

# Multiplex *gyrB* PCR Assay for Identification of *Acinetobacter baumannii* Is Validated by Whole Genome Sequence-Based Assays

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## Highlights of the Study

- The multiplex *gyrB* PCR assay (GBA) is simple for diagnosis of *Acinetobacter baumannii*.
- GBA has not been validated against a gold standard – DNA-DNA hybridization or its sequence-based substitutes (SBS).
- We compared GBA with SBS – a *k*-mer-based search of sequence reads using the Kraken 2 program, and average nucleotide identity – with a complete concordance of the results.

## Keywords

*Acinetobacter baumannii* · *gyrB* PCR · Whole-genome sequencing · Kraken 2 program · Average nucleotide identity · DNA-DNA hybridization

## Abstract

**Objective:** A multiplex *gyrB* PCR assay has been used to diagnose *Acinetobacter baumannii*. However, this assay has not been validated against the gold standard DNA-DNA hybridization assay, which is a laborious method. DNA-DNA hybridization assay is now replaced by whole genome sequence (WGS)-based methods. Two such methods are a *k*-mer-based search of sequence reads using the Kraken 2 program and average nucleotide identity (ANI). The objective was to validate the *gyrB* PCR assay with WGS-based methods. **Subjects and Methods:** We cultured 270 sequential *A. baumannii* isolates from the rectal swabs of 32 adult patients. The identity of the isolates was determined by *gyrB* PCR. The sequences of 269 isolates were determined by Illumina sequencing and the taxonomy was inferred by the

Kraken 2 program and ANI. **Results:** All the 269 isolates were confirmed as *A. baumannii* by Kraken 2 and ANI. **Conclusion:** The *gyrB* PCR assay is now validated for easy identification of *A. baumannii* in comparison with gold standard WGS-based assays.

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## Introduction

*Acinetobacter baumannii* causes ventilator-associated pneumonia, blood-stream infection, urinary tract infection, meningitis, wound infection, and skin and soft tissue infections, mostly in patients admitted to the intensive care unit [1, 2]. *A. baumannii* is a member of the *Acinetobacter calcoaceticus*-*Acinetobacter baumannii* (ACB) complex, which consists of six species: *A. calcoaceticus*, *A.*

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*baumannii*, *A. nosocomialis*, *A. pittii*, *A. seifertii*, and *A. dijkshoorniae* [3]. Except *A. calcoaceticus*, all other species are associated with human disease [3]. There are differences in the epidemiology, antimicrobial resistance, and clinical outcomes of infections due to the different species of the ACB complex. *A. baumannii* causes the majority of infections [4, 5]. Therefore, the correct identification of *A. baumannii* is critical. However, as all members of the ACB complex are closely related, their species-level differentiation is not possible by phenotypic tests [6]. Various automated systems are used for species-level identification in clinical microbiology laboratories (reviewed in reference [6]). These automated systems – Vitek 2, Vitek MS, BD Phoenix, Biolog, Microscan Walkaway, and Accelerate Pheno – have weaknesses in the accurate identification of *A. baumannii*. Matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF) from different studies showed a 100% sensitivity, a 91.7–99% specificity, and a 70–100% concordance with other methods for identification of *A. baumannii* [6].

Several nucleic acid sequence-based tests have been developed and evaluated for the identification of *Acinetobacter* spp. (reviewed in [6]). 16S rRNA sequencing is effective for identification at the genus level, and *rpoB* sequencing is appropriate for species differentiation. Amplification of 16S rRNA, 16S–23S ITS and *recA* followed by restriction analysis can be used for the identification of *Acinetobacter* spp. But these methods have not been thoroughly evaluated. The *bla*OXA-51 like gene-based PCR assay detects *A. baumannii* isolates [7], but the occurrence of this gene in a plasmid in some *A. baumannii* isolates [8] poses the potential risk of transmission of the plasmid to non-*Acinetobacter* spp. bacteria, resulting in a false-positive identification.

