Efficacy Of Enrofloxacin Against Transmissible Resistance Gene (Qnrs And Aac (6')-Ib-Cr) - Containing Escherichia Coli Isolates And Clones

*Murat CENGİZ, Erdem ARSLAN

Laboratory of Molecular Pharmacology, Department of Pharmacology and Toxicology,Faculty of Veterinary Medicine, Uludag University, 16059, Nilufer, Bursa, Turkey

Abstract

Aim of this study was to investigate the effects of qnrS1 and aac (6')-Ib-cr on susceptibility, some pharmacodynamics of enrofloxacin against molecularly constructed and natural isolates of E. coli from animals. The MPCs and mutation frequencies of the drug were determined using the agar dilution method. Time-kill assays were used to determine the antimicrobial activity of the drug against E. coli strains. MPCs of enrofloxacin for qnrS1- and aac(6´)-Ib-cr-containing transformants increased from 0.128 µg/ml to 2 and 4 µg/ml. MPC:MIC ratio of enrofloxacin for transformants and E. coli isolates were 4 and 8, respectively. Mutation frequencies were markedly higher for transformants and isolates compared to control strain. Mutation frequencies diminished up to 6.2×10^{-18} by increasing the concentrations used in the assay. The results of this study showed that enrofloxacin is less active against E. coli strains in case of additional mutations in QRDR. The concentration-dependent bactericidal effect of enrofloxacin is only observable by the eight times the MIC and increasing concentrations did not alter the bactericidal activity. The combination therapy can be considered to fight with resistant E. coli instead of monotherapy and artificially constructed E. coli strains, which exhibit similar reactions against ENR with natural E. coli isolates, can be used to test the pharmacological efficacy of the combinations.

Key words: E. coli, qnrS, aac(6´)-Ib-cr, mutant prevention concentration, time-kill

Introduction

Due to their excellent in vitro activity, flouroquinolones (FQs) are commonly used in the treatment of animal infections worldwide. Enrofloxacin (ENR) is the first FQ which is approved for use in the treatment of animals. With the extensive consumption of FQs in human and veterinary medicine, dissemination of FQ-resistant Escherichia coli (E. coli) isolates has become a major problem in infection control and treatment in the last decade worldwide (Sukul and Spiteller, 2007).

FQs are synthetic antimicrobials displaying concentration depended bactericidal effect by inhibiting the bacterial to-poisomerase enzymes. Mutations in the quinolone resistance-determining region (QRDR) resulting in structural alterations in target enzymes are an important mechanism of FQ resistance. Plasmid-mediated quinolone resistance (PMQR) genes also contribute to the resistance to FQs by either altering the molecular structure of FQ target enzymes or enzymatic inhibition of FQs. The first reported PMQR mechanism was the qnr gene, which encodes a protein that protects type II topoisomerases from quinolones (Martinez-Martinez et al., 1998). The aac (6')-Ib-cr

 * Corresponding author: Adres: Uludağ Üniversitesi Veteriner Fakültesi Farmakoloji ve Toksikoloji Anabilim Dalı, Görükle Kampusu, 16059, Bursa, Türkiye. E-mail: cengizm@uludag.edu.tr Telephone: +902242941323 Fax: +902242941202

gene is one of the major PMQR genes and is a variant of aac (6')-Ib which has two codon changes (Trp-102→Arg and Asp-179→Tyr). The aac (6')-Ib-cr gene encodes aminoglycoside acetyltransferase enzyme which is capable of modifying some FQs and reducing their activity via acetylation.

QRDR mutations and PMQR genes have been widely reported worldwide (Ferreira et al., 2010; Fortini et al., 2010; Akiyama and Khan, 2012). Cengiz et al. (2013) demonstrated a difference on the bactericidal activity of ENR against gyrA mutants and qnr-containing E. coli isolates even at the identical MICs. This suggests that genetic variations may have different effects on pharmacological activity of antimicrobials and survival of resistant sub-populations. Therefore, aims of this study was to investigate the effects of transmissible quinolone resistance genes (aac (6')-Ib-cr and qnrS1) on susceptibility, mutant prevention concentration (MPC), mutation frequency (MF) and efficacy of bactericidal activity of ENR against molecularly constructed and natural isolates of E. coli harbouring various QRDR mutations and PMQR genes.

Materials and Methods

Bacterial strains

Previously characterized five E. coli isolates collected from animals (Dalhoff and Schimitz, 2003), three transformants and one control strain (E. coli ATCC 25922) were used in this study.

Molecular cloning

Cloning of aac(6´)-Ib-cr and qnrS1 genes were performed as previously described by Emrich et al. (2010). qnrS1 gene was PCR amplified using primer pairs qnrs1 (F- ATACAAGCTTGATGGAAACCTACAATCATACA, R- ATACAAGCTTTTAGTCAGGATAAACAACAAT), the resulting PCR product was digested with HindIII and ligated to the specific HindIII site of pUC19.

