Assessment of epsilometer test over molecular detection for quinolone resistance in *Escherichia coli* and *Klebsiella pneumoniae* clinical isolates: A predictable schedule on routine basis

Ali PorMohammad[1,2], Alka Hasani[1,2*], Forough Shams[1,2], Mohammad Aghazadeh[2], Mohammad Reza Nahaie[2], Mohammad Ahangarzadeh Rezaee[2], Akbar Hasani[3]

[1] Research Center of Infectious Diseases and Tropical Medicine, Faculty of Medicine, Tabriz University of Medical Sciences, Tabriz, Iran
[2]Department of Medical Microbiology, Faculty of Medicine, Tabriz University of Medical Sciences, Tabriz, Iran
[3]Department of Clinical Biochemistry and Laboratory Sciences, Faculty of Medicine, Tabriz University of Medical Sciences, Tabriz, Iran
* Corresponding author: dr.alkahasani@gmail.com

Abstract: Background: Uprising quinolone resistance in *Enterobactericeae* isolates is a concern and its detection on daily basis necessitates a reproducible and specific method. Molecular methods are accurate, however, needs expertise and are cost effective in hospital based care setting. We aimed at evaluation of epsilometer test (E- test) for detection of quinolone resistance in *E.coli* and *Klebsiella pneumoniae* isolated in a University teaching and treatment center in Northwest Iran. **Materials and Methods**: One hundred and thirty four isolates including *E.coli* and *K.pneumoniae* isolates found resistant to either ciprofloxacin or nalidixic acid or both on disk agar diffusion were subjected for evaluation of Minimum inhibitory concentration (MICs) by E-test for as per the guidelines of clinical and laboratory standards institute (CLSI) and the manufacturer (Liofilchem, Italy). The resistance or susceptibility to quinolones was confirmed by the presence of *gyrA* (yielding a 648-bp product), and *parC* (yielding a 395-bp product) in these isolates polymerase chain reaction (PCR). **Results**: Among 63 *K.pneumoniae* and 71 *E.coli* isolates resistant to ciprofloxacin or nalidixic acid or both antibiotics on disk diffusion, 61.9% *K.pneumoniae* and 92.9% *E.coli* were found to have ciprofloxacin MIC in the resistance level, while for all MIC of nalidixic acid was >256 µg/ml. Among *K.pneumoniae* isolates, 93.65% were positive for *gyrA*, and 88.88% for *parC*. Almost similar observation was revealed in *E.coli* isolates with *gyrA* being detected in 98.59 % isolates and *parC* in 97.1% isolates. Among quinolone resistant isolates confirmed by MIC equal or above the resistance breakpoint, presence of these two genes provided definite evidence. **Discussions:** E-test offers a simple, inexpensive, rapid, and easier alternative for detection of quinolone resistance, thus it is recommended that in the light of high resistance, prior to treatment regimen, the status of resistance to be accurately defined for suitable selection of antibiotic and prevention of microbial resistance.

[Ali PorMohammad, Alka Hasani, Forough Shams, Mohammad Aghazadeh, Mohammad Reza Nahaie, Mohammad Ahangarzadeh Rezaee, Akbar Hasani. **Assessment of epsilometer test over molecular detection for quinolone resistance in *Escherichia coli* and *Klebsiella pneumoniae* clinical isolates: A predictable schedule on routine basis.** *Life Sci J* 2014;11(12s):1027-1031]. (ISSN:1097-8135). http://www.lifesciencesite.com. 222

Key words: Epsilometer, quinolone resistant, *Escherichia* coli, *Klebsiella pneumonia*

1.Introduction:

Quinolones are clinically useful and widely used antibiotics for the treatment of bacterial human and animal infections (1-3).Their wide use has triggered increased bacterial resistance worldwide(1). In Iran, recently increasing percentage of quinolone-resistant *E. coli* isolates have been giving cause for concern and in other countries start to rise the percentage of quinolone resistant *E. coli* isolates at the start of the new century (4). The DNA gyrase is molecular target in bacteria that consists of A and B subunits (5-7). Quinolone resistance mechanisms in gram-negative bacteria include chromosomal mutations, impermeability, active efflux, target modification, antibiotic neutralization, reduce levels of quinolone accumulation in the cells (1, 8-13). Genetic determination of FQs(fluoroquinolones) resistance is generally linked to chromosomal mutations affecting the quinolone resistance-determining regions (QRDR) of DNA gyrase (*gyrA* and *gyrB*) and topoisomerase IV (*parC* and *parE*) (1). Mutations in *gyr*A are the most common mechanism involved in quinolone resistance among bacteria (3).

