ORIGINAL ARTICLE

Identification of *B. pseudomallei* by 16S rDNA Sequencing*

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ABSTRACT

Background. Rapid and accurate identification of *Burkholderia pseudomallei* facilitates the early diagnosis and administration of appropriate treatment for melioidosis. However, current phenotypic methods are often inadequate. Identification system, based on 16S rRNA gene sequence, the MicroSeq® 16S rDNA Bacterial Identification Kit (AB Applied Biosystems, Foster City, CA, USA), has been developed for sequencing partial as well as the entire 16S rRNA gene to confirm suspected strains of *Burkholderia pseudomallei*. As its accompanying database is not exhaustive, a library of 16S rRNA sequences from local *B. pseudomallei* strains was established for inclusion in the database. The objective of the study was to develop a 16S rRNA gene sequence library for the identification of *B. pseudomallei* and use the sequences obtained to analyse the results from a suspected strain of *B. pseudomallei*.

Methods. We used the MicroSeq® Full Gene 16S rDNA Bacteria Identification Kit (AB Applied Biosystems, Foster City, CA, USA) to obtain sequences from 2 clinical isolates of *B. pseudomallei* and an ATCC 11668 strain. Consensus sequences were derived and submitted for incorporation in the Applied Biosystems MicroSeq® Analysis Software (AB Applied Biosystems, Foster City, CA, USA) database. This sequence, when compared with the GenBank database, showed only 1 base difference (99.9%) with the 16S rDNA sequence of a known *B. pseudomallei* strain BPU91839. We recently utilised the MicroSeq® 500 16S rDNA Bacterial Identification Kit (AB Applied Biosystems, Foster City, CA, USA) to identify a clinical isolate of *B. pseudomallei* which was incorrectly identified using an established commercial system, API 20NE. This kit sequences the first 500 base pairs of the 16S ribosomal RNA gene. The obtained sequence was compared with known 16S rDNA bacterial sequences from the GenBank database as well as our library of *B. pseudomallei* 16S rDNA sequences.

Results. Analysis of the first 500 base pairs of the 16S rRNA gene yielded a similarity score of 99% to 100% for at least one sequence of *B. pseudomallei* in GenBank and showed identical DNA sequence alignment with our consensus *B. pseudomallei* sequences.

Conclusion. Conventional phenotypic methods supplemented with commercially available systems may provide ambiguous identification of *B. pseudomallei*. This report demonstrates the utility of 16S rRNA gene sequences analysis for confirming the identity of suspected strains of *B. pseudomallei*.

Keywords: 16S rRNA gene, *Burkholderia pseudomallei*, DNA sequencing, misidentification

INTRODUCTION

Burkholderia pseudomallei (*B. pseudomallei*), a gram-negative bacillus, is the aetiological agent of melioidosis, which is a potentially fatal infectious disease in humans. It is an environmental saprophyte that is found in moist soil and water in parts of Southeast Asia, northern Australia, Central and South America.[1-3] Exposure to *B. pseudomallei* is acquired through inhalation or cutaneous inoculation.[3] Almost any organ may be infected during the course of disease, which may be localised or disseminated. Currently, diagnosis of the disease is through isolation of the organism by culture followed by identification using biochemical tests. This process may take several days. In addition, atypical strains may be misidentified by commercial identification systems.[4]

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The bacterial 16S ribosomal RNA gene is highly conserved in all bacteria of the same genus, yet its variable regions contain signatures unique to a species and provide useful information about the relationships between them.[5] Thus, sequencing the gene has become the gold standard for speciation of unknown or clinically unidentifiable bacteria.[6,7] With the discovery of polymerase chain reaction (PCR) and DNA 16S rRNA sequencing, bacteria that are difficult to identify have also been successfully speciated.[8] Further, this technique also allows the identification using only a small amount of bacterial DNA.

