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Research Paper

Molecular mechanism of fluoroquinolones resistance in *Mycoplasma hominis* clinical isolates

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Abstract

To evaluate the molecular mechanism of fluoroquinolones resistance in *Mycoplasma hominis* (MH) clinical strains isolated from urogenital specimens. 15 MH clinical isolates with different phenotypes of resistance to fluoroquinolones antibiotics were screened for mutations in the quinolone resistance-determining regions (QRDRs) of DNA gyrase (gyrA and gyrB) and topoisomerase IV (parC and parE) in comparison with the reference strain PG21, which is susceptible to fluoroquinolones antibiotics. 15 MH isolates with three kinds of quinolone resistance phenotypes were obtained. Thirteen out of these quinolone-resistant isolates were found to carry nucleotide substitutions in either gyrA or parC. There were no alterations in *gyrB* and no mutations were found in the isolates with a phenotype of resistance to Ofloxacin (OFX), intermediate resistant to Levofloxacin (LVX) and Sparfloxacin (SFX), and those susceptible to all three tested antibiotics. The molecular mechanism of fluoroquinolone resistance in clinical isolates of MH was reported in this study. The single amino acid mutation in ParC of MH may relate to the resistance to OFX and LVX and the high-level resistance to fluoroquinolones for MH is likely associated with mutations in both DNA gyrase and the ParC subunit of topoisomerase IV.

Key words: *Mycoplasma hominis*, quinolones, drug resistance, mutation.

Introduction

Mycoplasma hominis (MH) is a Gram negative microorganism that lacks a typical bacterial cell wall. It is commonly found in the genitourinary tract of pregnant and non-pregnant women. The colonization rates for MH range between 20% and 30% worldwide (Sweih *et al.*, 2012; Garcia-de-la-Fuente *et al.*, 2008). MH can cause a wide range of diseases, such as genital infections in adults and may be involved in infections in premature and low birth-weight infants with clinical manifestations of septicaemia (Gonzalez-Jimenez *et al.*, 2006; Karabay *et al.*, 2006; Koshiba *et al.*, 2011). This tiny microorganism can also invade in central nervous system, joint, peritonitis, pyelonephritis and respiratory tract (Garcia *et al.*, 2007; Kacerovsky *et al.*, 2009; Koshiba *et al.*, 2011; Krijnen R.*et al.*, 2006; Myers *et al.*, 2010; Pascual 2010). Barykova (2011) reported that MH infection may be involved in Prostate Cancer development.

MH was considered as a bacterium that is intrinsically resistant to quinolones like nalidixic acid but susceptible to fluoroquinolones, an important class of wide-spectrum antibacterial agents that target two enzymes essential for bacteria viability, the DNA gyrase and topoisomerase IV. However, the MH clinical strains isolated recently exhibited high-level resistance to various fluoroquinolones (Krausse *et al.*, 2010; Pereyre *et al.*, 2006; Kenny *et al.*, 2001). The molecular mechanism of fluoroquinolones resistance in clinical MH isolates remains to be elucidated. In present study, the quinolone resistance-determining regions (QRDRs) of DNA gyrase (gyrA and gyrB) and topoisomerase IV (parC and parE) in China clinical isolates were analyzed, and the molecular mechanism

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of fluoroquinolone resistance in MH clinical isolates was reported.

Materials and Methods

Bacterial strains and antibiotics

Strains

15 MH clinical isolates identified by a kit produced by LIVZON Pharmaceutical Group Inc (China) with different phenotypes of resistance to quinolone antibiotics were obtained from urogenital specimens in Liaoning North Hospital, Shenyang City, China, from 2009 to 2010. MH reference strain ATCC 23114(PG21) was offered from Livzon Pharmaceutical Group INC and served as reference control.

Quinolone antibiotics

Ofloxacin (OFX), Levofloxacin (LVX) and Sparfloxacin (SFX) used in this study were provided by Livzon Pharmaceutical Group INC.

Determinations of minimal inhibitory concentrations

MH was cultured and its susceptibility to quinolone antibiotics was tested as described previously (Karabay *et al.*, 2006). The minimal inhibitory concentration (MIC) was determined as the lowest concentration of an antibiotic at which (i) the medium color did not change during the 24 h incubation at 37 °C and (ii) microscopic examination of the bottom of the plate well showed no growth of mycoplasma. The susceptibility breakpoints for MH to OFXLVX and SFX were with the MIC values of ≤ 1 (mg/L), and the resistant breakpoints for MH to OFXLVX and SFX were with MIC values of > 4 (mg/L). If the value of MIC was between the two breakpoints, then the M*H* strain was defined as intermediate (Meng *et al*., 2009).