The resistance patterns associated with *gyrA* mutations are generally characterized by higher MICs of nalidixic acid than other FQs (10, 12, 14). Two mechanisms were involved in this peculiar pattern of resistance: one was an altered gyrase due to a transition from G to A at position 242 of the *gyrA* gene, leading to a Gly81Asp substitution in the gyrase A subunit, while the other was an impaired accumulation (12). In recent years several methods have been developed for antimicrobial susceptibility testing. Disk agar diffusion is a traditional and routine

method of antimicrobial sensitivity testing. E-test provides a rapid and convenient means for determining minimal inhibitory concentration (MIC) for a variety of antimicrobial agents. Studies have shown that E-test shows good agreement with reference "agar dilution" susceptibility testing methods (15). MIC determining methods like E-test, although provide quantitative measurement of antimicrobial sensitivity because of their cost and limited availability in developing countries, their application is not as frequent as disk diffusion method (16). Thus, we aimed at assessing epsilometer test over molecular detection for quinolone resistance in *Escherichia coli* and *Klebsiella pneumoniae* clinical isolates.

2. Materials and methods:

This study was carried out on the routine clinical specimens including, blood, wound, urine, endotracheal tube and various body fluids, being sent from various wards and intensive care units (ICU) and sent to Division of Microbiology during a period of six months from June- November, 2013. The samples were processed for the isolation and identification of *E.coli* and *K. pneumoniae* according to phenotypic methods as described elsewhere (17).

Antibiotic susceptibility testing was performed on all isolates using the Kirby – Bauer method and quinolone resistance was confirmed by ciprofloxacin and nalidixic acid E-tests as described by the CLSI (2009)(18). The interpretive breakpoints for the minimum inhibitory concentrations (MICs) to nalidixic acid were ≤8 µg /ml as susceptible and ≥32 µg/ml as resistant. The interpretive breakpoints for the MICs to ciprofloxacin were ≤1 µg/ml as susceptible and ≥4 µg/ml as resistant(19). ATCC 25922 *E. coli* reference isolate served as the standard drug-susceptible control for disk diffusion and MIC measurements.

The strains were preserved at −70°C in nutrient broth containing 15% v/v glycerol (20).

The gene *gyrA* was amplified with primers 5_TTAATGATTGCCGCCGTCGG-3 and 5-TACACCGGTCAACATTGAGG-3 (yielding a 648-bp product) and *parC* was amplified with primers 5-AAACCTGTTCAGCGCCGCATT-3 and 5-GTGGTGCCGTTAAGCAAA-3 (yielding a 395-bp product) to amplify the quinolone resistance-determining region (QRDR) present in all clinical isolates(21, 22).The annealing temperature was same as reported previously but with slight modifications (21).

3. Results:

Among 134 isolates, 71 *E.coli* were isolated from wound (n=9), blood (n=12) and urine (n=50); and *K.pneumoniae* isolates were isolated from wound (n=26), blood (n=19) and urine (n=16) *E.coli* was the predominant organism in the urine specimen than *K.pneumoniae*. On the other hand, *K.pneumoniae* was the most frequently isolated bacteria in blood cultures, however, the association was not found statistically significant. No significant difference was observed in the prevalence of either or both pathogens from other clinical specimens. On disk agar diffusion assay, *E.coli and K.pneumoniae* isolates obtained from urine specimens were found resistant (n=125; 88.65%) or intermediately resistant (n=16; 11.34%) to nalidixic acid, while 134 (71.05%) isolates, including 63 (47.01%) *K.pneumoniae* and 71 (52.98%) *E.coli*, irrespective of clinical source, were observed resistant to ciprofloxacin by disk agar diffusion method. In order to quantify this quinolone resistance, the MIC of ciprofloxacin and nalidixic acid was determined by E-test (Table 1).

Among *K.pneumoniae* isolates, all were resistant to nalidixic acid, but ciprofloxacin susceptibility varied: 19 (30.15%) isolates were ciprofloxacin-susceptible (MICs ≤1 µg/ml), 5 (7.93%) ciprofloxacin-intermediate (MICs ranged from 1-4 µg/ml), and 39 (61.9%) ciprofloxacin-resistant isolates (MICs ≥ 4 µg/ml). Among 71 *E.coli*, only one of them (1.4%) was nalidixic acid susceptible (MICs ≤8 µg/ml), while 5 (7.04%) were ciprofloxacin-susceptible (MICs of ≤1 µg/ml), and 66(92.95%) isolates were ciprofloxacin-resistant (MICs ≥ 4 µg/ml).

