Tropical Biomedicine 28(3): 646–650 (2011)

Reduced susceptibility of Malaysian clinical isolates of *Burkholderia pseudomallei* to ciprofloxacin

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Received 18 February 2011; received in revised form 6 June 2011; accepted 5 July 2011

Abstract. Ciprofloxacin, a quinolone with good intracellular penetration may possibly be used for treatment of melioidosis caused by *Burkholderia pseudomallei*, but problems with resistance may be encountered. Amino acid substitutions in *gyrA/gyrB* have given rise to fluoroquinolone resistance in various microorganisms. Using published primers for *gyrA* and *gyrB*, PCR was performed on 11 isolates of *B. pseudomallei* with varying degrees of sensitivity to ciprofloxacin, followed by DNA sequencing to detect possible mutations. Results showed an absence of any point mutation in either gene. Local isolates have yet to develop full resistance to ciprofloxacin and probably other mechanisms of resistance may have been involved in the decreased sensitivity to ciprofloxacin.

INTRODUCTION

Quinolones are broad-spectrum antibacterial agents, used in both clinical and veterinary medicine. Their extensive use has resulted in the rapid development of bacterial resistance to quinolones. To date, two main mechanisms of quinolone resistance have been established: alterations in the targets of quinolones, and decreased accumulation inside the bacteria due to impermeability of the membrane and/or an overexpression of efflux pump systems. Both of these mechanisms are chromosomally mediated (Ruiz, 2003).

In Gram negative bacteria, the target of quinolones and their fluorinated derivatives is the DNA gyrase, and the function of this enzyme is to catalyze the negative supercoiling of DNA. DNA gyrase is a tetrameric enzyme composed of two A subunits and two B subunits, encoded by *gyrA* and *gyrB* genes respectively (Horowitz & Wang, 1987). Quinolones block the activities of DNA gyrase by stabilizing an enzyme-DNA complex, which also functions as a barrier to the movement of other proteins. Amino acid changes in the DNA gyrase alter the structure of the quinolone binding site near the interface of the enzyme and DNA, and resistance is caused by reduced drug affinity for the modified enzyme-DNA complex (Hooper, 1999). Amino acid substitutions involved in the development of fluoroquinolone resistance have been described for *gyrA/gyrB* in various species of microorganisms (Viktorov *et al.*, 2008).

The use of fluoroquinolones in the treatment of melioidosis has been controversial but ciprofloxacin is useful because of its prolonged post-antibiotic effect and good intracellular penetration (Puthucheary & Vadivelu, 2002). With recent studies showing that the endemic zone of melioidosis has expanded to include the majority of the Indian subcontinent and southern China (Currie *et al.*, 2008), attention is needed to identify antimicrobials useful as prophylactic or therapeutic agents. This study was carried out to determine if

Malaysian clinical isolates of *Burkholderia pseudomallei* have developed resistance to ciprofloxacin due to alterations in *gyrA/gyrB*.

MATERIALS AND METHODS

Bacterial strains

In a previous study from our institution, 184 clinical isolates of *B. pseudomallei* had been tested by the Etest (AB Biodisk, Solna, Sweden) method using Mueller-Hinton agar (Oxoid, Basingstoke, UK) for their susceptibilities to a range of antimicrobials including ciprofloxacin. For ciprofloxacin, the MICs range was found to be 0.012-3 mg/L, with 32 out of 184 isolates (17.4%) showing intermediate resistance (Sam *et al*., 2010). In the absence of data for *B. pseudomallei*, the MIC results were interpreted according to the breakpoints for *Enterobacteriaceae* from the Clinical and Laboratory Standards Institute (CLSI, 2009) with sensitive, $\leq$1; intermediate, 2; and resistant, $\geq$4 in mg/L as breakpoints for ciprofloxacin. As there are no CLSI guidelines on breakpoints for *B. pseudomallei*, it can be argued that these breakpoints for *Enterobacteriaceae* used to interpret resistance for ciprofloxacin can be controversial. Quality control was performed using *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853. We selected 6 of these intermediate resistant isolates which were from blood cultures, based on a range of sensitivities to ciprofloxacin; MICs 2.0 mg/L (n=3), 1.5 mg/L (n=3), and 5 sensitive isolates; MICs 1.0 mg/L (n=4), 0.25 mg/L (n=1), of which 3 were from blood and 2 from pus cultures. *Burkholderia pseudomallei* NCTC 13178 was included as control. *B. pseudomallei* K96243 and *Salmonella* sp. were used as positive controls in the *gyrA* and *gyrB* studies, respectively. Bacterial strains were cultured under aerobic conditions at 37ºC overnight on Luria-Bertani agar (Difco™ LB Agar, Lennox).

DNA extraction and PCR

DNA was extracted by using the boiling method. A loopful of bacterial colonies grown overnight were suspended in 100µl of sterile double distilled water and boiled at 100ºC for 30 minutes. The suspension was then cooled on ice and centrifuged at 13000 rpm to remove cell debris. The supernatant was extracted and kept in -20ºC until use.

