Antibiogram and Plasmid profiling of clinical multidrug resistant Escherichia coli

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Abstract

Introduction: Multiple antibiotic resistances among common bacterial pathogens have been established as an alarming public health problem elsewhere. This study was aimed to explore prevalence of Extended-spectrum beta-lactamase (ESBL) producers among multi-drug resistant (MDR) and plasmid profiling patterns of Escherichia coli isolated in Kathmandu Model hospital, Nepal over three months period.

Methods: Altogether 49 isolates were randomly selected among the reported MDR E. coli and retested for their susceptibility pattern and ESBL production by Kirby-Bauer disc diffusion test. Minimum Inhibitory Concentration of the isolates towards (fluro) quinolones was determined by agar dilution method. Plasmids from isolates were extracted by alkaline lysis method.

Results: All isolates were completely resistant to Amoxycillin but sensitive to Imipenem. A very high prevalence (44, 89.8%) of ESBL producers was detected. Most of the isolates were resistant to commonly used antibiotics such as Quinolones, Tetracyclines and Cotrimoxazole. Among Quinolones, the Minimum Inhibitory Concentration (MIC) range for Nalidixic acid, Ciprofloxacin and Ofloxacin were 512 - >4093, 8 - 4096 and 1 - 256 µg/ml respectively. All 31 (63.3%) plasmid-harboring isolates contained a >33.5 kb sized plasmid. Among them, seven isolates possessed multiple (2 – 7) plasmids. Overall, twelve different resistance patterns were observed among the bacteria. Based on the patterns, the high molecular weight plasmid seemed to contain most of the resistance genes.

Conclusion: It is suspected that multi drug resistance and ESBL production in E. coli with resistance to Quinolones may be due to their high molecular weight plasmids. So, continuous antibiotic susceptibility test and surveillance of the plasmid and chromosome of E. coli is essential as plasmid analysis has been applied to determine the evolution and spread of antibiotic resistance among isolates.

Keywords: Escherichia coli, Antibiotic resistance pattern, Multidrug resistant (MDR), Extended spectrum Beta-lactamases (ESBL), Plasmid profiling

Introduction

Prevalence of multidrug resistance (MDR) among clinical isolates varies greatly worldwide and in different geographic areas with increase in drug resistance over time because of the indiscriminate use of antibiotics in clinical practice[1]. Among the bacteria, Gram negative bacteria including *Escherichia coli* have recently become more resistant to commonly used antibiotics. Those carrying R-plasmids are the most serious problem among antibiotic resistant organisms because of their rapid spreading nature[2].

E. coli that exists as a normal flora in human intestine is an opportunistic gram-negative, facultative anaerobic pathogen of *Enterobacteriaceae* family. E. coli is major causes of nosocomial and community-acquired infections in humans as well as one of the most frequently isolated

organisms in clinical specimen. E. coli have been associated with a number of disease including severe and sometimes fatal infections such as pleonephritis, septicemia, meningitis, endocarditis, urinary tract infection, epidemic diarrhoea of adults and children[3,4].

Pathogenic isolates of E. coli that have a relatively large potential for developing resistance to multiple drugs is of main concern because of its ability to produce Extended spectrum Beta-lactamases (ESBL). ESBLs or Ultra-broad-spectrum β-lactamases are plasmid-mediated bacterial enzymes that confer resistance to a broad range of B-lactams (penicillins, first-, second-, and third generation cephalosporins, and aztrenonam) by hydrolyzing these antibiotics. These enzymes are descended by genetic mutation from native B-lactamases found in gram negative bacteria. ESBLs are often acquired by transfer of genetic-related information from one organism to another and codes for resistance determinants to other antimicrobial agents; hence, multidrug (MD) resistance is expected of ESBL-producing isolates[5].

In the present situation, the emergence of MDR *E coli* isolates is a big threat for the antibiotic therapy. These MDR isolates are adding up resistance to antibiotics day by day. Therefore our prime research focus is to screen ESBL-positive resistance profile for β-lactamase, quinolones resistance and plasmid profiling of those emerging threats.