Among *K.pneumoniae* isolates, 59 (93.65%) isolates were observed to have *gyrA*, while *parC* was positive in 56 (88.88%). *gyrA* was revealed in 70 (98.59 %) *E.coli* isolates and 69 (97.18%) of them were positive for *parC*.

Interestingly, among 19 *K.pneumoniae* isolates found ciprofloxacin-susceptible, we detected *gyrA* in 18 (94.73%), and *parC* in 15(78.94%) isolates. Amongst 5 *K.pneumoniae* ciprofloxacin-intermediate isolates 4(80%) had shown presence of *gyrA*, 3(60%) *parC*, and all 39 *K.pneumonaie* ciprofloxacin-resistant isolates were *gyrA* and *parC* positive, and between 5 *E.coli* ciprofloxacin-susceptible isolates, we detected 4 (80 %) isolates to possess *gyrA*, 3 (60%) and *parC*, while all *E.coli* ciprofloxacin-resistant isolates (n=66) disclosed to have *gyrA* and *parC* genes (Table 1)

4. Discussion:

FQs are widely used antibiotics for urinary or intestinal tract infections, to nosocomial infections, to agricultural usage. The prevalence of resistance among *E.coli* also varies, between species and geographical regions, but uprising quinolone resistance in *Enterobactericeae* isolates is a concern. Mechanisms underlying FQ resistance, long thought to be confined to vertical inheritance, are now being

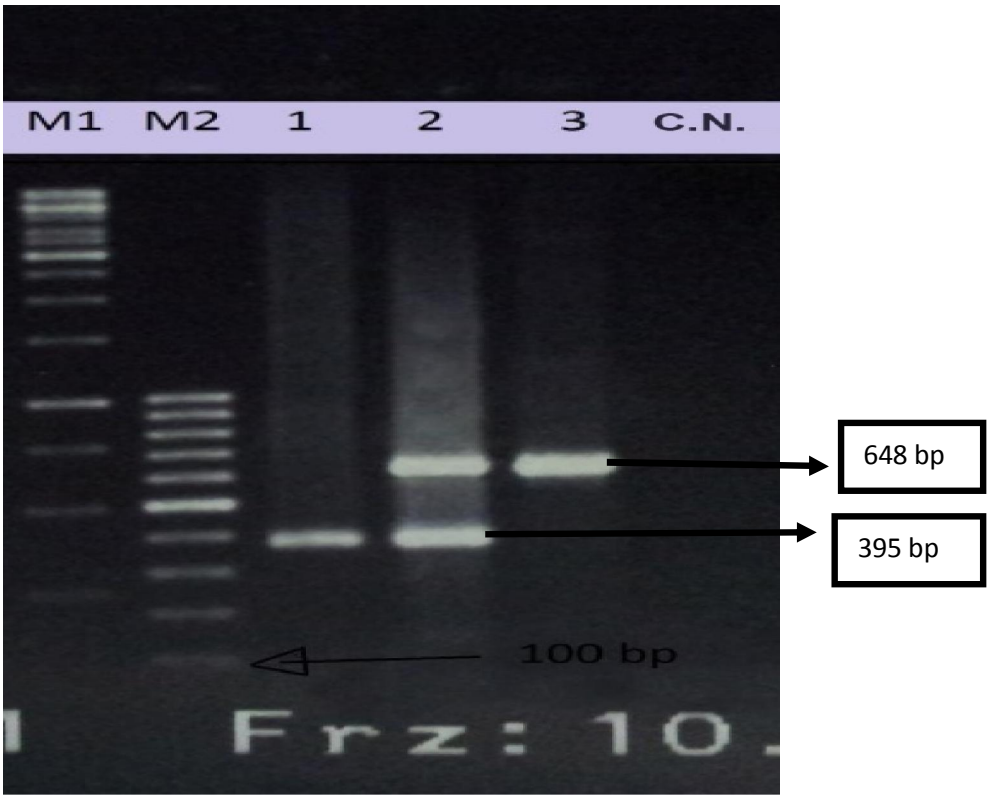
spread horizontally, either as in the plasmid-borne qnr genes of enteric bacteria or as in the transformation of chromosomal DNA, which was observed in streptococci (23).