A multiplex PCR based on *gyrB* for differentiation of *A. baumannii* and *A. nosocomialis* was developed. As there are sequence differences in the *gyrB* gene among different species of *Acinetobacter*, the design of *gyrB*-specific primers has allowed differentiation of species in the *gyrB* PCR assay [9, 10]. Lee et al. [11] analyzed 495 clinical isolates of *Acinetobacter* by multiplex *gyrB* PCR and Vitek 2. The accuracy rates were found to be 90.5% for *gyrB* PCR and 76.6% for Vitek 2. In comparison with additional assays such as *rpoB* and 16S rRNA sequencing, *gyrB* PCR showed a 100% concordance in identifying species within the ACB complex. In the development of *gyrB* multiplex PCR for the detection of *A. baumannii*, species identified by rDNA restriction analysis and a phenotypic method of Bouvet and Grimont were used as the gold standard for comparison [9]. The multiplex *gyrB* PCR assay seems to

be a simple assay for the identification of *A. baumannii*. However, the performance of this assay has never been compared against a gold standard method. The DNA-DNA hybridization (DDH) has been considered as the gold standard for bacterial species identification. But it is labor-intensive and error-prone. However, as the whole-genome sequencing of bacteria is available, numerous overall genome relatedness indices (OGRI) were developed to replace DDH [12]. Average nucleotide identity (ANI) is an example of OGRI and is considered as the gold standard for species differentiation [12]. Generally, ANI values of 95–96% are accepted for species delineation, corresponding to the traditional 70% DDH threshold [12]. Another method comparable to ANI is Kraken 2 program, a *k*-mer-based approach which provides a fast taxonomic classification from the sequence data [13].

We conducted a study on the sequential rectal colonization of adult patients with *A. baumannii* in the Intensive Care Unit of Mubarak Al Kabeer Hospital, Kuwait, the partial results of which have been published [14, 15]. We utilized this opportunity to evaluate the multiplex *gyrB* PCR assay with the whole-genome sequence-based Kraken 2 taxonomy for the identification of *A. baumannii*, and ANI.

## Patients and Methods

### Patients

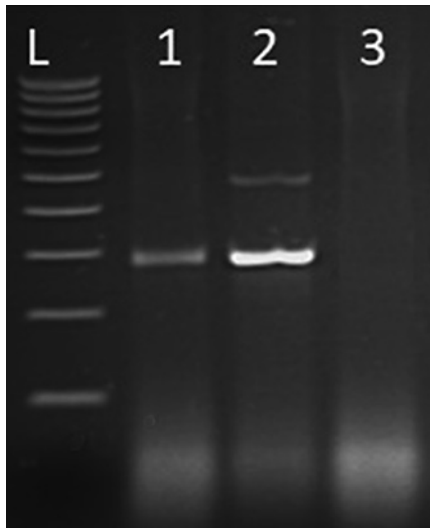
The relevant details have been published previously [14, 15]. Briefly, the study consisted of 32 adult patients. Their median age was 65 years, with an interquartile range of 69.75–50.25 years. The male to female ratio was 1:1. These patients were colonized long-term in the rectum by *A. baumannii* after hospital admission (positive cultures on  $\geq 5$  consecutive samplings). Rectal swabs were collected on the day of admission, the third day after admission, and then twice weekly until the patient was discharged or dead. The duration of follow-up of patients for sequential rectal specimens varied from 14 to 343 days. The period of study was from March 2015 to June 2016. The number of sequential isolates per patient varied from 5 to 16, and the isolates were multidrug resistant.

### Identification of Bacteria

Rectal swabs were initially enriched in an acetate-containing broth and then subcultured on to *Acinetobacter* CHROMagar (CHROMagar). Different morphotypes of typical red colonies were screened by API 20NE and confirmed as *A. baumannii* by multiplex *gyrB* PCR using three primers [9]. The appearance of two bands – 294-bp and 490-bp – indicated *A. baumannii* and a single band of 294-bp suggested *A. nosocomialis*. We included *A. nosocomialis* and *A. pittii* as controls in the assay.