The amplication of QRDRs of MH by PCR

The primers were designed to amplify the QRDRs of *gyrA*, *gyrB*, *parC* and *parE* of MH as previously described (Bébéar *et al.*, 1998). All primers were synthesized by Shanghai Sangon Biological Engineering Technology & Services Co., Ltd. and the sequences were listed in Table 1.

The genomic DNA of MH strains were extracted with routine molecular biological procedures. Briefly, the precipitates from 1 mL of culture were harvested by centrifugation at 15,000 *g* for 30 min. The precipitates were treated with proteinase K at 55 °C for 2 h in digestion buffer, and the DNA was extracted using the phenol-chloroform method. The DNA extracts were stored at -70 °C for PCR amplification and sequencing. The PCR was performed with the parameters of 10 min at 92 °C, followed by 40 cycles of 1 min at 94 °C, 1 min at 57 °C, and 1 min at 72 °C, then with a final extension of 10 min at 72 °C. PCR products were analyzed by electrophoresis in 2% agarose gels.

Sequences analysis of QRDRs of MH

The PCR products were directly subjected to sequence analysis using a 384 Well Thermal Cycler. The nucleotide sequences of the PCR products were compared to those of the reference MH strain PG21 (GenBank accession no. NC_013511) by performing BLASTn at http://www.ncbi.nlm.nih.gov/BLAST.

Results

The MICs of three fluoroquinolones (SFX, OFX and LVX) against *MH* was determined by the Broth dilution method, and the results were presented in Table 2. The sequence of QRDRs of MH strain ATCC 23114 was found to be identical with the sequence of wild-type strain PG21 in GenBank. While for the clinical isoltes, there were some mutations found in *gyrA*, *gyr B*, *parC* and *pare* (Table 2), which might related to the fluoroquinolones resistance.

Mutations in the genes of gyrA and gyrB

There were 7 SFX-resistant isolates that were found to carry nucleotide substitutions (Ser153-Leu) in *gyrA*, corresponding to the *E. coli* Ser83-Leu changes. Meanwhile, one of 7 SFX-resistant isolates was found to have another amino acid substitution, the Ser66-Ile. The other 8 clinical strains, including those resistant to OFX, LVX, or interme-

Table 1 - The designed primers for the amplication of MH QRDRs by PCR.

Name	Primer sequences	Amplicon size (gene)
MH3	forward 5'-TATGGTATGAGTGAACTTGG-3'	
MH4	reverse 5'-AATTAGGGAAACGTGATGGC-3'	349-bp (gyrA)
MH6	forward 5'-CTTCCTGGAAAATTAGCAGAC-3'	
MH7	reverse 5'-CTGTGCCTAAGGCGTGAATCA-3'	223-bp (gyrB)
MH11	forward 5'-CGTCGGATTTTATATTCAATG-3'	
MH13	reverse 5'-GGTGATTCCTTTAGCACCGTT-3'	348 bp (parC)
MH27	forward 5'-CTTTCAGGAAAATTAACTCCT-3'	
MH28	reverse 5'-ATCAGTGTCAGCATCTGTCAT-3'	297 bp (parE)

Table 2 - Amino acid changes in GyrA, GyrB, ParC, and ParE in MH clinical isolates and reference strain.

MIC (μg/mL) of strain	Mutation position of*						
	OFX	LVX	SFX	GyrA	GyrB	ParC	ParE
1	64.00	64.00	16.00	Ser83- Leu	-	Lys134-Arg Ser80-Ile	-
2	32.00	16.00	8.00	Ser83-Leu	-	Lys134-Arg	-
3	32.00	16.00	8.00	Ser83-Leu	-	Lys134-Arg	-
4	64.00	32.00	16.00	Ser83-Leu Ser66-Ile	-	Lys134-Arg	-
5	16.00	16.00	8.00	Ser83-Leu	-	Lys134-Arg	-
6	16.00	16.00	8.00	Ser83-Leu	-	Lys134-Arg	-
7	16.00	16.00	8.00	Ser83-Leu	-	Lys134-Arg	-
8	32.00	16.00	4.00	-	-	Lys134-Arg	Asp426-Asn
9	8.00	4.00	2.00	-	-	Lys134-Arg	-
10	8.00	4.00	2.00	-	-	Lys134-Arg	-
11	16.00	16.00	0.50	-	-	Ser80-Ile	-
12	2.00	2.00	0.50	-	-	-	-
13	4.00	4.00	1.00	-	-	Lys134-Arg	Arg447-Lys
14	0.50	0.50	0.03	-	-	-	-
15	0.50	0.25	0.03	-	-	-	-
ATCC 23114[#]	0.50	0.25	0.03	-	-	-	-

