACAGTTATAAGGTTTCAGGTG	*A. calcoaceticus*	This study
D19	CCGCTATCTGTATCCGCAGTA	*A. calcoaceticus*	This study
D16	GATAACAGCTATAAAGTTTCAGGTGGT	GS3	This study
D8	CAAAAACGTACAGTTGTACCACTGC	GS3	This study
Sp2F	GTTCCTGATCCGAAATTCTCG	*A. baumannii*	8
Sp4F	CACGCCGTAAGAGTGCATTA	*A. baumannii* and GS13TU	8
Sp4R	AACGGAGCTTGTCAGGGTTA	*A. baumannii* and GS13TU	8

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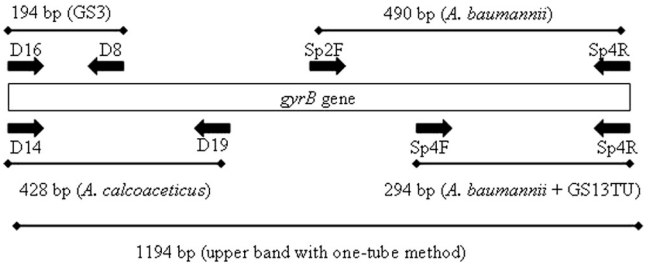

FIG. 1. Schematic diagram showing the annealing positions, species specificities, and expected sizes of PCR products by using primer pairs shown in Table 1.

using ARDRA as well as the simplified phenotypic identification scheme for *Acinetobacter* species devised by Bouvet and Grimont (3, 13). In addition, the following type strains were used: *A. baumannii* ATCC 19606^T, *A. bereziniae* ATCC 17924^T, *A. calcoaceticus* ATCC 23055^T, *A. johnsonii* ATCC 17909^T, *A. junii* ATCC 17908^T, *A. lwoffii* ATCC 13509^T, *A. radioresistens* SEIP 12.81, GS3 ATCC 19004^T, GS6 ATCC 17979^T, and GS9 ATCC 9957^T.

Template DNA for PCR was isolated by using the DNeasy kit (Qiagen, Hilden, Germany) or a 1-μl loopful of a colony from an agar plate was suspended in 100 μl PCR-grade water, boiled for 10 min, snap-cooled, and briefly centrifuged. The *gyrB* gene was amplified and sequenced using primer pair D14/gyrB-2 from nonduplicate *A. calcoaceticus* (n = 21) and GS3 (n = 24) isolates (Table 1). *gyrB* sequences were aligned and compared to those previously obtained for *A. baumannii* and GS13TU (8). Twenty-two primers were identified and evaluated for species specificity using the established multiplex PCR parameters (8). One primer pair each for *A. calcoaceticus* (D14/D19) and GS3 (D16/D8) was chosen and tested against the test organisms as a multiplex PCR using *Taq* PCR master mix (Qiagen) with a final concentration of 0.2 μM for each primer (Table 1). Using these four primers, *A. calcoaceticus* amplified a single 428-bp amplicon and GS3 amplified a single 194-bp amplicon (Fig. 1 and 2a). No PCR products were amplified using these primers in all other species tested.

Primers D14, D19, D16, and D8 were also tested as a multiplex in the presence of established primers Sp2F, Sp4F, and Sp4R at a final concentration of 0.2 μM for each primer (Table 1 and Fig. 2b). With this multiplex, an additional 1,194-bp PCR product was amplified with some, but not all, GS13TU, GS3, and *A. calcoaceticus* isolates (Fig. 1 and Fig. 2b, lanes 4 and 5). The 1,194-bp amplicon is amplified only when the seven primers are used as a multiplex. There is no 1,194-bp amplicon with the D14, D19, D16, and D8 multiplex. The other *Acinetobacter* species tested (*A. beijerinckii*, *A. bereziniae*, *A. guillouiae*, *A. haemolyticus*, *A. johnsonii*, *A. junii*, *A. lwoffii*, *A. radioresistens*, *A. schindleri*, *A. ursingii*, *A. venetianus*, and *Acinetobacter* genomic species 6, 9, 14, and 15TU) did not amplify a PCR product.

