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Research Article

Carriage of Class 1 and 2 Integrons in Quinolone, Extended-Spectrum-β-Lactamase-Producing and Multi Drug Resistant *E.coli* and *K.pneumoniae*: High Burden of Antibiotic Resistance

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Abstract

Purpose: The study aimed at assessing any association between quinolone resistance, MDR and ESBL production and their relation with the presence of integrons in *Esherichia coli* and *Klebsiella pneumoniae.*

Methods: *E.coli* and *K.pneumoniae* isolated from various clinical infections were fully identified and analyzed for being quinolone resistant. These isolates were further tested for ESBL production, multi drug resistance and carriage of integrons.

Results: In total, 135 isolates were confirmed as quinolone resistant. *K.pneumoniae* was observed as potent ESBL producer in comparison to *E.coli*. Ciprofloxacin resistance in both organisms was related significantly with the presence of integron class 1, co-presence of class 1 and 2 as well as to the presence of ESBL production ($p< 0.001$). However, nalidixic acid resistance was related significantly ($p< 0.01$) with only integron class 1 and to the presence of ESBL production. Class 1 and 2 integrons were found in 73.5% of MDR isolates with 13.2% of them possessing both *intI*1 and *intI*2 genes.

Conclusion: Prevalence of quinolone resistance together with ESBL production and MDR in *E.coli* and *K.pneumoniae* has contributed to the emergence of antibacterial resistance burden. The higher integron prevalence in our isolates advocates the potentiality of these isolates as a source for dissemination of resistance determinants.

Introduction

In contradiction of nalidixic acid, which is used only for urinary infections, the fluoroquinolones (FQ) have a broad range of therapeutic indications and in fact, were a major therapeutic advance of the 1980s.[1] Nevertheless, recent years have witnessed FQ resistance in *Escherichia coli* (*E.coli*) and other Enterobacteriaceae,[2] contingent on multiple mutations that diminish the affinity of its topoisomerase II and IV targets in various ways.[3] Alarmingly, however, is the upward trend observed in last two decades in the co- occurrence of ciprofloxacin resistance with resistance to beta-lactam antibiotics in *E.coli* and *Klebsiella pneumoniae*.[4-7] Furthermore, emergence of multi drug resistance (MDR) and Extended-Spectrum β-Lactamase (ESBL) production in *E.coli* and *Klebsiella pneumoniae*[8] in chorus with FQ resistance has knockdown the infrastructure of therapy substantially.[9] As regards to development of antibiotic resistance, the dissemination of resistance genes among bacterial strains is being debated frequently. One type of dynamic force which is perceived as a major crisis is located on the bacterial chromosome or a plasmid, and named as an integron helps bacteria to acquire novel combinations of resistance genes[10,11] and disseminate them along with the emergence of MDR strains.[10,12-14] Five integron classes related to antibiotic resistance have been described based on the homology of their integrase genes and Class 1 integrons are the most commonly found in nosocomial and community environments, followed by class 2 ones.[15] The prevalence of integrons is high among gram-negative isolates from patients in Europe.[14] Similar reports are available from Asian and Middle East countries.[16-18]

Our hospital is a tertiary University teaching institution, and a reference health care center for North West Iran. Similar to any hospital elsewhere worldwide, *E. coli* and *K. pneumoniae* are the two most isolated organisms from patient's samples in the hospital's microbiology section and for the last five years an upward trend in the

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incidence of quinolone resistance was observed in these clinical isolates. Thus, this study was taken up to uncover the level of association between quinolone resistance, MDR and ESBL production with the presence of integrons, in *E.coli* and *K.pneumoniae* isolated from a selected high risk groups of hospital admitted patients and those being referred as outpatients. To the best of our knowledge, this is the first report on the association between presence of integrons in quinolone resistant, ESBL production and MDR *E.coli* and *K.pneumoniae* from North West Iran.

