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Isolation and characterization of Plasmid DNA from clinically isolated *E.coli* strain at Pravara rural hospital, Loni, India

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Abstract

Urinary Tract Infection (UTI) is one of the most common infectious diseases and people of all age-groups and geographical locations are affected. Multiple resistance to antimicrobials drugs arising in *Escherichia coli* isolate may complicate therapeutic management of UTI. The present study aimed to isolate and characterize plasmid DNA from Clinical isolated *E. coli*. Biochemical tests confirm that the hospital isolate is *E.coli* and antibiotic susceptibility test results shows that it is MDR strain. Molecular techniques such as plasmid DNA isolation and PCR amplification of integrons genes were used to confirm MDR. The results showed that the size of plasmid DNA is more than 52 Kb and the size of successfully amplified integron genes (I and II) were 175 and 250 bp respectively. Hospital isolate was confirmed as *E.coli* MDR on the basis of existence of integron genes.

Keywords: *Escherichia coli*, biochemical test, antibiotic susceptibility test, plasmid DNA, PCR

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Introduction

Urinary tract infections (UTI's) are the most common infectious diseases in childhood. As much as 90% of community-acquired and 50% of nosocomial UTI's are caused by *Escherichia coli* (Pitout and Laupland, 2008). *E. coli* are the predominant facultative organism in the human gastrointestinal tract. Pathogenic forms of *E. coli* can cause a variety of diarrhea diseases in hosts due to the presence of specific colonization factors, virulence factors and pathogenicity associated genes which are generally not present in other *E. coli*. As extra intestinal pathogenic *E. coli*, including uropathogenic *E. coli* (UPEC), yearly affects a large proportion of the population; they are a major target of antimicrobial therapy. Thus, increased usage of antimicrobials has become the main driving force in generating and maintaining resistance bacteria (World Health Organization, 2001). In recent years, the threat caused by the acquisition of antibiotic resistance by pathogenic bacteria has been growing. Antibiotic resistance and, in particular, multidrug resistance (MDR) are major problems for the empirical treatment of patients (Aziz et al., 2008). Increased antimicrobial usage leads to the evolution of drug-resistant bacteria. It has been shown that, once evolved, resistance genes can spread through the world's bacterial populations irrespective of the pattern of antimicrobial use in an area. Therefore, mechanisms other than selection pressure might exist for maintaining a resistant bacterial pool. Resistance genes are disseminated by plasmids or by transposons and also can be integrated into DNA elements designated integrons (Valverde et al., 2009; Tato et al., 2010). Although integrons by themselves are not mobile, due to their presence in plasmids and

transposons, they can be transferred horizontally (Shohreh et al., 2008; Cambray et al., 2010; Findeiss et al., 2010). For these reasons integrons are major mechanisms for the spread and maintenance of multi-drug resistance (Farshad et al., 2008; Heringa et al., 2010; Stalder et al., 2012). Integrons may carry one or more genes in the form of tandem gene cassettes. Because the integron system has the ability to create novel combination of resistance genes, it may be a dynamic force in the evolution of multi-drug resistant (MDR) bacteria. In view of the emergence of MDR strains and their increased involvement in infections and outbreaks worldwide, combining antimicrobial susceptibility testing and molecular fingerprinting has become an important function in public health issues (Salimi et al., 2010).

Thus in the present study, clinically isolated MDR strain was confirmed as *E.coli* by performing different biochemical tests and has been studied for susceptibility to antimicrobial agents (Antibiotics). Furthermore, plasmid DNA was isolated that is responsible for carrying the integron gene and the existence of integrons in resistant isolates was assessed by PCR.

Materials and Methods

Bacterial strain and growth media

Clinically isolated *E.coli* strain from urine sample was obtained from Microbiology Department of Pravara Rural Medical College, Loni, India. This isolate was grown on LB-agar or LB- broth at 37°C. Culture was maintain on LB agar slants and kept at 4°C for further use.

Physiological and biochemical examination

Four to five suspected colonies from bacterial plate were picked, cultured and then identified by the various biochemical tests. Biochemical tests were performed to confirm *E. coli* using Gram staining, Catalase test, Indole, Methyl red, Voges-Proskauer test, Urease production, Simon citrate agar, and various sugar fermentation tests (Table 1).