In a study that carried out by Seyedipor and his coworkers in Tehran, Iran (24), of 52 *K.pneumoniae* isolates, 23 (44.23%) were found resistant to quinolones. In another quinolone-resistance surveyed in Iran in 140 *E.coli* isolates showed that 116 (82.8%) and 63 (45%) were nalidixic acid and ciprofloxacin resistance respectively on PCR (25). In the present investigation relation between results obtained from PCR and E- test did not show compatibility and was similar to other research carried out on the presence of *gyrA* and *parC* genes and detection of quinolone resistance by E-test, in *Campylobacter jejuni* and *Campylobacter coli* in Egypt (26). This is in agreement with the fact that mere presence of quinolone resistance genes is not a mandatory evidence of such resistance. Mutation study carried by other authors shows significant relation between *gyrA* and *parC* and quinolone resistance in isolates (16, 21, 24-26).

Table 1: Relation between presence of quinolone resistance on E- test and PCR

	MIC		Gene	
	CIP*	NA*	*gyrA*	*parC*
E.coli (n=71)				
1	0.025	-	-	-
1	0.08	8	+	-
1	0.25	>256	+	+
1	0.75	>256	+	+
1	1	>256	+	+
2	16	>256	+	+
32	>32	>256	+	+
32	>32		+	+
K.pneumoniae (n=63)				
1	0.019	-	+	+
1	0.025	-	+	+
1	0.032	-	+	+
2	0.047	-	+	+
2	0.064	>256	+	+
1	0.064	-	+	+
1	0.064	-	+	-
1	0.094		+	+
1	0.19		-	-
1	0.38	48	+	+
1	0.38	-	+	-
1	0.5	-	+	-
1	0.64	32	+	+
1	0.75	-	+	+
1	1	>256	+	+
2	1		+	+
1	1.5	>256	-	-
2	1.5		+	+
1	2		+	-
1	2.5		+	+
1	4		-	-
1	8		-	+
9	>32	>256	+	+
20	>32		+	+
6	>32	>256	+	+
2	>32	12	+	+

CIP :Ciprofloxacin; NA: Nalidixic acid



M1 (Marker 1kb)
M2 (Marker 100 bp)
Lane 1: *parC* (395 bp)
Lane 2: *parC* and *gyrA* (648 bp and 395 bp respectively)
Lane 3: *gyrA* (395 bp)
C.N: Control negative (PCR without DNA)

Figure 1: Electrophoresis of PCR product on 2% agarose gel

Acknowledgement

This work was supported by grant from the Research Center of Infectious diseases and Tropical Medicine, Faculty of medicine, Tabriz University of Medical Sciences, Tabriz, Iran (Grant No.92-10). Dr.Behrouz Naghili and Dr.Mojtaba Varshochi, Professors of Infectious Diseases are being thanked for their perpetual support for burn patients.We thank Ms. Zeila KhajeMohammadi for her technical assistance in the collection of clinical isolates and phenotypic workup.This manuscript is part of MSc thesis of first author (Thesis No. 92/2-3/1)

References:

1. Ambrozic Avgustin J KR, Zerjavic K, Orazem T, Grabnar M. Emergence of the quinolone resistance-mediating gene aac(6')-Ib-cr in extended-spectrum-beta-lactamase-producing *Klebsiella* isolates collected in Slovenia between 2000 and 2005. Antimicrob Agents Chemother. 2007;514171-4173.
2. Chu DTW, and P. B. Fernandes. Structure-activity relationships of the fluoroquinolones. Antimicrob Agents Chemother. 1989;33:131–5.
3. Rosen T. The fluoroquinolone antibacterial agents. Prog Med chem. 1990;27:235–95.
4. Karami A NK, Sorouri R, Ranjbar R, Khalilpo A. Use Of a Mama-Pcr Method To Detect Gyra Mutations In Nalidixic Acid Resistant Clinical Isolates Of Escherichia Coli.. Iranian J Publ Health. 2008;37:42-7.
5. Gellert M, K. Mizuuchi, M. H. O'Dea, and H. A. Nash. DNA gyrase: an enzyme that introduces superhelical turns into DNA: Proc. Natl. Acad.Sci. USA.1976: 3872–3876.
6. Horowitz DS, and J. C. Wang. Mapping the active site tyrosine of Escherichia coli DNA gyrase. J. Biol. Chem.1987;262:5339–44.
7. Maxwell A, and M. Gellert. The DNA dependence of the ATPase activity of DNA gyrase. J Biol Chem. 1984;259:14472–80.