The MicroSeq® Full Gene 16S rDNA Bacterial Identification Kit (AB Applied Biosystems, Foster City, CA, USA) has been developed for sequencing the entire 16S rRNA gene.[9,10] As only 500 basepairs (bp) are required for bacterial identification, the system was further simplified. MicroSeq® 500 16S rDNA Bacterial Identification Kit has been developed for sequencing the first 527 bp of the 16S rRNA gene.[11] To determine the species, this sequence is compared to a reference library of 16S rRNA sequences such as the MicroSeq® Analysis Software (AB Applied Biosystems, Foster City, CA, USA). As the current software did not include sequence data for identifying *B. pseudomallei*, the purpose of this study was to use the MicroSeq® Full Gene 16S rDNA Bacteria Identification Kit to create a reference 16S rRNA gene sequence for local isolates of *B. pseudomallei*. We then used the MicroSeq® 500 16S rDNA Bacterial Identification Kit to confirm the identity of a biochemically atypical strain of *B. pseudomallei* by comparative analysis with the reference sequence.

METHODS

Clinical Isolates and Conventional Identification Methods

B. pseudomallei clinical isolates DB 45770 and DB 51360 were used for the reference sequence. These blood specimens were identified by conventional phenotypic biochemical methods and confirmed by the API 20NE system (bioMerieux Vitek, Hazelton, MO, USA).

Genotypic Identification and 16S rDNA Sequencing

Genomic DNA was extracted from the two clinical isolates and an ATCC 11668 *B. pseudomallei* (China) strain with the DNeasy Tissue™ Kit (Qiagen, Hilden, Germany) by washing a loopful of bacterial cells with 1ml of sterile distilled water. The 16S rRNA gene was amplified from bacterial genomic DNA by PCR with the PCR module of the MicroSeq® Full Gene 16S rDNA Bacteria Identification Kit according to the manufacturer's instructions (AB Applied Biosystems, Foster City, CA, USA). Next, 1ml of the genomic DNA extract was diluted in 49ml of distilled water before adding to 50ml of MicroSeq® 16S rRNA gene PCR master mix. The mixture was amplified using a GeneAmp PCR System 9600 (AB Applied Biosystems, Foster City, CA, USA). The thermal cycling parameters consisted of an initial incubation step at 95°C for 10 min, followed by 30 cycles each of 30s at 95°C, 30s at 60°C and 45s at 72°C and a final extension at 72°C for 10 min. The PCR gene products were purified by using Qiaquick® Gel Extraction Kit (Qiagen, Hilden, Germany). For cycle sequencing reactions preparation, 45ml of purified PCR product was diluted with 60ml of water and 7ml of diluted PCR product was aliquoted into each of the 12 strip tubes containing sequencing brew of 12 16S sequencing primers (5F, 338F, 515F, 776F, 1087F, 1174F, 357R, 531R, 810R, 1104R, 1193R and 1540R). Subsequently, the reactions were cycle sequenced on GeneAmp PCR System 9600 (AB Applied Biosystems, Foster City, CA, USA) using the sequencing module of the same MicroSeq® kit. The parameters used consisted of a 96°C denaturation step for 10s followed by an annealing and extension step from 65°C in which temperature decreased by 1°C after every 6 cycles until it reached 55°C, for a completion of 66 cycles altogether. The DNA sequences were determined by capillary electrophoresis using the ABI PRISM 377 DNA sequencer (AB Applied Biosystems, Foster City, CA, USA). For microbial identification, the DNA sequences obtained from the bacterial isolates were compared to the ATCC strain by multiple sequence alignment using the analysis software, DNAStar, as well as the GenBank database through the NCBI (National Centre for Biotechnology Information server) gapped BLASTN 2.0.5 program and the Sanger Center.

Utility of 16S rDNA Sequencing

A 66-year-old patient presented to another hospital with severe left-sided pneumonia. The blood culture isolate, EB29389, with ambiguous conventional results from a commercial identification system was identified genotypically using the MicroSeq® 500 16S rDNA Bacterial Identification Kit according to the manufacturer's instructions of PCR amplification of the first 527 bp of the 16S rRNA gene. Next, 1ml of a 1:10 dilution of the genomic DNA extract was added to 24ml of distilled water before 25ml of MicroSeq® 500 16S rRNA gene PCR master mix was added into 0.2-ml MicroAmp PCR tubes. The mixture was amplified, purified and cycle sequenced with the same parameters as above, except that for the cycling

Table 1. API 20NE profiles of the reference strains.

