The Journal of Antibiotics (2009) 62, 109–111
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NOTE

Effect of lactoferricin on fluoroquinolone susceptibility of uropathogenic *Escherichia coli*

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The Journal of Antibiotics (2009) **62,** 109–111; doi:10.1038/ja.2008.22; published online 23 January 2009

Keywords: *Escherichia coli*; fluoroquinolones; lactoferricin B; MIC; resistance

Members of the *Enterobacteriaceae* family are mainly involved in the etiology of urinary tract infections, and *Escherichia coli* is by far the most common microorganism isolated from about 50% of all nosocomial and 90% of outpatients' urinary tract infections.[1] Quinolones are effective antibacterial agents that are commonly used as antimicrobials in the management of urinary tract infections, owing to which the rates of antimicrobial resistance among *E. coli* strains have increased greatly during the past two decades.[1,2] Bacteria are able to develop resistance by point mutations in chromosomal genes codifying DNA gyrase and topoisomerase IV targeted by quinolones.[3] Other mechanisms involve mutations affecting the accumulation of fluoroquinolones in the bacterial cell, such as the expression of outer membrane proteins and alteration in the lipopolysaccharide.[3] Furthermore, plasmid-mediated resistance has also been identified: *qnr* gene products capable of protecting DNA gyrase and AAC(6′)-Ib-cr, a variant aminoglycoside acetyltransferase, providing enzymatic antibiotic inactivation.[3]

The emergence of bacterial strains exhibiting resistance against conventional antibiotics has encouraged the search for novel antimicrobial strategies. Among the compounds that are currently under investigation for their therapeutic potential are a number of antimicrobial peptides.[4] The positively charged antimicrobial peptide lactoferricin B (Lfcin B), a 25-amino-acid peptide released from the N-terminal part of bovine lactoferrin (Lf) by gastric pepsin cleavage, has recently received attention due to its broad host defense properties against bacteria, fungi and parasites.[5,6] Lfcin B is active toward Gram-positive and Gram-negative species, including *E. coli*. This peptide contains many hydrophobic and positively charged residues, which enable its interaction with negatively charged biological membranes. In *E. coli*, depolarization and large effects on the integrity of cytoplasmic membrane have been shown.[6,7]

Lf and Lfcin B have been shown to be effective synergistic agents when used in combination with antibiotics.[6] Among Gram-negative bacteria, Lf enhances the sensitivity of *Pseudomonas aeruginosa* to chloramphenicol,[8] of *Stenotrophomonas maltophilia* to rifampin,[9] of *Salmonella enterica* to erythromycin[10] and of *E. coli* to novobiocin.[11] Lfcin B has been shown to act synergistically with erythromycin,[12] and a synergistic growth-inhibitory activity by bovine Lf lysate and gentamicin toward *E. coli* was observed.[13]

In this study, we primarily analyzed the susceptibility of uropathogenic *E. coli* strains to fluoroquinolones. As *E. coli* fluoroquinolone resistance has been associated with reductions in virulence traits and shifts from the phylogenetic group B2 toward groups A, B1 or D,[2] we then compared antibiotic resistance with the strains belonging to phylogenetic groups and with the occurrence of some capsular determinants that can be considered as cell protection genes. In succession, to gain insight into the interference of natural peptides with susceptibility to fluoroquinolones, we examined whether Lfcin B could influence the activity of norfloxacin and ciprofloxacin toward these strains.

E. coli strains were isolated from the urine of subjects attending a private medical practice at BIOS s.p.a. Microbiology Laboratory and Hospital, Umberto I BIT 05 Microbiology Laboratory, and identified using standard methods. The strains were stored in 15% glycerol at −80 °C and subcultured in Brain Hearth Infusion broth (Oxoid, Rome, Italy) at pH 6.8 for further analysis. *E. coli* ATCC 25922 was used as the bacterial reference strain.

The phylogenetic grouping of *E. coli* strains was determined by PCR. The three candidate genes were *chuA* 279-bp, *yjaA* 211-bp and an anonymous DNA fragment designated TSPE42 152-bp. The phylogroup classification (A, B1, B2, D) was made on the basis of the presence of specific PCR-amplified fragments according to Kanamaru *et al.*[14]

E. coli were screened by PCR for cell protection genes associated with three capsule groups. The tested genes were *kpsMT*K1 (153 bp), *kpsMT* II (272bp) and *kpsMT*K5 (159 bp). Primer sequences, PCR mixtures and conditions were as published by Johnson and Stell.[15]

Antimicrobial susceptibility tests were carried out according to the National Committee for Clinical Laboratory Standards guidelines[16] for the antibiotics cephalotin, cefotaxime, amoxicillin, gentamicin,

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Received 25 July 2008; accepted 4 December 2008; published online 23 January 2009

ciprofloxacin, norfloxacin, nitrofurantoin and co-trimoxazole, by the automated microdilution method Vitek2 (Biomérieux, Rome, Italy).

