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Ribotyping of *Burkholderia mallei* isolates

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Abstract

In this study, the subspecies differentiation of 25 isolates of *Burkholderia mallei* was attempted based on their ribotype polymorphisms. The isolates were from human and equine infections that occurred at various times around the world. DNA samples from each isolate were digested separately with *Pst*I and *Eco*RI enzymes and probed with an *Escherichia coli*-derived 18-mer rDNA sequence to identify diagnostic fragments. Seventeen distinct ribotypes were identified from the combined data obtained with the two restriction enzymes. The results demonstrate the general utility of ribotyping for the subspecies identification of *B. mallei* isolates.

Keywords: *Burkholderia mallei*; Ribotyping; Genotyping; DNA polymorphisms; Ribosomal; Subspecies identification

1. Introduction

In recent years, the scientific basis for the identification of microorganisms has undergone a shift in emphasis from the traditional reliance on biochemical and microscopic identification of phenotypic characteristics to techniques based on nucleotide sequence heterogeneities [1, for review]. Some of these techniques have been used to distinguish strains at the subspecies level, and thereby provide a sound basis for the epidemiological tracking of the likely source of an outbreak. These approaches typically rely on some variation of a DNA "fingerprint"; a unique or diagnostic hybridization pattern arising from the amplification or probing of repetitive sequences occurring in polymorphic regions of the genome.

Ribotyping is one such fingerprinting approach. Bacterial ribosomal RNA (rRNA) operons comprise a family of highly conserved genes, each of which is flanked by regions of DNA with much greater variability than that encoding the rRNA operons themselves. Restriction fragment length polymorphisms (RFLPs) arising from sequence differences in the flanking restriction sites, or from insertions, deletions or recombinations within the rDNA-containing fragments, can be identified by probing restriction-digested, size-fractionated and immobilized DNA fragments with labeled homologous DNA sequences. An advantage of ribotyping is that it enables genetic analysis of an organism without prior knowledge of its genomic DNA sequence. In addition, it can be a sensitive means to identify genetic heterogeneity in a readily interpretable pattern.

In the present work, the subspecies discrimination of 25 isolates of *Burkholderia mallei* was approached through polymorphisms identified by ribotyping, using *Pst*I and *Eco*RI restriction enzymes. Ribotyping was previously used by others [2–8] to characterize isolates of the related organism *Burkholderia pseudomallei*, the

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causative agent of melioidosis, which is a significant public health problem in Southeast Asia and Northern Australia. A total of at least 22 different ribotypes were described from *B. pseudomallei*.

There are no previously reported *B. mallei* DNA polymorphisms known to us. We believed the previous success with *B. pseudomallei* suggested the utility of ribotyping for subspecies discrimination of *B. mallei*.

B. mallei is a Gram-negative rod-shaped obligate parasite that causes Glanders primarily in equines, but also in humans. Cats, dogs and many other mammals can be infected under experimental conditions, while hamsters [9] and mice [10] are the most common laboratory models with which to study *B. mallei*. Mortality is very high, there is no vaccine, and a chronic form of the disease sometimes develops that can exacerbate into the acute form even after many years [11]. Glanders has disappeared from most regions of the world, leaving only enzootic foci in Asia and eastern Mediterranean countries, and sporadic human cases among those whose occupations involve direct contact with infected equines or work with the organism in laboratories. The organism has received increased attention recently because it was designated by the US Centers for Disease Control and Prevention as a Category B Bioterrorism Agent, (http://www.scchealth.org/docs/doche/bt/cats.html). Also, it has been reported recently that German saboteurs maliciously injected *B. mallei* into animals during World War I [12,13]. Other published reports include the construction of *B. mallei* strains containing multiple antibiotic resistance genes [14], a study of the correlation of antibiotic resistance with infectivity [15] and its alleged intentional release in Afghanistan [16]. These reports suggest the importance of developing a reliable means for the forensic discrimination of various isolates of the organism, which was the objective of this work.

2. Materials and methods

2.1. Sources and growth of bacteria

Table 1 summarizes the available information on the strains used in this study. At the time of publication, arrangements were being made for safe deposit of these isolates with the American Type Culture Collection (www.atcc.org).

2.2. DNA isolation

Isolates were streaked on Luria Broth (LB) plates supplemented with 4% glycerol and grown at 37 °C for 1–2 days. Individual colonies were inoculated into 5 ml LB + 4% glycerol liquid medium and grown at 37 °C for 1–3 days. Suspended cells (5 ml) were centrifuged at 5000*g* for 15 min and the resulting pellet was vigorously resuspended and washed in 4 ml TS buffer (0.05 M NaCl, 0.02 M Tris, pH 8). Vigorous resuspension was apparently critical to obtain digestible DNA and was presumably related to the removal of the polysaccharide capsule. Some isolates did not yield a clear interface between the pellet and the supernatant, which was rectified by increasing the TS volume to 25 ml. Cells were centrifuged and the pellets resuspended as before in 4 ml TS buffer. Following another centrifugation, cells were resuspended in 0.6 ml saline, to which 1.2 ml sucrose-RNase-lysozyme solution was added (a stock solution contained 2.0 ml of 1 mg/ml boiled RNase, 44 mg lysozyme, 8.6 gm RNase-free sucrose, and 19.0 ml TES4 buffer [0.05 M each of NaCl, ethylenediamine tetraacetic acid, and Tris, pH 8]). This suspension was incubated at 37 °C for 15 min, then at 55–60 °C for 3 min. To this solution was added (with gentle swirling) 0.6 ml 3.5% Sarkosyl (Sigma) in TES4, followed by a 20-min incubation at 55–60 °C. Pronase (Sigma) solution was prepared at 9 mg/ml in TES4 buffer and incubated at 37 °C for 60 min (autodigestion). An aliquot (0.25 ml) of this solution was added to the lysate followed by an overnight incubation at 37 °C. Two phenol/chloroform extractions were performed by adding 1 ml water, 2.5 ml water-saturated phenol and 1.25 ml chloroform to the lysate, shaking gently and incubating on ice for at least 30 min prior to centrifugation at 5000*g* at 4 °C for 15 min. Following the second extraction, the aqueous layer was removed and extracted with 1.25 ml chloroform only. Following centrifugation of the chloroform extract, the top (aqueous) layer was removed and 1.5 volumes of ice-cold isopropanol were added. This mixture was inverted gently to precipitate the DNA. Precipitated genomic DNA was removed with a bent glass pipet, washed in ice-cold 100% ethanol, dried briefly, and dissolved in 1 ml of TE buffer (10 mM Tris and 1 mM EDTA, pH 8.0) in a sterile tube. DNA concentrations were estimated based on comparisons with known standards in an agarose gel electrophoresis.

2.3. DNA analysis

DNA was digested with restriction enzymes according to the enzyme suppliers' recommendations. Restriction-digested DNA and ^{32}P-labeled DNA molecular weight standards were size fractionated through a 20 cm long, 0.6% (w/v) agarose gel in 40 mM tris-acetate and 1 mM EDTA (TAE) at 30–60 V for 16–32 h, according to the fragment sizes to be resolved and visualized by autoradiography. Southern transfer of gels to nylon membranes was performed according to Sambrook et al. [17]. Molecular weight standards (1–12 kb ladder from Invitrogen, Carlsbad, CA) and 1.5–48.5 kb Lambda DNA mono cut mix from New England Biolabs (Beverly, MA) were labeled with [^{32}P]ATP. The 1–12 kb ladder standards were