

Use of a MAMA-PCR Method to detect gyrA Mutations in Nalidixic Acid Resistant Clinical Isolates of *Escherichia coli*

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(Received 29 Jul 2007; accepted 30 Dec 2007)

Abstract

Background: *Enterobacteriaceae* are a large group of bacteria widely distributed in nature. *Escherichia coli* is the most common cause of urinary tract infection. Two amino acid substitutions, in GyrA, are commonly responsible for quinolone resistance in *E. coli*. The aim of this study was molecular survey of nalidixic acid resistance *E. coli* isolated from patients in the codones of 83 and 87 gyrA genes.

Methods: During 5 months (January to June 2005) of Molecular Survey of Nalidixic Acid Resistance, one hundred and twenty-one *E. coli* isolates from urine samples of patients referred to clinical laboratory of Baqiyatallah Hospital were cultured. Differential tests were done for diagnosis of *E. coli*. An economical and time-efficient mismatch amplification mutation assay (MAMA) PCR was developed to detect mutations in the chromosomal gyrA gene causing these substitutions.

Results: In nalidixic acid antibiogram test, 55 cases (45.5%) were sensitive, 63 cases (52%) were resistant and 3 cases (2.5%) were intermediate. Results of PCR and MIC were similar to antibiogram. There was not any mutation in the sensitive samples but there were performed five mutations on the 85, 81, 107, 97 and 87 codones of resistance samples. The codone number 87s mutation is one of the main mutations of nalidixic acid resistance.

Conclusion: Depending on results of this study and comparison with other studies, trend of resistance of *E. coli* is increasing. Therefore, we recommend control of antibiotic misuse and application of MIC and PCR tests (if possible) prior to treatment for suitable selection of antibiotic and prevention of microbial resistance.

Keywords: *Escherichia coli*, Nalidixic acid resistance, MAMA PCR, gyrA gene

Introduction

Escherichia coli is one of the most common causes of bacterial infections and, in Iran, increasing numbers of quinolone-resistant *E. coli* have been giving cause for concern since the late 1990s. Furthermore, the number of reports of quinolone-resistant *E. coli* in other countries continues to rise at the start of the new century (1, 2).

The most frequent mechanism of resistance to quinolone in *E. coli* includes alterations in genes that encode subunits of the quinolone targets DNA gyrase (in gyrA and gyrB genes) and topoisomerase IV (in parC and parE) (3-5). These alterations involve mainly mutations located in the quinolone resistance-determining region (QRDR) of the gyrA gene and its homologous region of the parC gene (4, 6, 7). In contrast, mutations in

gyrB and parE genes are of minor importance and are rare contributors to quinolone resistance (5, 8).

In *E. coli*, mutations in the quinolone resistance determining regions (QRDRs) of the gyrA and parC genes, at nucleotide positions 248 and 259/260 of gyrA resulting in Ser-83 and Asp-87 alterations and mutations at nucleotide position 238/239 and 250/251 of parC resulting in Ser-80 or Glu-84 changes, have been reported to be mainly responsible for quinolone resistance (5, 9, 10).

Although several different methods, such as restriction fragment length polymorphism (RFLP), single-strand conformational polymorphism (SSCP) analysis and sequencing of the relevant gene regions, have been used to detect such mutations

(7,11), the procedures are labour intensive and time consuming.

In this study, we developed a simple rapid PCR mismatch amplification mutation assay (MAMA PCR) to detect the significant mutations in both chromosomal *gyrA* of *E. coli* isolates.

Materials and Methods

Bacteria and MICs One hundred and twenty-one *E. coli* isolates from urinary samples collected from Baqiyatallah hospital in Tehran from January to June 2005 were used in the study. The MICs of nalidixic acid (NAL) and other antimicrobial agents were determined by agar dilution testing according to the National Committee for Clinical Laboratory Standards (NCCLS) performance guidelines (12). In nalidixic acid antibiogram test, 55 cases (45.5%) were sensitive, 63 cases (52%) were resistant and 3 cases (2.5%) were intermediate.

Primer design and MAMA PCR protocol

The rationale behind MAMA PCR is that a single nucleotide mismatch at the 3' extremity of the annealed reverse primer renders *Taq* polymerase unable to extend the primer. So, the absence of the specific PCR product (coupled with a positive internal PCR control) reveals a deviation from the wild-type DNA sequence. In this study, we introduced another nucleotide alteration near the 3' end of the MAMA primer to enhance the 3' mismatch effect. The MAMA primers for mutation detection are shown in Fig. 1. Other primers used are as follows: Forward primer *gyrA*(P1), 5'-gat aca gta gag gga tag cgg-3' (position: upstream of gene); Reverse Primer *gyrA* (P2), 5'- gtt ata cgg aaa tcc gtc tgg c -3' (position: 360–382).for each template four PCR carry out. In each PCR, a forward primer and a MAMA primer were used in a PCR for mutation detection. These primers generate a short PCR product from the wild-type gene, but fail to produce a product from any gene with a mutation at the location covered by the mismatch positions on the MAMA primer. A third, control primer that is expected to anneal efficiently to

all gene alleles was used in conjunction with the forward primer to generate a longer PCR product as an internal control.