Lab. No.	API Profile	%	Identity
DB 45770	5156577	97.6	*B. pseudomallei*
DB 51360	1156577	99.9	*B. pseudomallei*

sequencing reactions preparation, 3ml of purified PCR product was diluted with 4ml of deionised water and 13ml of Forward or Reverse Sequencing Mix was added into the tube. For microbial identification, the DNA sequences obtained from the bacterial isolates were compared with our consensus *B. pseudomallei* sequences as well as the GenBank database through the NCBI (National Center for Biotechnology Information server) gapped BLASTN 2.0.5 program.

RESULTS

Presumptive Identities of Clinical Isolates by Conventional Methods

The two *B. pseudomallei* clinical isolates DB 45770 and DB 51360 were identified to the genus and species level by conventional clinical microbiological methods and the API 20 NE system (Table 1).

16S rRNA Gene Sequence Analysis and Identification of Consensus Sequence

Multiple sequence alignment of the 16S rDNA sequences derived from DB 45770, DB51360 and the ATCC 11668 showed that the sequences were fully conserved. Consensus sequence (1507 bp) of the *B. pseudomallei* 16S rRNA gene was constructed. The closest match in the GenBank database was the sequence from *B. pseudomallei* ascension no. BPU 91839, which differed from our sequences by 1 bp (99.9%). The sequence from *B. pseudomallei* K96243 (Thailand) from the Sanger Center (Contig 133) also differed by 1 bp but at a different position. The consensus sequence was then submitted for incorporation into the MicroSeq® 16S rDNA sequence library with the confirmed identification of *B. pseudomallei*.

Identification of Ambiguous and Suspected Strains of B. Pseudomallei *by Commercial Identification Systems and 16S rRNA Gene Sequence Analysis*

The blood and endotrachael secretion cultures from the 66-year-old patient with severe left-sided pneumonia grew an oxidase-positive, Gram-negative bacillus. *B. pseudomallei* was suspected because of the antibiogram (resistant to aminoglycosides and polymixin B). However, the mucoid colony appearance was atypical and the microorganism was identified as *C. violaceum* (Profile number: 1150514) by the API 20 NE identification system. The first 527 bp of the 16S rRNA gene showed complete sequence alignment with our consensus *B. pseudomallei* sequences. When submitted to the GenBank database, the sequence yielded a similarity score of 100% with *B. pseudomallei* BPU 91839.

DISCUSSION

Melioidosis often presents as acute life-threatening or chronic infections. More recently, *B. pseudomallei* has also been recognised as a potential agent for bioterrorism. Thus, the diagnosis of melioidosis has implications for specific patient management and potentially, public health. Traditional phenotypic methods for identification are time-consuming and may yield ambiguous results that may misidentify *B. pseudomallei*, leading to a wrong diagnosis. Therefore, genotypic methods involving sequencing the conserved and phylogenetically informative 16S rRNA gene could complement any conventional phenotypic test.[9]

One limitation of using this method to identify *B. pseudomallei* is that the 16S rRNA sequence of *B. mallei* is indistinguishable from *B. pseudomallei*. *B. mallei*, which causes glanders in horses, can be transmitted to humans. Unlike melioidosis, glanders in humans is uncommon and occurs primarily in persons in close contact with sick animals or through laboratory-acquired infection. Another potential limitation of commercial identification kits is that the database could be limited due to a lack of uncommon strain characterization and this may require that more of such sequences be included in the genetic databases.

CONCLUSION

In this study, we successfully utilised 2 different kinds of commercial kits, the MicroSeq® Full Gene 16S rDNA Bacteria Identification Kit (AB Applied Biosystems, Foster City, CA, USA) and the MicroSeq® 500 16S rDNA Bacterial Identification Kit (AB Applied Biosystems, Foster City, CA, USA), to genotypically establish the identification of suspected strains of *B. pseudomallei* isolated from clinical specimens. The bacterial DNA sequences of the 16S rRNA gene were assembled with sequence analysis software from the MicroSeq® system and confirmed with public databases, as the MicroSeq® 16S ribosomal DNA sequence library in its original form did not contain any *B. pseudomallei* sequence.

We then affirmed the utility of this sequence data by confirming the identity of an atypical *B. pseudomallei* strain, which could not be identified by biochemical methods. The MicroSeq® 16S rDNA sequence library has been enhanced by the addition of the *B. pseudomallei* consensus sequence and in considering the results, 16S rDNA sequencing may become a reliable tool for identification of atypical *B. pseudomallei* in clinical laboratories in the future.

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