Methods

Bacterial strain: Out of many samples from urinary tract infected patients, forty-nine isolates were randomly selected among the reported MDR E. coli at Kathmandu Model Hospital, Kathmandu from February 2010 to April 2010. The samples were further retested in the Laboratory of Kantipur College of Medical Science by sub-culture in MacConkey agar, analyzing their colony characteristic as well as by Gram staining and biochemical tests. The pure culture of MDR isolates were preserved in 20% glycerol containing Tryptic Soya broth and kept at -70°C until subsequent tests were performed.

Antimicrobial susceptibility testing: Antimicrobial Susceptibility test was performed by kirby-Bauer disk diffusion method following Clinical and Laboratory Standard Institute guidelines.[6] Isolates were introduced to 12 commonly used antibiotics namely Amoxycillin, Chloramphenicol, Ciprofloxacin, Cotrimoxazole, Doxycycline, Gentamycin, Imipenem, Nalidixic acid, Nitrofurantoin, Norfloxacin, Ofloxacin and tetracycline. Isolates showing resistance to three or more different classes of antibiotics were considered as MDR.

Screening and confirmation of ESBL producers: MDR isolates were screened for possible ESBL production using ceftazidime (30µg) and ceftriaxone (30µg) following CLSI, 2010. Confirmation of ESBL production was determined by combined disk (CD) assay using MASTDISCTM ID ESBL detection discs consisting of ceftazidime (30µg) and ceftazidime (30µg) plus clavulanic acid (10µg). Isolates with zone of inhibition diameter for either ceftazidime ≤22mm or ceftriaxone ≤25mm or both were considered as the possible ESBL producing strains and an increase in zone diameter of ≥5mm in the presence of clavulanic acid was concluded as confirmed ESBL producers.

Determination of Minimum Inhibitory Concentration (MIC): Among quinolones, MICs of naldixic acid, ciprofloxacin and ofloxacin were determined by agar dilution method following CLSI, 2010. Secondary antibiotics standards provided by Nepal Medicine Council, Babbarmahal were used for MIC determination.

Extraction and profiling of plasmid DNA: Plasmid DNA was isolated by alkaline lysis method [7], separated on 0.8% agarose gel electrophoresis at 120 V for 1 hrs and stained by SYBR safe stain. Plasmid profile were created by grouping strains possessing the same number of plasmid bands and molecular mass or a part of profile containing a core plasmid[3].

Data analysis: Data were analyzed by using WHONET 5.6.

Results

Of the 49 clinical MDR E. coli isolates screened for antimicrobial agents, all were found to be resistant to Amoxicillin (100%), Ciprofloxacin (100%) and Nalidixic acid (100%) but sensitive to Imipenem (100%). A higher percent of sensitivity was also found to Nitrofurantoin (97.95%). A very high prevalence (89.8%) of ESBL producers was detected among these MDR isolates. The MIC of fluoroquinolones showed that all isolates were resistant to nalidixic acid and ciprofloxacin where as only 2 (4.08%) isolates were susceptible to ofloxacin (Table 1).

Table 1. Minimum Inhibitory Concentrations for MDR E. coli isolates (n=49)

	Antibiotic name	Ciprofloxacin	Nalidixic acid	Ofloxacin
	Breakpoints	S≤1 R≥4	S≤16 R≥32	S≤2 R≥8
M	1µg/ml	0	0	1
I	2µg/ml	0	0	1
C	4µg/ml	0	0	1
	8µg/ml	1	0	0
V	16µg/ml	1	0	1
A	32µg/ml	5	0	6
L	64µg/ml	6	0	32
U	128µg/ml	6	0	4
E	256µg/ml	4	0	3
S	>256µg/ml	26	49	0
	No of resistant isolates	49	49	47
	MIC Range	8 to 4096	512 to >4096	1 to 256

Among these 49 isolates, 31 (63.27%) harbored plasmids ranging in molecular size from 2.05 kb to >33.5 kb. On the basis of plasmid band and number of visible bands, seven different plasmid profile groups for the antibiotic-resistant strains were identified. The number of strains per plasmid profile group vary from 1-11 (Table 2). The most common antibiotic resistance pattern was AmxNalNorCipOflTetDoxCot followed by AmxGenNalNorCipOflTetDoxCot, AmxNalNorCipOflDoxCot and other R-type resistance patterns (Table 2). Strains showing the resistance pattern AmxGenNalNorCipOflTetDoxCot contained the highest number of plasmids.