8. Crumplin GC. Mechanisms of resistance to the 4-quinolone antibacterial agents. J Antimicrob Chemother. 1990;26:131–44.
9. Denis A, and N. J. Moreau. Mechanisms of quinolone resistance in clinical isolates: accumulation of sparfloxacin and of quinolones of various hydrophobicity, and analysis of membrane composition. J Antimicrob Chemother. 1993;32:379–92.
10. Heisig P, and R. Tschorny. Characterization of fluoroquinolone resistant mutants of *Escherichia coli* selected in vitro. Antimicrob Agents Chemother. 1994;38:1284–91.
11. Kumagai Y, J. I. Kato, K. Hoshino, T. Akasaka, K. Sato, and H. Ikeda. Quinolone-resistant mutants of *Escherichia coli* DNA topoisomerase IV parC gene. Antimicrob Agents Chemother. 1996;40:710-4.
12. Vila J, J. Ruiz, F. Marco, A. Barcelo, P. Goni, E. Giralt, and T. Jimenez De Anta. Association between double mutation in gyrA gene of ciprofloxacin-resistant clinical isolates of *Escherichia coli* and MICs. Antimicrob Agents Chemother. 1994;38:2477–9.
13. Yoshida H, M. Nakamura, M. Bogaki, H. Ito, T. Kojima, H. Hattori, and S.Nakamura. Mechanism of action of quinolones against *Escherichia coli* DNA gyrase. Antimicrob Agents Chemother. 1993;37:839–45.
14. Willmott CJR, and A. Maxwell. A single point mutation in the DNA gyrase A protein greatly reduces binding of fluoroquinolones to the gyrase-DNA complex. Antimicrob Agents Chemother. 1993;37:126–7.
15. Anand Manoharan RP, V. Shankar, Kurien Thomas & M.K. Lalitha. Comparison of disc diffusion & E test methods with agar dilution for antimicrobial susceptibility testing of Haemophilus influenzae. Indian J Med Res. 2003;117:81-7.
16. F. BD. The E-test challenged with selected strains. Diagn Microbiol Infect Dis 1992;15:465–8.
17. Lehman MCD MGe. *Enterobacteriaceae* In: Textbook of diagnostic microbiology. 4th, Saunders, Elsevier, Maryland Heights, Missouri. 2011. 427-50.
18. National Committee for Clinical Laboratory Standards (1998) Performance Standards for Antimicrobial Susceptibility Testing; 8th Informational Suppl. NCCLS document M100-S8. National Committee for Clinical Laboratory Standards, Wayne, PA.
19. C.Forbes BA SD, Weissfeld AS Enterobacteriaceae In: Bailey PMT and Scott's Diagnostic Microbiology. 13th, Elsevier Mosby. 2014. 307-15.
20. Sambrook J RD. Molecular cloning: a laboratory manual Cold spring harbor laboratory press Cold Spring Harbor, New York; 2001.
21. Bansal S TV,. Contribution of mutations in DNA gyrase and topoisomerase IV genes to ciprofloxacin resistance in *Escherichia coli* clinical isolates. Int J Antimicrob Agents. 2011;37:253-5.
22. Vasilaki O NE, Ikonomidis A, Sofianou D, Frantzidou F, Alexiou-Daniel S, et al,. Emergence of the plasmid-mediated quinolone resistance gene qnrS1 in *Escherichia coli* isolates in Greece. Antimicrob Agents Chemother. 2008;52: 2996-7.
23. Balsalobre MAAC, M., Santos, P. M, Penati, M. A., Demetrio, C. G. B. Ruminal kinetics of irrigated Tanzaniagrass under three post grazed stubbles intensities. Rev Bras Zootec. 2003;32(6):1747-62.
24. Seyedpour SM, Eftekhar F. Quinolone Susceptibility and Detection of qnr and aac(6')-Ib-cr Genes in Community Isolates of *Klebsiella pneumoniae*.Jundishapur J Microbiol. 2014;7(7): e11136.
25. Firoozeh F1 ZM, Soleimani-Asl Y,. Detection of plasmid-mediated qnr genes among the quinolone-resistant *Escherichia coli* isolates in Iran. J Infect Dev Ctries. 4014;8(7):818-22.
26. Mayar M. Said HE-M, Fawkia M. El-Beih, et al. Detection of gyrA mutation among clinical isolates of *Campylobacter jejuni* isolated in Egypt by MAMA PCR. J Infect Dev Ctries 2010;4(9):546-54.

11/26/2014