The extracted DNA was subjected to amplifications by PCR using published primers specific for quinolone resistance-determining regions of *gyrA* (forward 5'-CTTCCGGATGTCCGCGATGG-3' and reverse 5'-CGACCTCGTTCAGGTTGTGC-3', gene product size 472 bp) (Viktorov *et al*., 2008) and *gyrB* (forward 5'-CAAACTGGCGGACTGTCAGG-3' and reverse 5'-TTCCGGCATCTGACGATAGA-3', gene product size 345 bp) (Ling *et al*., 2003). Amplifications were carried out in a MyCycler™ thermal cycler (Bio-Rad Laboratories, Inc.) using the following protocol: for *gyrA*, an initial denaturation step at 95ºC for 5 min, followed by 35 cycles of denaturation at 95ºC for one min, annealing at 60ºC for 30 sec and extension at 72ºC for 2 min, with a final extension at 72ºC for 5 min.

For *gyrB*, an initial denaturation step at 95ºC for 5 min, followed by 35 cycles of denaturation at 94ºC for one min, annealing at 52ºC for one min and extension at 72ºC for one min, with a final extension at 72ºC for 5 min.

PCR amplicon purification and nucleotide sequencing

The products were separated by agarose gel electrophoresis, stained with ethidium bromide and visualized using a UV transilluminator (Ingenius, Syngene Bio Imaging). The bands containing PCR amplicons were sliced and purified using MEGAquick-spin™ PCR & Agarose Gel DNA Extraction System (Intronbio). The purified products were sent for sequencing (Bioneer Corp). Results were analysed using Geneious Pro 4.7.5 (Biomatters Ltd). The *gyrA* and *gyrB* sequences were aligned with *B. pseudomallei* K96243 (GenBank Accession no. NC_006350) to detect any possible mutations in the genes.

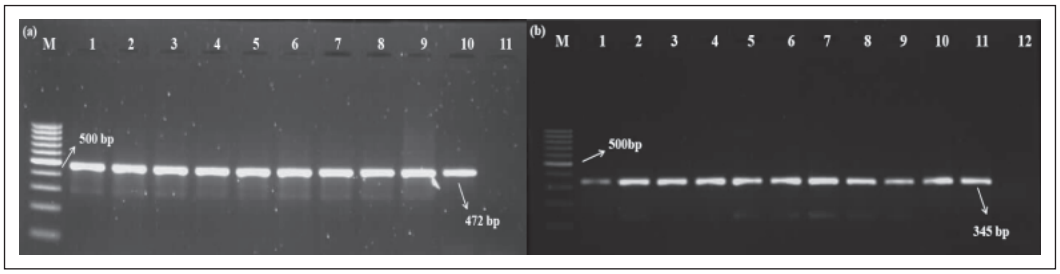

Figure 1. (a) Amplification of *gyrA* gene
Lane M: 100 bp DNA marker, Lanes 1-7 & 9: *B. pseudomallei*, Lane 8: *B. pseudomallei* NCTC 13178, Lane 10: positive control, *B. pseudomallei* K96243, Lane 11: negative control (sterile MiliQ water)
(b) Amplification of *gyrB* gene
Lane M: 100 bp DNA marker, Lanes 1-8 & 10: *B. pseudomallei*, Lane 9: *B. pseudomallei* NCTC 13178, Lane 11: positive control, *Salmonella* sp., Lane 12: negative control (sterile MiliQ water)

RESULTS AND DISCUSSION

In a study by Higgins *et al.* (2003), both low- and high-level ciprofloxacin resistance of *Pseudomonas aeruginosa* was associated with a Thr83→Ile substitution in *gyrA*, which was also the most common *gyrA* mutation. Another study on *P. aeruginosa* also confirmed that a single alteration in *gyrA* (Thr83→Ile) was the most frequently detected alteration, while other mutations were also found at positions Asp-87 and Gln-106 (Lee *et al.*, 2005). On the other hand, a study on *Salmonellae* showed that the most common mutation in *gyrA* was at codon 87 in which a G→A transversion led to substitution of asparagine for aspartate (Asp87→ Asn), followed by mutation at codon 83 (C→T transversion) resulting in Ser83→Phe (Ling *et al.*, 2003). Though not as prevalent as mutations in *gyrA*, ciprofloxacin resistant isolates of *P. aeruginosa* also had alterations in *gyrB* with alteration Glu468→Asp being the most common. Alterations in *gyrB* were found to play a complementary role in the acquisition of resistance to fluoroquinolones (Lee *et al.*, 2005). Conversely, Ling *et al.* (2003) did not detect any mutation in *gyrB* of *Salmonellae*.

A total of 349 nucleotides of the *gyrA* and 269 nucleotides of the *gyrB* were determined in our study. No sequence variation was detected in either *gyrA* or *gyrB* of the 10 intermediate resistant isolates and the sensitive isolate, indicating that there were no genetic alterations in either gene.