Cloning of aac(6´)-Ib-cr gene was performed in a two-step procedure. In the first step, aac(6´)-Ib-cr gene was PCR amplified using primers aac(6')-Ib-cr2 (F- ATACAAGCTTGACAAAGTTAGGCATCACAAAGT, R- ACTATAAGCTTTTAGGCATCACTGCGTGTTC). Then the resulting DNA fragment was digested with HindIII and inserted into specific HindIII site of pUC19 with using T4 DNA Ligase. In the second step, the resulting recombinant plasmid was used as template for a second PCR

amplification using primer pairs of aac(6´)-Ib-crΔlacZ (F-CTTTGTGATGCCTAACTTTGTTTTTTGCGTTGCT-CATAGCTGTTTCCTGTGTGAAATTG, R-ACAAAGT-TAGGCATCACAAAGTACAGCATCGTGACCAA-CAGC). Verified PCR fragment then used directly for transformation of the respective E. coli recipient strains (E. coli ATCC 25922) that were called as MtX for aac(6´)-Ib-cr and MtS for qnrS1. Transformation was performed by using Transform Aid Bacterial Transformation Kit (Fermentas), as described by the manufacturer. Transformants were selected on 50 μg/ml ampicillin containing Luria-Bertani (LB) agar plates.

Mutant prevention concentration and mutation frequency

The MPC of ENR was determined by the method of Blondeau et al. (2009). Briefly, each E. coli isolate and control strain was freshly grown from stock stored at -25 °C. Strains were incubated overnight at 37 °C in 100 ml of Mueller-Hinton Broth (MHB). Cultures were centrifuged at 9000 rpm for 20 minutes. The supernatant was discarded and pellet was re-suspended in 3 ml of MHB to reach > 10^{10}cfu/ml. A 100 μl of this culture was used to inoculate Plate Count Agar (PCA) plates containing 1xMIC-32xMIC range of ENR. The plates were examined every 24 hours for growth of E. coli. The MPC was determined as the concentration that allowed no growth of bacteria at the end of 96 hours incubation.

MFs were determined by the method of Ricci et al. (2006). The parent strains grown overnight in antibiotic-free broth were concentrated by centrifugation and resuspended in sterile broth to give a range of inocula (10^6 to 10^{10}cfu/ml). Agar plates containing ENR at 0.5, 1, 2 and 4 times the MIC were inoculated with 100 μl (10^5 to 10^9 CFU) of each cell suspension and incubated at 37°C for up to 96 h.

Time-kill assay

Time-kill experiments were performed as described by Begic et al. (2009). A liquid overnight bacterial culture of bacterial strains was diluted with MHB. The following ENR concentrations were tested: 0, 0.5, 1, 2, 4, 8, 16 and 32 times the MIC. Bactericidal activity was defined as a ≥ 3 log decrease in cfu/ml relative to the bacterial concentration at 0 min.

Results

In the present study, the transfer of transmissible genes to reference strain increased the MICs of ENR against mo-

Table 1. Resistance profiles and mechanisms of E. coli, mutant prevention concentrations of enrofloxacin and mutant frequencies of the strains and time-kill assays