Table 2: Antibiotic resistance pattern, plasmid contents and plasmid size of MDR isolates

Antibiotic resistance pattern	Plasmid profile	No. showing pattern	Molecular weight
AmxGenNalNorCipOflTetDoxCot	6	1	>33.50,>33.50,10.13,4.24,2.05,
	5	2	>33.50,9.39,6.68,5.06,2.05
	4	1	>33.50,>33.50,5.06,2.05
	3	1	>33.50,9.39,6.68
	2	1	>33.50,6.68
	1	3	>33.50
AmxNalNorCipOflTetDoxCot	3	1	>33.50,9.39,6.68
	2	2	>33.50,6.68
	1	2	>33.50
AmxNalNorCipOflTetDox	3	1	>33.50,9.39,6.68
	2	2	>33.50,6.68
	1	1	>33.50
AmxGenNalNorCipOflTetDoxChlCot	6	1	>33.50,>33.50,9.39,6.68, 3.12,2.05
	2	1	>33.50,10.13
	1	1	>33.50
AmxNalNorCipOflDoxCot	3	1	>33.50,10.13,6.68
	2	2	>33.50,10.13
AmxNalNorCipOflDoxChlCot	2	1	>33.50,10.13
AmxNalNorCipTetDox	1	1	>33.50
AmxNalCipDoxTetCotChl	5	1	>33.50,9.39,6.68,5.06,4.24
AmxNalNorCipOflCot	1	1	>33.50
AmxNalNorCipOflTetDoxChlCot	1	1	>33.50
AmxNalNorCipOflTetDoxCotNit	1	1	>33.50
AmxGenNalNorCipOflTetDoxCot	2	1	>33.50,>33.50

Note: Lane 1 and Lane 7 super mix DNA Ladder (marker). Lane 4 >33.50,>33.50,9.39,6.68,5.06, 4.24(Eco 41); Lane 5 >33.50, 6.68 (Eco 29); Lane 11 (Eco 44) and Lane 13 >33.5 Kb (Eco 26) and Lane 2, Lane 3, Lane 6, Lane 8,Lane 9, Lane 10, Lane 12, Lane 14 and Lane 15 all are negative.

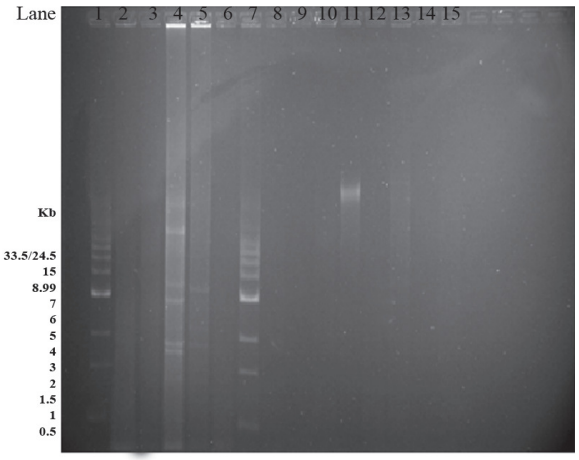

Figure 1. Agarose gel electrophoresis showing different plasmid bands

Discussion

Studies on antimicrobial sensitivities among commonly isolated microorganisms, mainly bacterial pathogens, has been conducting elsewhere. For developed countries, the advent and access of sophisticated molecular techniques accelerated them into new horizon of molecular biology of bacterial resistance. However, in our context, almost all of the basic researches used to focus on mere observations of antibiotic susceptibility tests. In these days,It's good that many of the published works these days are focusing on more specific microbial groups of clinical and non-clinical relevance. In this study, we tried to explore the relation between antibiotic susceptibility of multi-drug resistant E. coli, especially ESBLs, to their plasmid profiles in a hospital setting.

Randomly selected 49 MDR E. coli isolates were taken in to our research lab and retested for their antibiotic sensitivity response. Sixteen different resistance patterns were observed among the isolates with antibiotics that are commonly used in Nepal to treat E. coli infections.