PCR experiments Template for PCR was prepared by the heat lysis method (13) except that bacteria were directly inoculated into 1.0 mL of LB broth in Eppendorf tubes for overnight culture. For each PCR, 1 µL of supernatant containing template DNA was added to a final volume of 20 µL containing: 1.5 µL forward primer, 1.5 µL MAMA primer or reverse Primer, 2 µL of 10 x Taq buffer, 1.5 µL of d NTPs, 1.5 µL Mgcl₂ and 0.5 µL of Taq DNA polymerase. [Amplification was carried out on a DNA Thermolyne programmed as follows: initial denaturation at 94 °C for 5 min and 30 cycles of denaturation at 94 °C for 5 min, annealing at 64 °C for 5 min and extension at 72 °C for 5 min, with a final step of 72 °C for 5 min. Large scale PCR was carried out on a DNA Multiblock System with the same programme. PCR products were visualized on horizontal 1.0% agarose gels in 0.5 x TBE buffer, loaded with 5 µL of reaction mix and stained with ethidium bromide after electrophoresis (Fig. 2).

Results

Detection of mutations in the QRDR of the *gyrA* gene, was performed in the 63 NAL_R and 55 NAL_S and 3 NAL_I strains by Four PCR for each template (5, 7). Results of PCR and MIC were similar to antibiogram (Table1).

The results defined five groups according to the mutations in *gyrA*. Group I: templates with PCR result of P1,2(+) , P1,3(+) , P1,4(+) , P1,5(-) or P1,2(+),P1,3(+),P1,4(-) , P1,5(+) and NAL MICs of 200 µgr/ml have two alterations, at Ser-83 & Asp-87 in GyrA. Group II: templates with PCR result of P1,3(-) , P1,4(+) , P1,5(-) or P1,2(+), P1,3(-) , P1,4(-) or P1,5(+) or P1,2(+),P1,3(-) ,P1,4(+) ,P1,5(-) and NAL MICs of 200 µgr/ml have Asp-87 substitutions in GyrA. Group III: templates with PCR result of P1,2(+), P1,3(+), P1, 4(-),P1, 5(-) and NAL MICs of 200 µgr/ml have Ser-83 substitutions in GyrA and sensitive

Table 1: Comparison Result of PCR with Result of MIC & Antibiogram

Sensitive to Nalidixic Acid antibiogram disk	Result of MIC (25-800) µrg/ml	Result of PCR				Number Of Templates
		P1&P2	P1&P3	P1&P4	P1&P5	
S	25	+	-	-	-	43
R	200	+	+	+	-	33
S	25	-	-	-	-	11
R	200	-	+	-	+	7
R	200	-	-	-	+	3
R	200	-	+	+	-	12
I	50	+	+	+	-	3
R	200	+	-	-	+	4
R	200	+	+	-	+	3
R	200	-	-	+	-	1
R	20	+	+	-	-	1
Total				121		



Fig. 2: Agarose gel of *E. coli* MAMA PCR products with primer P1& P2.

Lane 1: DNA molecular weight standard 100 bp.

Lane 2-13: Lanes 6, 7,8,9,10,11 were positive (450bp) & Lanes 3,4,5,12,13 were negative.

Discussion

No amino acid changes were detected in GyrA protein in the 55 NAL_S isolates, whereas at least one amino acid substitution in the GyrA protein (position 83 and/or 87) was detected in NAL_R strains in study sequencing.

Two amino acid substitutions (Ser-83 & Asp-87), in GyrA, are commonly responsible for quinolone resistance in *E. coli*. Y. Sáenz et al. revealed that the Ser-83→Leu substitution in the GyrA protein of *E. coli* strains was the substitution most frequently identified, as previously reported. (6, 10, 11, 16) Tavio et al. reported the Ser-83→Ala change in a human *E. coli* clinical isolate and also described the Ser-83→Val

substitution in an in vitro mutant selected under antibiotic pressure (17).

In respect of changes in position 87, Y. Sáenz et al. revealed that the Asp-87→Tyr and Asp-87→Gly single substitutions in the GyrA protein were associated with a lower ciprofloxacin MIC (0.06 mg/L) than the Asp-87→Asn change (MICs 0.25–0.5 mg/L). The substitutions of Ser-83 for Leu, Ala or Val have resulted not only in the loss of the hydroxyl group of serine and thus the ability to form hydrogen bonds, but also in the replacement by an aliphatic chain. All the changes in Asp-87 for Asn, Tyr, Gly or His involve the loss of a negatively charged amino acid. These changes suggest that the ability to form hydrogen

bonds and the negative charge at these positions seem to be important for quinolone interactions with the DNA gyrase-DNA complex (17).

Targeting the wild-type sequence is a more comprehensive tactic than targeting a particular mutation. However, a warning is pertinent. Although the approach described in this paper will detect a number of different mutations in the wild-type sequence at the position of interest, it has limitations. First, it does not identify the nature of the mutation. Therefore, it is not a substitute for DNA sequence analysis. Secondly, changes at the third base position of a codon would be detected by our version of MAMA, but these will not necessarily result in an amino acid substitution in the gene product because of the degenerate nature of the genetic code. For example, any change at the third base position of *gyrA* codon 83 (TCG) would not alter the amino acid in GyrA, i.e. Ser-83. Similar considerations apply to other codons. Hence, detection of such silent mutations by our version of MAMA could, in principle, lead to wrong conclusions being drawn about amino acid substitution in the gene product. However, nucleotide changes at the third base positions of the four codons targeted in this study have, to our knowledge, not been reported.

In conclusion, the MAMA PCR method proposed reliably detects the mutations in *gyrA* that are commonly responsible for resistance to quinolone displayed by *E. coli*, and the method is suitable for profiling and characterizing a large number of isolates in resistant outbreaks.

Acknowledgements

This work was supported by a grant from Baqiyatallah University of Medical Sciences, Iran.

The authors declare that they have no Conflict of Interests.

Referentes

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