E. coli strain	Genotype	MIC_{ENR} (µg/ml)	MPC_{ENR} (µg/ml)	Mutation frequency (cfu/ml)				Time-kill (conc./log reduction)	
				0.5xMIC	1xMIC	2xMIC	4xMIC	8 h	24 h
ATCC25922	-	0.032	0.128	4.8×10^{-10}	5.1×10^{-16}	5.2×10^{-18}	-	8xMIC/3 $\log_{10}$ 16xMIC/≥ 4 $\log_{10}$	MIC/3 $\log_{10}$ 2xMIC/≥ 4 $\log_{10}$ 4xMIC/≥ 5 $\log_{10}$ 8xMIC/≥ 6 $\log_{10}$
MtS	qnrS1	0.512	2	4.4×10^{-8}	3.8×10^{-10}	5.2×10^{-14}	-	4xMIC/≥ 3 $\log_{10}$ 8xMIC/≥ 4 $\log_{10}$ 32xMIC/≥ 5 $\log_{10}$	2xMIC/≥ 3 $\log_{10}$ 4xMIC/≥ 4 $\log_{10}$ 8xMIC/≥ 6 $\log_{10}$
MtX	aac(6´)-Ib-cr	1	4	4.3×10^{-9}	5.5×10^{-11}	4.9×10^{-14}	-	4xMIC/3 $\log_{10}$ 8xMIC/4 $\log_{10}$	MIC/≥ 3 $\log_{10}$ 4xMIC/≥ 4 $\log_{10}$ 8xMIC/≥ 5 $\log_{10}$
MtSX	qnrS1 aac(6´)-Ib-cr	1	4	2.3×10^{-8}	4.2×10^{-12}	3.1×10^{-13}	-	4xMIC/3 $\log_{10}$ 8xMIC/4 $\log_{10}$	2xMIC/≥ 3 $\log_{10}$ 4xMIC/≥ 4 $\log_{10}$ 8xMIC/≥ 5 $\log_{10}$
E101	qnrA1 aac(6´)-Ib-cr	32	256	4.1×10^{-10}	6.1×10^{-12}	2.4×10^{-14}	6.0×10^{-15}	2xMIC/3 $\log_{10}$ 8xMIC/4 $\log_{10}$ 16xMIC/5 $\log_{10}$	MIC/≥ 3 $\log_{10}$ 4xMIC/≥ 4 $\log_{10}$ 16xMIC/≥ 5 $\log_{10}$ 32xMIC/≥ 6 $\log_{10}$
E103	qnrS1 aac(6´)-Ib-cr	128	1024	6.3×10^{-9}	7.8×10^{-12}	4.9×10^{-16}	1.2×10^{-18}	8xMIC/4 $\log_{10}$ 16xMIC/5 $\log_{10}$	MIC/≥ 3 $\log_{10}$ 4xMIC/≥ 4 $\log_{10}$ 16xMIC/≥ 5 $\log_{10}$ 32xMIC/≥ 6 $\log_{10}$
E247	qnrS1 oqxB gyrA (S83L, D87Y) parC (S80I)	128	1024	4.1×10^{-12}	6.1×10^{-15}	3.9×10^{-17}	5.9×10^{-18}	4xMIC/3 $\log_{10}$ 8xMIC/≥ 4 $\log_{10}$ 16xMIC/≥ 5 $\log_{10}$	MIC/≥ 3 $\log_{10}$ 4xMIC/≥ 4 $\log_{10}$ 16xMIC/≥ 6 $\log_{10}$
E248	qnrS1	1	8	8.1×10^{-8}	4.0×10^{-13}	6.6×10^{-15}	2.4×10^{-16}	4xMIC/3 $\log_{10}$ 8xMIC/≥ 4 $\log_{10}$ 32xMIC/≥ 5 $\log_{10}$	MIC/≥ 3 $\log_{10}$ 4xMIC/≥ 4 $\log_{10}$ 8xMIC/≥ 5 $\log_{10}$
E308	qnrS1 gyrA (S83L, D87N) parC (S80I) parE (S458A)	128	1024	3.3×10^{-8}	7.2×10^{-11}	5.7×10^{-14}	6.2×10^{-18}	8xMIC/≥ 4 $\log_{10}$	MIC/≥3 $\log_{10}$ 4xMIC/≥ 4 $\log_{10}$ 8xMIC/≥ 5 $\log_{10}$

lecularly constructed E. coli strains (table 1). Individually, qnrS1 and aac(6´)-Ib-cr caused 16 and 32-fold an increase in the MICs of ENR, respectively. Coexistence of the genes increased the MIC of ENR from 0.032 µg/ml to 1 µg/ml (32-fold). MPCs of ENR ranged from 4 to 8 times the MIC and MPCs for E. coli isolates were markedly higher than MPCs for transformants and control stain (table 1). Transmissible genes (qnrS1, aac(6´)-Ib-cr) elevated MPCs of ENR from 0.128 µg/ml to 2 and 4 µg/ml for the transformants (16 and 32-fold). For some E. coli isolates (E103, E247, E308), MPCs of ENR were 8000 times the MPC for control strain.

The results of time-kill assays were presented in table 1. In time-kill assays, ENR did not achieve total bacterial elimination (≥ 6 log) within 8 hours and ENR concentrations required for bacterial elimination within 24 hours were lower than those for 8 hours. ≥ 5 log reduction against qnrS-containing MtS and E. coli E248 was achieved at maximum concentration used in the assays (32xMIC). However, ENR achieved total bacterial elimination only for MtS among transformants in 24 hours at 0.25 times the concentration used in 8 hours. An increase in bactericid-

al activity of ENR against transformants did not occur in 24 hours at all concentrations over ≥8 times the MIC. The same concentrations of ENR (8 times MIC) caused ≥ 4 log reduction against qnrS+aac(6´)-Ib-cr-containing MtSX and E. coli E103. Although, MtSX was inhibited 1 log less than E. coli E103 in the end of time-kill assay. QRDR mutations caused 1 log decrease in the inhibition of E. coli isolates (E247, E308) compared to MtS.
Table 1.