Table 1. Biochemical characterization of *E. coli*

Biochemical test		Reaction
Catalase		-
Simmon's Citrate		-
Indole Production		+
Methyl Red		+
Voges- Proskauer		_
Urease		+
Oxidase		-
Acid from sugar	(a) Glucose	+
	(b) Mannitol	+
	(c) Lactose	+
	(d) Sucrose	+

Determination of antibiotic resistance pattern

Antibiotic susceptibility testing was conducted on Muller-Hinton agar using disc diffusion by Beathy method (Beathy et al., 2004) method using commercial antibiotic discs purchased from Hi-Media Laboratories. The discs were placed on Muller-Hinton agar previously seeded with 0.5ml of test culture grown for 24 hr. The antibiotics used were Ampicillin (10 mcg), Chloramphenicol (30 mcg), Doxycycline hydrochloride (30 mcg), Gentamycine (10 mcg), Amikacin (30 mcg), Co-thimoxozole (10 mcg) and Cefalexin (30 mcg).

DNA isolation and PCR

Genomic DNA was isolated from *E.coli* by boiling method (Solberg et al., 2006). In brief bacteria were harvested from 1.5 ml of an overnight LB broth culture, suspended in sterile distilled water, and incubated at 95°C for 10 min following centrifugation of the lysate, the supernatant was stored at -20°C as a template DNA stock. Whereas, plasmid DNA from overnight grown *E.coli* culture was isolated by one step plasmid method (Huang, 1991). In brief, 0.5 ml overnight grown culture was mixed with 0.5 ml of Phenol:Chloroform:isoamyl alcohol (25:24:1) and vortexed at maximum speed for 1 min. The

mixture was centrifuged and upper aqueous layer containing DNA was precipitated with 0.6 ml of chilled isopropanol. The obtained DNA pellet was washed with 70% ethanol, air dried and dissolved in appropriate volume of TE buffer. DNA sample was treated with RNAse. The isolated genomic and Plasmid DNA was confirmed by running on 0.7% agarose gel at 100V. Integrons were detected using PCR with degenerate primers designed to hybridize to conserved regions of integron encoded integrase genes Int1 and Int2. Sequences of the primers were as follows: Forward Primer: 5'TGC GGG TYA ARG ATB TKG ATTT 3'; Reverse Primer: 5'CAR CAC ATG CGT RTA RAT 3' where B= C or G or T, K= G or T, R=A or G and Y= C or T (Farshad et al., 2008). These primers were purchased from Chromus Biotech (Bangalore, India). PCR amplification was carried out in 50 µl reaction mixture containing 2 µl template DNA (200 ng), 1 µM of each primer, 2.0 mM $MgCl_2$, 0.2 mM of dNTPs and 1.25 U *Taq* Polymerase. PCR was performed as follows: initial denaturation at 94°C for 5 min followed by 30 cycles consisting of denaturation at 95°C for 30 sec, annealing at 55°C for 30 sec, extension at 72°C for 45 sec and final extension at 72°C for 10 mins. Expected sizes of amplicons for Integrons were ascertained by electrophoresis in 1% of agarose gel with an appropriate molecular size marker (100 base pair DNA ladder, Himedia).

Results

Biochemical tests

Biochemical test results shows Positive for Indole, Methyl Red, Citrate Utilization, Triple sugar with H2S and gas production, sugar ferments (Glucose, Mannitol, Sucrose and Lactose), and Catalase test. Vogas-Proskouer and Oxidase test shows negative results. Thus, the results of biochemical tests confirmed that the hospital isolate is *E.coli* and its Gram negative nature was confirmed by Gram staining.

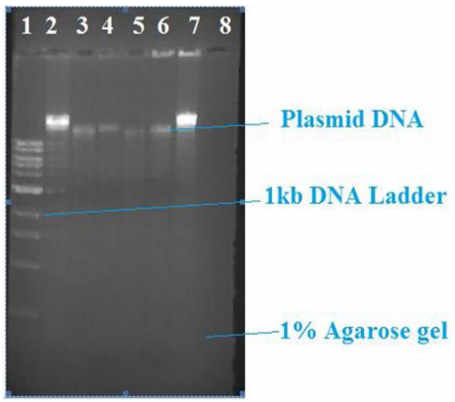
Antibiotic susceptibility

The hospital isolate strain *E.coli* shows resistant to Gentamicin, Amikacin, Deoxycycline hydrochloride, Co-thimoxozole, Cefalexin, Ampicillin, and Chloremphinocol. Hence it was designated as multidrug resistant (MDR) strain.

Extraction of genomic and plasmid DNA

Extraction of genomic DNA from *E.coli* MDR was isolated successfully by rapid boiling method. The plasmid DNA shows a clear single band with the size more than 51kb (Fig. 1).