All isolates were resistant to Amoxicillin, Nalidixic acid and Ciprofloxacin similar to recent studies conducted elsewhere[3,8,9]. We observed more resistance patterns compared to that by Daini OA and Adesemowo A, Gobernado et al., and Ozbakir G et al.[3,10,11]. We included four commonly used quinolones, viz., Nalidixix acid, Norfloxacin, Ofloxacin and Ciprofloxacin. Except two, all isolates were resistant to those four quinolones. This finding elucidated that these bacteria are being resistant to all generations of quinolones tested. These antibiotics are used for years in Nepal and bacteria are gaining resistance to all gyrase inhibitors – broad spectrum antibiotics. Most of the isolates were resistant to Tetracyclines and Cotrimoxazoles too. This result is similar to other findings elsewhere that the ESBL producers are resistant to quinololes as well[12,13,14]. Interestingly, most of them were sensitive to Chloramphenicol that is rarely used in these days and Nitrofurantoin to occasionally treat Urinary tract infections[15]. Only the drug of choice seemed to be Imipenem - a monolactam. However, it can easily be generalized that the ESBLs may gain resistance to the monolactam sooner or later similar to their aggressive response to the gyrase inhibitor – the quinolones. Therefore, this study explored an urgency of finding other effective anti-ESBL antibiotics in the area of clinical microbiology.

Comparatively higher prevalence (89.80%) of positive ESBL producers was found in the current research work. These ESBL producers showed highly resistance to quinolones (96-100%), tetracycline (68%), Doxycycline (93%) and Cotrimaxazole (77%), and low resistance to Gentamycin (32%) and Chloramphenicol (16%). All the isolates were susceptible to Imipenem and highly sensitive too Nitrofurantoin (98%). Ahmed *et al.,* 2010; Lagace-Wien *et al*., 2007; and Vaidya, 2011 reported similar study of co-existence of ESBL with resistance to Cotrimoxazole, Tetracycline, Doxycycline, Gentamycin, Ciprofloxacin and degree of sensitivity to Imipenem and nitrofurantoin[16-18].

In our study, plasmids of the molecular size ranging from 2.05 to >33.5 kb were isolated from 63.27 % of total MDR *E coli*. Among them, seven isolates possessed multiple (2 – 7) plasmids. Overall, twelve different resistance patterns were observed among the bacteria. Plasmids of different molecular size ranging from 0.12 kb to 65 in clinical isolates of E. coli had reported in the work of Daini *et* al., 2008 and Hamad, 2009[3,19]. Daini *et* al., 2008 concluded that different plasmids often coexisted in the same host cell[3]. Previous studies have demonstrated that multiple antibiotic resistances are associated with higher molecular weight plasmids[20,21]. Plasmids of same molecular size can be found in many strains. Atmost isolates in this research showed a common higher molecular weight plasmid (>33.50 kb). Based on the resistance patterns, the high molecular weight plasmid seemed to contain most of the resistance genes.

Plasmids were not observed in 18 (36.73%) of our MDR isolates. The resistance in those isolates might probably be

chromosomal borne. In many bacteria, the mobile genetic elements, transposon carried resistance genes[22]. However, the broad spectrum of resistance can't be possessed by transposons alone. These plasmids less isolates might be Hfr strains.

In conclusion, MDR and ESBL-producing E. coli constitute a major health problem in Nepal. Drug resistant E. coli harbored plasmid of varying size and molecular weight. Higher molecular weight plasmid may be associated with MDR and ESBL-producing E. coli. So, increased periodic review and formulation of antibiotic policy to control acquisition of drug resistance and appropriate routine antimicrobial susceptibility testing with evaluation of phenotypic confirmatory ESBL by MIC and genotypic detection to confirm ESBL producers and detection of other β-lactamases, and surveillance of the plasmid and chromosome of E. coli is essential as plasmid analysis has been applied to determine the evolution and spread of antibiotic resistance among isolates.

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Conflict of interest: None declared