Discussion and Conclusion

PMQR genes confer low-level quinolone resistance (Strahilevitz et al., 2009). Transfer of qnr genes increases the MICs of FQs up to 125-fold according to the previous studies (Oloffson et al., 2007; Fortini et al., 2011). Whereas, aac(6´)-Ib-cr gene has lower potential than qnr genes to increase MICs of FQs (Dalhoff and Schimitz 2003; Robicsek et al., 2006; Strahilevitz et al., 2009; Emrich et al., 2010; Brailes et al., 2011; Chodhury et al., 2011). The results of this study showed that transfer of qnrS1 and aac(6´)-Ib-cr genes to E. coli reference strain caused 16 and 32-fold an increase in

the MICs of ENR, respectively.

MPC testing is technically more demanding than MIC testing (1010cfu⁄ml versus 105cfu⁄ml respectively) and may also offer some value for guiding optimal antimicrobial therapy as it provides practical information on drug concentrations necessary to restrict mutant growth (Dalhoff and Schimitz, 2003). MPC:MIC ratio of FQs vary depending on bacterial strain and its genotype and may reach up to 136 according to the previous studies (Randall et al., 2004; Wetzein, 2005; Oloffson et al., 2007). The results of this study showed that MPC:MIC ratio of ENR for transformants and E. coli isolates were 4 and 8, respectively. MPC:MIC ratio of ENR reached up 32-fold for qnrS+aac(6´)-Ib-cr-containing MtSX and 8000-fold for E. coli isolates compared to the control strain. Briales et al. (2011) reported that MPC:MIC ratio of FQ may increase up to 4000-16000 in the presence of QRDR mutations and PMQR genes in an E. coli strain. Mutation frequencies ranged from 5.1×10^{-16} to 3.8×10^{-10} at 1xMIC and were markedly higher for transformants and isolates compared to control strain. Mutation frequencies diminished up to 6.2×10^{-18} by increasing the concentrations used in the assay. Sub-MIC concentrations of FQs increase mutation frequencies in E. coli (Ozawa and Asai, 2013). In this study, transformants and E. coli isolates had markedly higher mutation frequencies than control strain at 1xMICs. This result supported that transmissible resistance genes increase the mutation possibility when MICs are used for the inhibition of bacteria. Brailes et al. (2011) also stated that the PMQR might enable mutant bacteria with low levels of FQ resistance to survive long enough for them to grow and emerge during FQ treatment. qnrS1-containing MtS had highest mutation frequency at 1xMICs. There is no significant difference between mutation frequencies of qnrS+aac(6´)-Ib-cr-containing MtSX and qnrS+aac(6´)-Ib-cr-containing E. coli E103 ($\sim \times 10^{-12}$). In this study, mutation frequencies decreased from 3.3×10^{-8} up to 6.2×10^{-18} in higher concentrations than MICs as reported previously (Kim et al, 2003; Drago et al., 2010).

In veterinary medicine ENR is administered by subcutaneous injection to cattle and intramuscular injection to pigs and orally to cattle, pigs turkeys and chickens, for the treatment of infections of respiratory and alimentary tract. The recommended doses are 2.5 to 5 mg/kg bw/day for 3 to 5 days (cattle and pigs) or 10 mg/kg bw/day for 3 to 10 days (chickens and turkeys) (EMA, 1998a). Cmax values of 0.45-2.7 µg/ml were attained 1 to 2 hours after administration

(EMA, 1998b). E. coli is a sensitive species given MIC values of 0.03 µg/ml against ENR (EMA, 1998a). Based on the results of this study, bactericidal activity of ENR can shortly be observed after administration against transmissible resistance gene-containing transformants and qnrS1-containing E. coli E248, but daily recommended doses (2.5-10 mg/kg bw/day) of ENR may not be caused desired inhibition after 8 and 24 hours of administration and mutation tendency of E. coli may increase. Jacobsen et al. (2012) reported that the cfu counts for isogenic qnrS-containing E. coli incubated in 4xMIC reduced during 5-24 hours and the cfu counts for 8-16xMIC remained stable during hours 5-24. In this study, 1 log reduction addition to the reduction recorded in 8 hours was observed in 24 hours. Bacterial inhibition remained stable only for qnrS1-containing E. coli E248.

In conclusion, mutation frequencies of E. coli strains reduced by the increasing of ENR concentrations. In the other hand, ENR is less active against E. coli strains in case of additional mutations in QRDR. The concentration-dependent bactericidal effect of ENR is only observable by the eight times the MIC and increasing concentrations did not alter the bactericidal activity. Therefore, dose optimization approach is not efficient to gain back the bactericidal activity of ENR which is one of the commonly used FQ in veterinary medicine. The combination therapy can be considered to fight with resistant E. coli instead of monotherapy and artificially constructed E. coli strains, which exhibit similar reactions against ENR with natural E. coli isolates, can be used to test the pharmacological efficacy of the combinations.

Acknowledgement

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