Materials and Methods

Bacterial isolates and Antibiotic susceptibility testing

During a period of nine months, 234 *E.coli* and *K.pneumoniae* isolated from various clinical infections were fully identified according to standard bacteriological procedures.[19,20] Duplicate isolates from the same patient were excluded. These isolates were subjected to routine antibiotic susceptibility testing performed by disc agar diffusion method.[21] The antibiotics included were gentamicin (10µg), amikacin (30µg), ceftriaxone (30µg), ceftazidime (30µg), imipenem (10µg), co-trimoxazole (1.25µg), nalidixic acid (30µg), ciprofloxacin (5µg), cefamandole (30µg) and ceftizoxime (30µg) (Mast Diagnostics, UK). MDR was defined as resistance to 3 or more different group of antibiotics. FQ and nalidixic acid resistance was confirmed for non-susceptibility by minimum inhibitory concentration (MIC) on E-test (Liofilchem) performed according to manufacturer's instructions with interpretative criteria of Clinical Laboratory Standards Institute (CLSI).[21] Any decrease in the zone sizes for the 3rd generation cephalosporins was used presumptively as ESBLs producer, and was confirmed later by CLSI criteria.[21] 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.[22]

Phenotypic ESBL confirmatory method

Antibiotic disks of ceftazidime (30 µg) with ceftazidime/clavulanic (30/10 µg), cefotaxime (30 µg) with cefotaxime /clavulanic acid (30/10 µg), cefpodoxime with cefpodoxime /clavulanic acid and aztreonam (30µg) (Mast Diagnostics, UK) were placed onto pre-inoculated Muller-Hinton agar plate with the test organism according to CLSI.[21] Regardless of the zone diameters, a >5 mm increase in a zone diameter for an antimicrobial agent tested in combination with clavulanic acid versus its zone size when tested alone, indicated a probable ESBL production.[21] ESBL producing strain *K. pneumoniae* ATCC 700603 and non-ESBL producing strain *E. coli* ATCC 25922 were used as positive and negative control in each test, respectively.

DNA extraction and integrase analysis

For DNA extraction, *E. coli* and *Kpneumoniae* were cultured in Lauria Bertani (LB) broth at 37°C overnight, and DNA was extracted by CTAB method.[23] For detection of integrons, amplification of the integrase genes of class 1, 2 and 3 integrons (*intI1* , *intI2* and *intI3*) with the Int1F / Int1R ,Int2F / Int2R and Int3F/ Int3R primers as multiplex PCR was performed as described earlier to yield a PCR product of 475bp, 789bp and 922 bp respectively.[24]

Data were analyzed using the statistical package for social sciences (SPSS 18.0, IBM SPSS, New York, USA). Contingency table analysis was done by a chi-square test or two-tailed Fisher's exact test where applicable. A p-value of less than 0.05 was considered as statistically significant. Pearson's correlation was used to calculate association between antibiotics for detection of ESBL.

Results

Bacterial isolates

Two hundred and thirty four isolates obtained from outpatients (n=88) and inpatients (n=146) including, 150 (64.1%) *E.coli* and 84 (35.89%) *K.pneumoniae* were taken into study. *E.coli* was the predominant organism in the urine specimen and isolated more frequently in outpatients than *K.pneumoniae*, the two-tailed P value equals 0.0268 and the association was not found statistically significant. On the other hand, *K.pneumoniae* was the most frequently isolated bacteria in blood cultures from inpatients, though association not considered to be significant. No significant difference was observed in the prevalence of either or both pathogens from other clinical specimens (Table 1).

Quinolone resistance and MDR

On disk 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. MICs of nalidixic acid ranged from 8 to >256mg/L and for ciprofloxacin from 0.032 to >32mg/L. MIC_{50} and MIC_{90} values of nalidixic acid for 125 *E.coli* and *K.pneumoniae* isolates were found in resistance breakpoints (both = 163.55mg/L). Intermediate resistant isolates on disk agar diffusion were further confirmed as susceptible with MIC being <16mg/L. Ciprofloxacin MIC_{50} and MIC_{90} was observed as 24.78mg/L. One *E.coli* isolate found intermediate resistant on disk agar diffusion assay was later confirmed resistant by E-test, thus in total 135 isolates were found as quinolone resistant. The clinical source of these 135 quinolone resistant isolates were urine [75 (55.55%) *E.coli*, 15 (11.11%) *K.pneumoniae*], blood [7 (5.85%) *E.coli*, 18 (13.33%) *K.pneumoniae*], and wound [6 (4.44%) *E.coli*, 7 (5.18%) *K.pneumoniae*]. One *E.coli* obtained from bronchial secretion was also FQ resistant.