### Genome Sequencing

Genomic DNA from *A. baumannii* isolates extracted using the DNeasy blood and tissue extraction kit (Qiagen) was shipped



**Fig. 1.** Agarose gel electrophoresis of PCR products from *gyrB* PCR assay. Ethidium bromide-stained gel was photographed under UV light. Lane L: 100 bp-ladder (Invitrogen), lane 1: *A. nosocomialis*, lane 2: *A. baumannii*, and lane 3: *A. pittii*.

to the Microbiology Diagnostic Unit Public Health Laboratory, Peter Doherty Institute for Infection and Immunity, The University of Melbourne, Australia, where whole genome sequencing was done. Sequencing libraries were prepared using the Nextera XT DNA sample preparation kit (Illumina), and the sequence read data were produced on the Illumina NextSeq instrument (paired end, 150 base reads). The Illumina sequence reads were de novo assembled using Spades v3.9 [16]. The resulting draft genome sequences were annotated using Prokka v1.12b [17]. Taxonomic classification of isolates was done using Kraken 2 [13]. Sequencing reads were classified using the PlusPF database and isolate classification was determined from the top scoring species level taxon.

ANI was also determined between each isolate and the reference *A. baumannii* type strain, ATCC 19606 (assembly accession: GCA\_009035845.1). An ANI value of  $\geq 95\%$  between the test organism and the reference organism indicated that both organisms belonged to the same species [18].

#### Data Availability

Raw reads for all isolates sequenced in this study have been deposited in the National Center for Biotechnology Information (NCBI) under Bioproject accession number PRJNA791537.

## Results

There were a total of 270 sequential *A. baumannii* isolates cultured from the rectal swabs of 32 patients. The identity was confirmed by the *gyrB* PCR assay. *A. baumannii* yielded two bands of 490-bp and 294-bp. The

control isolates of *A. nosocomialis* (previously genospecies 13 TU) and *A. pittii* (previously genospecies 3) [10] yielded a single band of 294-bp and no band, respectively (Fig. 1).

Sequence data were produced for 269 isolates; the E5 isolate failed to revive after storage and was not sequenced. The sequence data sets ranged from 2,756,308 reads to 6,071,244 reads per isolate. Read depth coverage was estimated to range between 101- and 265-fold coverage; this, in conjunction with an average read quality score ranging from 32.8 to 33.9 and average trimmed read length ranging from 146 to 149, indicated that data quality was consistent and suitable for analysis. All 269 isolates were identified as *A. baumannii* using Kraken 2 (highest average match of about 70%) with a 100% concordance with *gyrB* PCR assay. ANI of each of 269 isolates with the reference *A. baumannii* isolate was  $>97\%$  confirming the result obtained with Kraken 2.

## Discussion

Even though *A. baumannii* is a nosocomial pathogen causing diseases of many systems, it is not known to cause disease in the intestinal tract [1, 2]. However, it colonizes the intestinal tract [19, 20]. Our isolates originated in the intestinal tracts of a group of patients who were sampled multiple times over a 15-month period. Two isolates each from 8 patients and three isolates each from 2 patients were identical by pulsed field-gel electrophoresis as analyzed by bionumerics [14], thus constituting a total of 22 identical isolates. Thus, of the 269 isolates subjected to the *gyrB* PCR assay, 247 isolates were non-identical from individual patients. The previously published antibiogram data [15] showed that the isolates were sufficiently heterogeneous for comparison among the diagnostic methods.

Multiplex *gyrB* PCR assay is a relatively simple and straightforward assay for the identification of *A. baumannii*. We have confirmed that this assay is specific for detecting *A. baumannii* in comparison with WGS-based gold standard assays.

## Conclusion

Multiplex *gyrB* PCR assay is found to be accurate for the diagnosis of *A. baumannii* in comparison with WGS-based gold standard assays.

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## Statement of Ethics

This study was carried out in accordance with the Declaration of Helsinki for experiments involving human subjects. Informed written consent was obtained from patient relatives who were caregivers. Approval for the study was granted by the Ethics Review Committee of the Health Sciences Center, Kuwait University, and the Ministry of Health, Kuwait (approval no. 112).

## Conflict of Interest Statement

The authors have no conflicts of interest to declare.

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## Author Contributions

M. John Albert: study design, data analysis and interpretation, and drafting of the manuscript; Ghayda Al Hashem: data collection; Vincent O. Rotimi: study design. All the authors approved the final version of the manuscript before submission.

## Data Availability Statement

Raw sequence data can be accessed from Bioproject Number PRJNA791537 at the National Center for Biotechnology Information (NCBI) website (<https://www.ncbi.nlm.nih.gov/>).