References

1. Livermore DM, Canton R, Gniadkowski M et al. CTX-M: changing the face of ESBLs in Europe. J Antimicrob Chemother 2007:59: 165-174.
2. Saeed A, Khatoon H and Ansari FA. Multidrug resistant Gram-negative bacteria in clinical isolates from Karachi. Pak J Pharm Sci 2009: 22: 44-48.
3. Daini OA and Adesemowo A. Antimicrobial susceptibility patterns and R-plasmids of clinical strains of Escherichia Coli. Aust J Basic Appl Sci 2008: 2: 397-400.
4. Okonko IO, Soleye FA, Amusan TA et al. Incidence of Multi-Drug Resistance (MDR) organisms in Abeokuta, Southwestern Nigeria. Global J Pharmacol 2009: 3: 69-80.
5. Khanfar HS, Bindayna KM, Senok AC et al. Extended spectrum Beta-Lactamases (ESBL) in Escherichia coli and Klebsiella pneumoniae: trends in the hospital and community settings. J Infect Dev Ctries 2009: 3: 295-299.
6. Clinical Laboratory Standards Institute. Performance standards for antimicrobial susceptibility testing. Twentieth informational supplement CLSI document M100-S20. Wayne, PA: CLSI, .2010.
7. Sambrook J, Fritsch EF and Maniatis T. Molecular cloning: a laboratory manual, 1989, 2nd edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
8. Karlowsky JA, Hoban DJ, DeCorby MR et al. Fluoroquinolone-resistant urinary isolates of Escherichia coli from outpatients are frequently multidrug resistant: Results from the North American urinary tract infection collaborative alliance-quinolone resistance study. Antimicrob Agents Chemother 2006: 50(6): 2251–2254.
9. Lim KT, Yasin R, Yeo CC et al. Characterization of multidrug resistant ESBL-producing Escherichia coli isolates from hospitals in Malaysia. J Biomed Biotechnol 2009: 22: 661-667.
10. Gobernado M, Valdes, Alos JI et al. Antimicrobial susceptibility of clinical Escherichia coli isolates from uncomplicated cystitis in women over a 1-year period in Spain. Rev Esp Quimioterap 2007: 20: 68-67.
11. Ozbakir G, Avcioglu NH and Bilkay IS. Determination of antibiotic resistance and plasmid DNA profiles of Esherichia coli strains isolated from clinical materials. Int J Agric Biol 2010: 12: 724-728.
12. Das RN, Chandrashekhar TS, Joshi HS et al. Frequency and susceptibility profile of pathogens causing urinary tract infections at a tertiary care hospital in western Nepal. Singapore Med J 2006: 47: 281-285.
13. Karki S, Rai GK and Manandhar R. Bacteriological analysis and antibiotic sensitivity pattern of blood culture isolates in Kanti Children Hospital. J Nepal Paediatr Soc 2010: 30: 94-97.
14. Jha BK, Singh YI, Khanal LK et al. Prevalence of asymptomatic bacteriuria among elderly diabetic patients residing in Chitwan. Kathmandu Uni Med J (KUMJ) 2009: 7: 157-161.
15. Sandegren L, Lindqvist A, Kahlmeter G et al. Nitrofurantoin resistance mechanism and fitness cost in Escherichia coli. J Antimicrob Chemother 2008: 62: 495-503.
16. Ahmed K, Thokar MA, Toboli AS et al. Extended spectrum β-lactamase mediated resistance in Escherichia coli in a tertiary care hospital in Kashmir. India. Afr J Microbiol Res 2010: 4: 2720-2728.

17. Lagace-Wiens PRS, Nichol KA, Nicolle LE et al. ESBL genotypes in fluoroquinolone-resistant and fluoroquinolone-susceptible ESBL-producing Escherichia coli urinary isolates in Manitoba. Can J Infect Dis Med Microbiol 2007: 18: 133-137.

18. Vaidya VK. Horizontal transfer of antimicrobial resistance by extended spectrum β-lactam producing Enterobactericeae. J Lab Physicians 2011:3: 37-44.

19. Hamad IS. Distribution of conjugative plasmids among some multiple antibiotic resistant E.coli strains isolated from patients of teaching hospital in Sulaimani city. J Duhok Univ 2009: 2 (Special Issue): 282-287.

20. McPherson P and Gealt M. Isolation of indigenous wastewater bacterial strains capable of mobilizing plasmid pBR325. Appl Environ Microbiol 1986: 51: 904-909.

21. Zulkifli Y, Alitheen NB, Raha AR et al. Antibiotic resistance and plasmid profiling of Vibrio parahaemolyticus isolated from cockles in Padang, Indonesia. International Food Research Journal 2009: 16: 53-58.

22. Son R, Nasreldin EH, Zaiton H et al. Characterisation of Vibrio vulnificus isolated from cockles (Anadara granosa): antimicrobial resistance, plasmid profiles and random amplification of polymorphic DNA analysis. FEMS Microbiol Lett 1998: 165: 139-143.