Among these quinolone resistant isolates, 56 *E.coli*, and 57 *K.pneumoniae* were recovered from inpatients and 16 *E.coli* and 6 *K.pneumoniae* from outpatients.

Of 72 quinolone resistant *E.coli* isolates, 57 (79.16%) were highly resistant to 3rd generation cephalosporins, non-susceptibility being in the range of 49.2% - 85.9% [cefamandole (85.9%), ceftriaxone (81.6%), ceftazidime (63.3%) and ceftizoxime (49.2%)] followed by gentamicin (61.9%) and co-trimoxazole (61.6%), while this trend was more heavily observed in *K.pneumoniae*, with cephalosporin non-susceptibility appearing in the range of 52.3%- 96.8% [cefamandole (96.8%), ceftriaxone and ceftazidime (88.8%), ceftizoxime (52.3%)] followed by gentamicin (63.9%) and co-trimoxazole (60.3%). Cefepime resistance was low in *E.coli* (28%) as well as in *K.pneumoniae* (23%). Forty seven of 63 (74.60%) *K.pneumoniae* and 59 of 72 (81.94%) *E.coli* were simultaneously resistant to other antibiotics appearing as multi drug resistant isolates (n=106; 78.51%). The most frequent phenotype pattern of MDR was ceftazidime-ceftriaxone-cefamandole-ceftizoxime-gentamicin- cotrimaxazole found in 56 (52.83%) isolates, followed by ceftazidime-ceftriaxone-cefamandole- ceftizoxime- amikacin- gentamicin-cotrimaxazole in 31 (29.24%) and ceftazidime-cefamandole-ceftriaxone in 12 (11.3%) isolates. Phenotypic resistance pattern cefamanadole-ceftriaxone-ceftizoxime-amikacin-gentamicin-imipenem phenotype was disclosed by 7 (6.6%) isolates.

Table 1. Type of clinical specimens and distribution of *E.coli* and *K.pneumoniae*

Clinical specimens	Inpatients		Outpatients	
	E.coli	*K.pneumoniae*	*E.coli*	*K.pneumoniae*
Blood	14	27	8	7
Urine	53	22	57	9
Wound	10	11	4	3
Endotracheal tube	1	3	0	0
Broncho- alveolar lavage	1	2	0	0
Catheter tip	1	0	0	0
CSF	0	0	0	0
Sputum	0	0	0	0
Body fluids	1	0	0	0
Total	81	65	69	19

Quinolone resistance and ESBL

When quinolone resistant *E.coli* and *K.pneumoniae* isolates were tested by disk agar diffusion method for being ESBL producers, *K.pneumoniae* was observed as potent ESBL producer (n=44/63; 69.84%) in comparison to *E.coli* (n=42/72; 58.33%). However, the two-tailed P value equaled 0.2096 and the association was not found statistically significant. Among the beta lactams used alone and in combination for detection of ESBL production, ceftazidime, cefotaxime, cefpodoxime and cefotaxime combinations with clavulanic acid correlated well for detection of ESBL production in both bacteria and this correlation analyzed by Pearson's correlation was found significant at the 0.01 level. However, ceftazidime was observed the most suitable substitute over cefpodoxime and cefotaxime alone and in combination with clavulanic acid ($p<0.05$) for detection of ESBL production. *E.coli* were highly resistant towards cefpodoxime (92%), aztreonam (78%), cefotaxime (69%), ceftazidime (66.1%) while, *K.pneumoniae* in comparison to *E.coli* revealed high resistance against cefpodoxime and aztreonam (90%) , ceftazidime (87%) and cefotaxime (85%). Table 2 shows the antimicrobial susceptibility of quinolone resistant *E.coli* and *K.pneumoniae*.

Quinolone resistance, MDR, ESBL production and Integrons carriage

Of 135 isolates, 97 (71.8%) isolates presented with integrons, while in others neither integrase genes of class 1 and 2 *(intI1*, *intI2*) nor 3 (*intI3*) was observed.