The new *gyrB* multiplex PCR can be used as a stand-alone multiplex PCR to identify *A. calcoaceticus* and GS3. Alternatively, it can be performed in the presence of the previously established primers in a single PCR tube to both identify and differentiate the 4 species of the Acb complex. The method is robust, cheaper than sequencing, and reproducible, it can yield

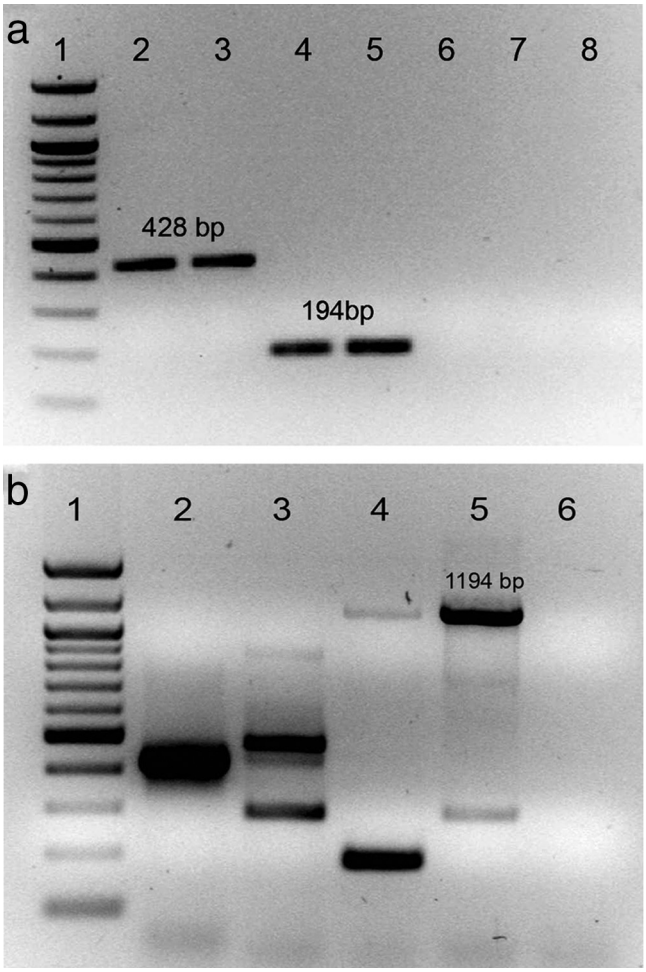

FIG. 2. (a) Example of an agarose gel showing *Acinetobacter* isolates for which the species were determined by multiplex PCR using *gyrB*-directed primers D14, D19, D16, and D8. Lanes: 1, 100-bp marker; 2 and 3, *A. calcoaceticus*; 4 and 5, GS3; 6, *A. baumannii*; 7, GS13TU; 8, pooled DNA from *A. lwoffii*, *A. junii*, *A. haemolyticus*, and *A. johnsonii*. (b) Example of an agarose gel showing *Acinetobacter* isolates for which the species were determined by PCR using *gyrB*-directed multiplex primers (D14, D19, D16, D8, Sp2F, Sp4F, and Sp4R). Lanes: 1, 100-bp marker; 2, *A. calcoaceticus*; 3, *A. baumannii*; 4, GS3; 5, GS13TU; 6, pooled DNA from *A. lwoffii*, *A. junii*, *A. haemolyticus*, and *A. johnsonii*. In this gel, GS3 and GS13TU amplify an additional 1,194-bp product.

a result in <2.5 h, and it enables the reliable identification of the clinically most relevant *Acinetobacter* species. Its simplicity means that it can be employed readily in most laboratories, where it should contribute to a better understanding of the epidemiology and clinical significance of the most important *Acinetobacter* species.

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