Note: SFX, sparfloxacin; LVX, levofloxacin; OFX, ofloxacin; *GyrA, GyrB, ParC, and ParE residue positions are based on the respective gene sequences for MH (4; this study). GyrA positions 136 and 153 corresponding to *E. coli* coordinates 66 and 83, respectively. ParC positions 91 and 145 corresponding to *E. coli* coordinates 80 and 134, respectively. ParE positions 432 and 453 corresponding to *E. coli* coordinates 426 and 447, respectively.
[#] -, identical to that in the reference strain, PG21(GenBank accession no. NC_013511).

diate to OFX, LVX and SFX, or susceptible to all three tested antibiotics did not present any change in amino acid residue encoded by gyrA, while presented changes in amino acid residue encoded by ParC and ParE.

As for the *gyrB* genes of 15 clinical isolates, three nucleotide mutations (G2173A, G2278A, and A2197T) respectively were found. The most popular substitution was G2173A, which was happened in 7 clinical isolates. However, these nucleotide mutations did not cause amino acid change in translation.

Mutations in the genes parC and parE

Eleven out of 13 isolates with resistance or intermediate resistance to OFX, LVX were found to harbor a Lys134-Arg substitution in ParC. Among these 11 isolates, 7 strains simultaneously carried the Ser83-Leu substitution in GyrA protein. Only 2 out of the 13 clinical isolates presented two alterations in ParE, the Asp426-Asn and Arg447-Lys mutations. The two isolates also had the Lys134-Arg mutations in ParC.

Discussion

The present study reported that the 13 quinolone-resistant clinical isolates were found to carry target mutations in either gyrA and parC (7 isolates with resistance to OFX, LVX and SFX) or parC (4 isolates with no-resistance to SFX and resistance or intermediate resistance to OFX and LVX). There were no significant alterations in GyrB amino acid, although several nucleotide substitutions can be detected in QRDR of *gyrB*. No mutations were found in clinical isolates with the phenotypes of resistant to OFX, intermediate resistant to LVX and SFX, and susceptible to all these three tested quinolone antibiotics. Several point mutations in the QRDR including the Ser83 Leu substitution in gyrA were required to observe significant reduced susceptibility to SFX, with values greater than 8 mg/L. In contrast, a single mutation in parC was enough to increase OFX and LVX MIC values up to 8 mg/L. This suggests that SFX interacts with the different QRDR targets (ParC and GyrA) whereas OFX primary target is parC. And this is consistent with previous findings (Bébéar *et al.*, 1999; Kenny *et al.*, 1999) who found mutations in parC and gyrA secondary to SFX exposure and mutations in parC only secondary to OFX exposure.

Our results also indicated that the Lys134-Arg substitution in ParC of MH might be related to its resistance to OFX and LVX. The Asp426-Asn (Mh8) and Arg447-Lys (Mh13) substitutions in ParE were detected, but the biological significance corresponding to these alterations remains elucidated.

The evolution of MH strains resistant to fluoroquinolones antibiotics was revealed in an in vitro studies reported previously (Bébéar *et al.*, 1997, 2000, 2003). The mechanism such a step by step resistant mutant in literature is unclear and need our further experiments to prove. The substitution of Ser83 in GyrA and Glu84 in ParC was con-

sidered to be associated with high-level resistance of MH against SFX. However, the Lys134-Arg substitution in ParC of MH in our results was firstly reported, which did not appeared in the induced experiment in vitro.

In conclusion, this study reports that the relation between fluoroquinolone resistance and mutation positions of QRDR of MH in clinical isolates. Our results indicate that the single amino acid mutation in ParC of MH may relate to the resistance to OFX and LVX and the high-level resistance to fluoroquinolones for MH is associated with mutations in both DNA gyrase and the ParC subunit of topoisomerase IV.

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