Integrase genes were carried by 62 (72%) bacteria producing ESBL, including 27 (65%) *E. coli* and 35 (77.7%) *K. pneumoniae*. Presence of class 1 integrons in *E.coli* was observed to be associated with the resistance of the isolates to ceftriaxone, ceftazidime, gentamicin and nalidixic acid while, class 2 integron presence was related to the non- susceptibility of isolates to imipenem, nalidixic acid and co-trimoxazole (Table 2). In contrast, presence of class 1 integrons in *K.pneumoniae* was associated with resistance towards imipenem, nalidixic acid, ceftazidime and gentamicin, while the resistance to gentamicin and co-trimoxazole was observed to be associated with the presence of class 2 integrons (Table 2), compared with 10 (22.2%) ESBL-negative isolates ($p<0.05$). Ciprofloxacin resistance (MIC ≥4 µg/ml) in both *E.coli* and *K.pneumoniae* was related significantly ($\chi2= 8.8$; $p< 0.01$) with the presence of integron class 1

and co-presence of integron class 1 and 2. This resistance was also significantly (χ^2= 14.983; p< 0.001) related to the presence of ESBL in isolates as compared to non ESBL production. On the other hand, nalidixic acid resistance in both *E.coli* and *K.pneumoniae* was related significantly (χ^2= 8.2; p< 0.01) with the presence of only class 1 integron. This resistance was also significantly (χ^2= 16.625; p< 0.001) related to the presence of ESBL as compared to non ESBL production.

The association existed between presence of integrons and drug resistance to cefamandole, ceftriaxone, ceftazidime, gentamicin, co-trimoxazole, nalidixic acid and ciprofloxacin (Table 2).

Table 2. Association of resistance to various antimicrobial agents and presence of integrase genes in *E.coli* and *K. pneumoniae* isolates

Antibiotics	Quinolone resistant *E.coli* (n=72)					Quinolone resistant *K.pneumoniae* (n=63)				
	intI1 (%)	*intI2* (%)	*intI1,2* (%)	ESBL (+)[a]	ESBL (-)[b]	*intI1* (%)	*intI2* (%)	*intI1,2* (%)	ESBL (+)[a]	ESBL (-)[b]
Ciprofloxacin	15 (26.7)	11 (19.6)	10 (17.8)	36 (64.2)	20 (35.7)	23 (37.8)	10 (16.3)	4 (6.5)	42 (68.9)	19 (31.1)
Nalidixic acid	17 (34)	7 (14)	10 (20)	30 (60)	20 (40)	8 (50)	3 (18.7)	1 (6.2)	12 (75)	4 (25)
Amikacin	3 (21.4)	3 (21.4)	6 (42.8)	14 (100)	----	4 (33.3)	3 (25)	3 (25)	9 (75)	3 (25)
Gentamicin	16 (34)	9 (19.1	9 (19.1)	34 (72.3)	13 (27.6)	19 (47.5	10 (25)	4 (10)	34 (85)	6 (15)
Cotrimoxazole	16 (32.6)	8 (16.3)	9 (18.3)	29 (59.1	20 (40.8)	21 (53.8)	10 (25.6)	5 (12.8	32 (82)	7 (17.9)
Cefamandole	21 (30.4)	11 (15.9)	10 (14.4)	42 (60.8)	27 (39.1)	24 (38)	11 (17.4)	4 (6.3)	44 (69.8)	19 (30.1)
Ceftizoxime	11 (30.5)	4 (11.1)	6 (16.6)	22 (61.1	14 (38.9	13 (37.1	7 (20)	4 (11.4	28 (80)	7 (20)
Ceftazidime	21 (40.3)	9 (17.3	7 (13.4)	37 (52.1	15 (21.1	23 (41%)	10 (17.8	4 (7.1%)	44 (78.5	12 (21.4
Ceftriaxone	20 (31.7)	11 (17.4)	10 (15.8)	39 (61.9	24 (38.1	23 (39.6	10 (17.2)	4 (6.9)	44 (75.8)	14 (24.1)
Imipenem	2 (18.2)	2 (18.2)	6 (54.5)	10 (90.9	1 (9.1)	2 (50)	0	2 (50)	4 (100)	0
Nitrofurantoin	5 (6.9)	0	0	3 (7.1)	1 (1.4)	6 (9.5)	0	0	4	0

a:ESBL producer, *b*: ESBL non producer

Class 1 (n= 43; 40.5%) and 2 (n= 21; 19.8%) integrons were found in 78 (73.5%) of 106 MDR isolates. Fourteen (13.2%) MDR isolates possessed both *intI1* and *intI2* genes.