Fig. 1. Agarose gel electrophoresis of Plasmid DNA from MDR *E.coli*. Lane1: 1Kb, DNA ladder; Lane 2-7: Plasmid DNA



Detection of Integrons by PCR

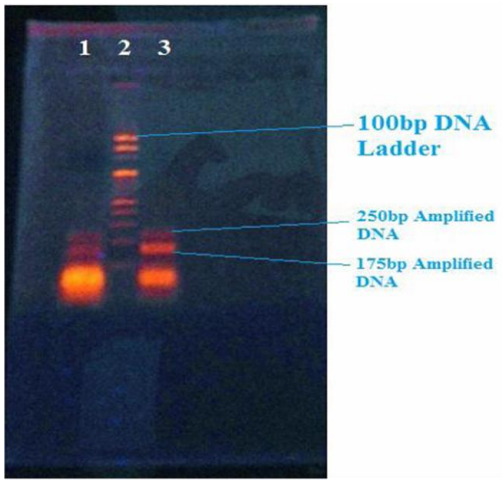
Isolated plasmid DNA was used for the detection of Integron genes and PCR results showed that integron genes were successfully amplified with 250 and 175 bp size (Fig. 2).

Discussion

Urinary tract infection is a common bacterial disease, often contributes to a frequent cause of morbidity in out-patients as well as hospitalized-patients (Agodi et al., 2010; Baral et al., 2012). Clinical experience has

indicated the presence of numerous cases of antibiotic resistance to common antibiotics by uropathogens in both developed and developing countries (Baral et al., 2012). Resistant to newer and more potent antimicrobials are no exceptions, making the therapeutic options very limited to certain antimicrobial agents like carbapenem, colistin and fosfomycin (Giamarellou, 2010). The updated knowledge and situation of the prevailing bacterial uropathogens that are multidrug resistant (MDR) is of prime importance for the proper use of antimicrobial drugs and the policy making to combat multidrug resistance in UTIs (Giamarellou, 2010). Worldwide *E. coli* is the major cause of UTIs and accounts for 75% to 90% of UTI isolates (Poisson et al., 2010). The increasing prevalence of infections caused by antibiotic-resistant bacteria makes the empirical treatment of UTIs difficult and outcome unpredictable.

Fig. 2. Amplification of Integrase genes of *E.coli* MDR. Lane1: Amplified integrase gene product (2.0 mM $MgCl_2$ conc); Lane 2: 100 bp DNA ladder; Lane 3: Amplified integrase gene product (2.5 mM $MgCl_2$ conc)



The present study provides the information about the isolation, identification and characterization of hospital isolate as *E.coli* and confirmed as MDR strain. The clinical isolate in the present study was obtained from Pravara Rural Hospital, Loni, India. The results for Gram nature and biochemical tests confirmed that the isolate was *E.coli* strain. Antibiotic susceptibility testing of this *E.coli* strain shows that it is resistant to Gentamicin, Amikacin, Deoxycycline hydrochloride, Co-thimoxozole, Cefalexin, Ampicillin, and Chloremphinocol and hence the strain showing resistance to more than 3 antibiotics is defined as multi drug resistance (Falagas et al., 2006). Further, to confirm this hospital isolate as MDR, we used molecular methods such as plasmid isolation and amplification of integrase genes. *E. coli* is a common enteric commensal of mammals and a common cause of human infections. As such, *E. coli* strains are routinely exposed to a wide range of antimicrobial agents. *E. coli* also has a very wide natural distribution (Selander and Levin, 2006) and a propensity for plasmid carriage. Resistance to various antibiotics is relatively common in clinical pathogens and also common in *E. coli* strains and it is frequently plasmid-mediated. In this study, we investigated the possible relationship between antibiotic resistance and genetic determinants (plasmids and integrons). Since MDR in *E.coli* is mainly due to large conjugative plasmid. It was observed that the size of isolated plasmid DNA from MDR strain is more than 52 kbp (Sanouri et al., 2008).

Integrons play an important role in antibiotic resistance of clinical *E.coli* strains because they are able to capture, integrate and express gene cassettes in coding antibiotic resistance (Bing et al., 2008). The prevalence of integrons ranging from 22-59% has been reported in clinical *E.coli* (Idrees et al., 2011). It is well known that integrons carry and transfer MDR genes in bacteria (Huang et al., 2012). In our isolate integrons were detected by amplification of integron genes as class I and Class II with 175 and 250 bps size respectively. These integron genes

may involve in resistance to certain antibiotics including Gentamicin, Amikacin, Deoxycycline hydrochloride, Co-thimoxozole, Cefalexin, Ampicillin, and Chloremphinocol. Nevertheless, the genetic association of these Class I and Class II integrons cassettes regions may be confirmed by sequencing.

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