Norfloxacin and ciprofloxacin, used in minimal inhibition concentration assays, were purchased from Sigma-Aldrich (Milan, Italy). Lfcin B (FKCRRWQWRMKKLGAPSITCVRRAF) was synthesized by GenScript Corporation (Piscataway, NJ, USA). The chemicals were dissolved in double-distilled water and stored at −20 °C until used.

The minimal inhibitory concentrations (MICs) of the drugs were determined using a standard microdilution technique in 1.0% Bacto Peptone Water (DIFCO Lab, Detroit, MI, USA), pH 6.8, with a log-phase inoculum of 1×10^4 CFU ml^{-1}. Polystyrene 96-well plates (Nunc, Rochester, NY, USA) were incubated at 37 °C up to 24 h. After incubation, the optical density was determined in each well at 590 nm. The MIC was defined as the lowest concentration of the drug at which bacterial growth was inhibited. All tests were carried out in triplicate and the results were averaged.

Synergy testing was performed to determine the *in vitro* ciprofloxacin and norfloxacin interactions with Lfcin B by the fractional inhibitory concentration (FIC) index.[17] The FIC index calculation was performed according to Vorland *et al.*:[12] synergy was defined as the condition when the FIC index was <0.5, partial synergism as when $0.5<\text{FIC}<1$, indifference as when $1<\text{FIC}<4$, and antagonism as when the FIC index was >4. Checkerboard test results represented the average of triplicate testing for each isolate.

Fifty-four *E. coli* strains isolated from the urine samples of clinical and community UTI patients were submitted to antimicrobial susceptibility tests performed routinely for Gram-negative bacteria. The results obtained were evaluated according to clinical criteria as the percentage of sensitive, intermediate or resistant strains. Among the isolates examined, a noticeable resistance to most of the drugs tested was observed (results not shown), and the prevalence averaged around 22.2% for norfloxacin and ciprofloxacin. In Table 1, the susceptibility of *E. coli* isolates exhibiting resistance to fluoroquinolones, of sensitive isolates and of the ATCC 25922 reference strain, as well as the presence of cell protection genes codifying for the expression of extracellular capsule polysaccharides and phylogenetic grouping, is reported. *E. coli* strains sensitive to fluoroquinolones exhibited MIC values 2–8-fold higher than those of the ATCC 25922 reference strain. Three quinolone-susceptible *E. coli* strains belonged to the phylogenetic group B2 and one to group D, whereas, among the resistant ones, three, four and five strains belonged to A, B2 and D groups, respectively. The three cell protection genes tested—*kpsMT*K1, *kpsMT* II, *kpsMT*K5—were randomly distributed in both quinolone-susceptible and -resistant strains; *kpsMT* II capsule gene was the most frequent.

Then trials were performed to assess the MICs of the peptide Lfcin B toward the growth of *E. coli* isolates. Lfcin B was serially twofold diluted, starting from the concentration of 50 down to 0.097 μg ml^{-1}. The results obtained showed that 12.5 μg ml^{-1} was the dose capable of completely inhibiting bacterial growth in all strains tested and 3.12 μg ml^{-1} corresponded to the sub-inhibiting concentration, because at this concentration, after 24-h incubation at 37 °C, no decrease in the optical density, as compared with Lfcin B-untreated controls, was observed.

To determine Lfcin B–fluoroquinolone interactions, the FIC index was evaluated for each strain by combining different concentrations of norfloxacin, ciprofloxacin and Lfcin B. Table 2 summarizes the synergy data. The combination of Lfcin B with the quinolones had synergistic or partial synergistic effects toward both resistant and sensitive strains. Synergy was associated with a decrease of two or more dilutions of MIC values and was observed for both quinolones in two resistant strains. Partial synergism was associated with a decrease up to two dilutions of MICs: ciprofloxacin showed partial synergism with Lfcin B against six *E. coli* isolates (two susceptible and four resistant strains) whereas norfloxacin showed partial synergism against four isolates (one susceptible and three resistant strains). Indifference between the antibacterial agents used and Lfcin B was observed in the remaining uropathogenic *E. coli* tested and in the reference strain, whereas antagonism was never detected. Hence, in response to this association, most of the strains showed a variation in MIC values: synergistic effect was observed for both drugs in 12.5% of strains, whereas partial synergism was observed for ciprofloxacin in 37.5% of strains and for norfloxacin in 20% of strains.

In agreement with literature data,[1] the results from this investigation showed that, in a noticeable percentage of uropathogenic *E. coli*

Table 1 Susceptibility to fluoroquinolones of *E. coli* strains and distribution of phylogenetic groups and *kpsMT*K1, *kpsMT*II and *kpsMT*K5 capsule genes

Strains s,[a] r[b]	*MIC (μg ml^{-1}) ciprofloxacin*	*MIC (μg ml^{-1}) norfloxacin*	*Phylogenetic group*	kpsMT*K1*	kpsMT*II*	kpsMT*K5*
ATCC 25922	0.05	0.05	B2	–	+	+
C86 s	0.78	0.78	B2	+	+	–
O39 s	0.39	0.19	B2	+	+	–
C38 s	3.12	3.12	B2	–	+	+
C43 s	0.39	0.39	D	+	–	–
C58 r	50	12.5	B2	–	+	+
O12 r	25	12.5	D	+	+	+
C24 r	50	100	D	–	+	+
C25 r	12.5	100	D	–	+	+
C20 r	50	25	B2	+	+	–
C31 r	50	50	A	+	–	–
C69 r	100	100	B2	+	+	+
C87 r	50	50	D	–	+	+
C104 r	100	100	B2	–	+	+
C111 r	50	50	A	–	–	–
C134 r	50	50	A	–	–	–
O25 r	200	200	D	+	+	–

Fluoroquinolone susceptibility: [a]sensitive strain, [b]resistant strain.