In respective to the organism, 21(35.6%) quinolone resistant and 11 (18.6%) MDR *E.coli* had *intI1* and *intI2* genes respectively. Nine (15.2%) *E.coli* harbored both *intI1* and *intI2* integrase genes. In comparison, 24 (51%) quinolone resistant and 11(23%) MDR *K.pneumoniae* isolates possessed *intI1* and *intI2* respectively and 5(10.6%) isolates had both *intI1* and *intI2* (Table 3). Class 3 integron was not found in any of the tested bacterial species.

In relation to harboring integrase genes, class 1 integrase gene was being possessed by 22 *K.pneumoniae* and 11 *E.coli.* In contrast, class 2 integrase gene (*intI2*) was possessed more frequently by *E.coli* (n=11) isolates over *K.pneumoniae* (n=5) and this association was significant (p<0.05).

Discussion

Our study analyzed 234 *E.coli* and *K.pneumonaie* isolates obtained from various clinical specimens from inpatients and outpatients, comprehensively including community and hospital associated infections for quinolone resistance, ESBL production, multidrug resistance, possession of integrase genes and the association between them.

The microbial etiology of urinary tract infections (UTI) has been well-established and reasonably consistent. *Escherichia coli* remains the predominant uropathogen (80%) isolated in acute community-acquired uncomplicated infections, followed by gram positive and other gram negative organisms.[25] In our study, *E.coli* was the predominant (82.4%) cause of community acquired UTIs, though 65.15% hospital associated UTIs were also due to this organism. We found *K.pneumoniae* to be principle cause of bloodstream infections. Similar reports are available which supports this finding.[26-28] Though these two bacteria are leading cause of clinical infections in hospital as well as community based patients,[9] however, increasing trend of antimicrobial resistance is a serious concern which has tempered the therapeutic options. Forty five (31.91%) of *E.coli* (34/110; 30.9%) and *K.pneumoniae* (11/31; 35.4%) obtained from urine specimens were resistant to nalidixic acid and those

(46.55%) recovered from other clinical specimens were resistant to ciprofloxacin. Several reports are available on the mechanism of quinolone resistance in either *E.coli* or *K.pneumoniae* or even both or quinolone resistance in ESBL producing bacteria; however, no report is available on the actual resistance of nalidixic acid and ciprofloxacin in these two isolates [29-35], except few published studies on urinary isolates,[36-40] whereby low prevalence has been reported as compared to our study. Study performed in our neighboring country reported 17% and 38% of *E. coli* isolates obtained from uncomplicated and complicated UTI respectively, were found resistant to ciprofloxacin.[40]

Table 3. Association of ESBL production, presence of integrase genes and MDR with quinolone resistance in *E.coli* and *K.pneumoniae* clinical isolates

		Ciprofloxacin resistance[a]		Nalidixic acid resistance[a]	
		E.coli	*K.pneumoniae*	*E.coli*	*K.pneumoniae*
ESBL producer		74.2[a] (26 /35)[b]	29 (11/38)	91.4 (32/35)	47.3 (18/38)
ESBL non producer		69.2 (9/13)	18.2 (2/11)	92.3 (12/13)	72.8 (8/11)
INTEGRON class	1	81.8 (9/11)	27.2 (6/22)	100 (11/11)	50 (11/22)
	2	63.6 (7/11)	20 (1/5)	81.8 (9/11)	80 (4/5)
	1,2	100 (9/9)	25 (1/4)	100 (9/9)	50 (2/4)
INTEGRON not detected		58.9 (10/17)	27.7 (5/18)	88.2 (15/17)	50 (9/18)
MDR		69.2 (27/39)	29.7 (11/37)	64.8 (37/39)	54 (20/ 37)
Non MDR		88.8 (8/9)	16.6 (2/12)	22 (2/9)	50 (6/12)

a Percentage of isolates
b Number of isolates/total number of quinolone resistant isolates