The absence of any point mutation in either the *gyrA* or *gyrB* gene in the 11 clinical isolates implies that local isolates of *B. pseudomallei* have not yet developed mutations that alter the amino acids encoded by both the genes due to selection pressure. Absence of fully resistant *B. pseudomallei* isolates also suggests that local isolates are still sensitive and only demonstrate intermediate resistance to ciprofloxacin. The emergence and spread of antimicrobial resistance in pathogenic bacteria takes many forms, but always represents a process of evolution in response to selective antimicrobial pressure. This selective pressure is most commonly generated by human production and use of antimicrobial agents. The use of fluoroquinolones in food animals also suggests non-human sources of selective pressure and transmission of resistance to humans (Rice, 2009).

To date, there have been no reports of mutations of *gyrA* conferring resistance to fluoroquinolones in pathogenic *Burkholderia* (Viktorov *et al.*, 2008). However, given that the tested isolates have intermediate resistance to ciprofloxacin, there may be other mechanisms playing a role in its reduced susceptibility such as the *qnr* gene product that protects the DNA gyrase from quinolone inhibition (Ruiz, 2003). Another possibility is the efflux pump contributing to resistance by removing the presence of antibiotics in the microorganism. Efflux systems which are able to accommodate a variety of unrelated antimicrobial agents,

including antibiotics, biocides, dyes, detergents, fatty acids, organic solvents, and homoserine lactones, are responsible for much of the intrinsic multidrug resistance in Gram-negative bacteria (Poole, 2001). In *B. pseudomallei*, intrinsic resistance to many antibiotics is attributed to multiple multidrug efflux pumps, such as AmrAB-OprA, BpeAB-OprB, and BpeEF-OprC (Chan *et al.*, 2004; Kumar *et al.*, 2006). In a study done by Mima & Schweizer (2010), BpeAB-OprB was found to be a multidrug efflux system which extrudes macrolides (clarithromycin and erythromycin), fluoroquinolones (ciprofloxacin, norfloxacin and ofloxacin), tetracyclines (doxycycline and tetracycline), acriflavine and to a lesser extent, chloramphenicol. The study also showed that BpeAB-OprB shares substrates with AmrAB-OprA, such as acriflavine, fluoroquinolones, macrolides and tetracyclines. Hence, although mutations in the *gyrA* and *gyrB* genes were absent, the reduced susceptibilities of the clinical isolates of *B. pseudomallei* used in this study may have been due to these efflux pumps.

Despite treatment with high-dose ceftazidime, which is the drug of choice, septicaemic melioidosis carries a mortality rate of 65% (Puthucheary *et al.*, 1992). In a study by Russell *et al.* (2000), ciprofloxacin prophylaxis and immediate therapy both raised the median lethal dose (MLD) of *B. pseudomallei* from 19 colony forming units (cfu) in untreated mice to 4 x 10^6 cfu. However, therapeutic ciprofloxacin only raised the MLD to 180 cfu. Hence, the authors suggest that fluoroquinolones may be useful for immediate therapy or as prophylaxis for individuals known to have been exposed, or at a high risk of exposure to melioidosis (Russell *et al.*, 2000).

In a different study, ciprofloxacin or ofloxacin given for a median of 15 weeks to treat 57 adult patients with melioidosis was associated with an unacceptably high failure rate of 29% (Chaowagul *et al.*, 1997). However, the ability of quinolones, and newer fluoroquinolones in particular, to accumulate within phagocytes suggests they may still prove useful for the treatment of melioidosis. Although human clinical experience and animal experimentation proved disappointing thus far, it may be prudent to further explore and/or re-examine the *in vitro* and *in vivo* activities of some of the newer fluoroquinolones (Estes *et al.*, 2010). It is also possible that ciprofloxacin may be a supplementary therapeutic option in selected areas with lower resistance (Sam *et al.*, 2010). At the same time, novel therapeutics are being developed for melioidosis treatment, such as immuno-antimicrobial therapy for *B. pseudomallei* infection where an alternative strategy to improve the efficacy of conventional antimicrobial therapy is to combine immunotherapy with antibiotic therapy. Besides that, the bacterial capsule, type 3 secretion system and other virulence factors may be used as potential therapeutic agents, as well as novel antimicrobials such as isocitrate lyase inhibitors (Estes *et al.*, 2010).

The continuous rise in the prevalence of quinolone-resistant bacteria can be attributed to the extensive use and to some degree misuse of these antibacterial agents in clinical and veterinary settings (Ruiz, 2003). This is of great concern and emphasizes that antibiotic usage requires close monitoring and surveillance to ensure no further development of resistance occurs to become a major threat to healthcare and compromise the efficacy of antibiotics.

Acknowledgements. This research was supported by IPPP research grant (PS254/2010A) and RG090-09HTM provided by University of Malaya.

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