Table 2 FIC index and interaction of fluoroquinolones in combination with Lactoferricin B towards *E. coli* strains

Strains s[a], r[b]	*Ciprofloxacin*		*Norfloxacin*	
	FIC index	*Interaction*	*FIC index*	*Interaction*
ATCC 25922	1.5	Indifference	1.5	Indifference
C86 s	0.7	Partial synergism	0.7	Partial synergism
O39 s	0.7	Partial synergism	1.5	Indifference
C38 s	1.5	Indifference	1.5	Indifference
C43 s	1.5	Indifference	1.5	Indifference
C58 r	0.3	Synergism	0.4	Synergism
O12 r	0.9	Partial synergism	0.9	Partial synergism
C24 r	0.7	Partial synergism	0.6	Partial synergism
C25 r	0.6	Partial synergism	1.5	Indifference
C20 r	1.5	Indifference	1.5	Indifference
C31 r	1.5	Indifference	1.5	Indifference
C69 r	1.5	Indifference	1.5	Indifference
C87 r	0.7	Partial synergism	0.8	Partial synergism
C104 r	1.5	Indifference	1.5	Indifference
C111 r	1.5	Indifference	1.5	Indifference
C134 r	0.4	Synergism	0.4	Synergism
O25 r	1.5	Indifference	1.5	Indifference

Abbreviation: FIC, fractional inhibitory concentration.
Fluoroquinolone susceptibility: [a]sensitive strain, [b]resistant strain.

isolates, a high level of resistance to fluoroquinolones was present. However, the investigation of *E. coli* phylogroups and cell protection genes related to pathogenicity failed to individuate phylogenetic traits or capsule genes associated with fluoroquinolone resistance. Data obtained with synergy tests showed that the cationic peptide Lfcin B used alone had a powerful inhibiting effect toward all uropathogenic *E. coli* isolates investigated, and that in 50% of the strains examined the combination of Lfcin B with fluoroquinolones determined a decrease of the MICs (synergism or partial synergism).

The overall results achieved on interaction between Lfcin B and fluoroquinolones in both *E. coli*-susceptible and -resistant strains are possibly due to the membrane-disorganizing nature of this peptide,[6,7] which leads not only to increased permeability through the bacterial cell wall but also to the dissipation of the proton-motive force, resulting in decreased activity of ATP-dependent multi-drug efflux pumps.[6] At sub-inhibiting concentrations, Lfcin B may affect the access/efflux of drugs, thus modifying the quinolone concentrations required to inhibit growth.

Furthermore, the results obtained allow the assumption that Lfcin B could act on *E. coli* strains, independently from their resistance or susceptibility to fluoroquinolones, causing different events: a synergistic or semi-synergistic action with consequent decrease of MIC values, or indifference. The fluoroquinolones exert their inhibitory action by forming a stable complex with the DNA and the target enzyme.[18] Thus, a clarification of the behavior shown by both sensitive and resistant *E. coli* strains in combination experiments can be related to both altered quinolone uptake and mutational events in different enzyme regions holding amino acids involved in the interaction with fluoroquinolones. A silent mutation in drug susceptibility could still influence the kind of interaction between the drugs and the enzyme: this could be the case of the sensitive strains C86 and O39, in which the response to the association of fluoroquinolones with Lfcin B is semi-synergistic with a consequent decrease of MIC values. Moreover, the presence of movable resistance DNA gyrase protective genes, such as *qnr*A, *qnr*B, *qnr*S and/or AAC(6′)-IB-cr,[3] in the *E. coli* strains could also influence the response to fluoroquinolones–Lfcin B association. To better understand the mechanisms of interaction between Lfcin B and these drugs, resulting in synergism, a molecular analysis of the major genes implicated in resistance will be required.

Interestingly, experimental data in mice showed that oral administration of Lf and derivative peptides is effective in reducing infection and inflammation at the level of the urinary tract, through the transfer of Lf or its peptides to the site of infection via renal secretion,[19] suggesting that Lfcin B–fluoroquinolone association might represent an approach to control the growth of uropathogenic *E. coli*.

Taken together, these results could be of interest as the association of fluoroquinolones with the antibacterial peptide Lfcin B could allow the use of these therapeutic agents at lower concentrations for a reasonable number of *E. coli* strains. Moreover, this association could allow extending the prescription of drugs that otherwise should be discarded because of the increased resistance of bacteria to them worldwide.[1–3]

ACKNOWLEDGEMENTS

This work was supported by grants from MIUR to Dr L Seganti and S Schippa.

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