Of 72 quinolone resistant *E.coli*, and 63 *K.pneumoniae* isolates, high resistance to 3rd generation cephalosporins was observed, non-susceptibility being in the range of 49.2% - 85.9% and 52.3%- 96.8% respectively, which is quite high. The importance of infections due to ESBL producing *E.coli* and *Klebsiella* species has been increasingly recognized in recent years.[41] A significant increase in the prevalence of fluoroquinolone resistance ($p<0.001$) was evident in their study conducted among the ESBL-*E.coli* and *K.pneumoniae* isolates over the 5 year study period. Another significant feature of their case – control report is fulfilling the criteria of MDR by 18.8% isolates. The only independent risk factor for MDR ESBL- *E.coli* and *K.pneumoniae* was infection with *K. pneumoniae*. Schwaber et al.[42] noted high levels of co-resistance (≥40%) among their isolates for all agents except amikacin and imipenem. Another research study analyzed 867 non-repeat isolates comprising 8 species, originating from the community and 23 European hospitals, and showed a significant relation between MDR and the presence of integrase genes, independent of species or origin.[13] Our study was in concordance with this research study which found 75.6% of their isolates as ciprofloxacin resistant and integron positive. We found 74.6% *K.pneumoniae* and 81.9% *E.coli* to be simultaneously resistant to other antibiotics appearing as multi drug resistant isolates, which suggest for limitations and precise use of antibiotics in our region. The present study showed the presence of class 1 and 2 integrons in 73.5% of MDR isolates and 13.2% of them possessed both *intI*1 and *intI*2 simultaneously. Our isolates had comparatively low imipenem resistance. Phenotypic resistance pattern disclosing cephalosporin resistance with aminoglycoside and imipenem phenotype was disclosed by 6.6% isolates.

Ciprofloxacin resistance was related significantly with the presence of integron class 1 and co-presence of integron class 1 and 2 together in our isolates. This resistance was also significantly related to the presence of ESBL producing isolates as compared to non ESBL production. Nalidixic acid resistance was related significantly with the presence of only class 1 integron in the isolates studied. This resistance was also significantly related to the presence of ESBL production. Our study had various limitations, with major one being study of risk factors for such a high resistance. Though we did not perform this, nevertheless we can assume exposure to antibiotics as one of major predisposing factor as our University based tertiary hospital is a core center for all North West region and patients first treated at their primary care center are referred for further treatment. Another factor may be misuse of antibiotics as antibiotics are available over- the- counter. In UK, nursing home residents had very high prevalence of gut carriage of MDR *E. coli*.[43] Exposure to antibiotics was high among residents, with carriers having spent significantly more days receiving trimethoprim or FQs in their published report. The presence of an ESBL determinant significantly curtails the number of antimicrobial agents, and limits therapeutic option. In addition, frequent links between ESBL genes and other resistance genes on the mobile DNA elements that are involved in their dissemination, ESBL producers often present as complex multidrug resistant phenotypes.[44-46]

Conclusion

Prevalence of quinolone resistance in *E.coli* and *K.pneumoniae* in our clinical setting has contributed to augmentation in antibacterial resistance. High resistance to cephalosporins in ESBL-producing pathogens restricts the possibilities for effective treatment of infections. Appropriate infection control measures should be aimed at minimizing the spread of ESBLs and this should be specified as a high priority. A hopeful prospect in our study was low resistance to carbapenem.

Prevalence of class I and II integron in our quinolone resistant isolates is a similar trend observed in other published studies, however, co- prevalence with ESBL and multi drug resistance is a striking feature of our study. This is a therapeutic concern and requires further investigation taking into account the associated risk factors and study of gene cassettes. Presence of integrons warns dissemination of antibiotic resistance. Additionally, in view of confirmation of high resistance towards nalidixic acid and FQ by MICs, it is necessary that these tests should be available in the hospital for exact detection of antibiotic resistance.

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Ethical Issues

Not applicable.

Conflict of Interest

No potential conflicts of interest.

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