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Molecular typing of Multidrug Resistant Uropathogenic *Escherichia coli* by Restriction Fragment Length Polymorphism

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Abstract

The present study investigates the prevalence, antibiotic susceptibility pattern, and molecular characterization of multi-drug resistant uropathogenic *Escherichia coli* (E. coli) isolated from patients. Antibiotic susceptibility testing (AST) revealed a significantly high resistant level in all fifty uropathogenic E. coli (UPEC) isolates against tested antibiotics (tetracycline, doxycycline, azithromycin, erythromycin, ciprofloxacin, levofloxacin, ampicillin, amoxicillin, amikacin and gentamycin) and was found as multidrug-resistant. The amplified products of housekeeping genes (adk, gyrB, icd, mdh, purA, recA, and fumC) and 16S rRNAgene of *E. coli* isolates were subjected to Restriction fragment length polymorphism (RFLP) analysis and correlated with antibiotic susceptibility pattern. Using this molecular marker technique, it was evident that genetic variability occurred among *E. coli* isolates from different infectious samples. In AMOVA analysis, the calculation of Nei's genetic diversity (H) and Shannon's information index(I) were observed maximum in group-I of housekeeping genes (H-0.2032, I-0.3265) thereby confirming remarkable polymorphisms in group-I isolates. The dendrogram obtained from Neighbor-joining and structural analysis revealed the splitting of isolates into three clusters with a clear distinction of sensitive/ low resistance strains from moderate resistance and high resistance strains. This study reports a clear correlation between genetic and phenotypic aspects of *E. coli* isolates which is quite evident from statistical analysis like AMOVA, structural analysis, and Principal Coordinate analysis. The data obtained from this study could be useful in designing strategies for better management of UTI disease.

Keywords: Uropathogenic *Escherichia coli*; Urinary tract infection; Antibiotic susceptibility; Genetic polymorphism; Restriction fragment length polymorphism.

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1. Introduction

Urinary tract infection (UTI) is one of the most common infections affecting the population of all age groups with characteristic symptoms, invasion, and multiplication of a significant number of bacteria *i.e.* 10^5 cfu/ml of bacteria within the urinary tract.[1] UTI remains a significant cause of morbidity in a population of all age groups and sex which increases expenditures related to health care.[2-4] Among all uropathogenic bacteria, *Escherichia coli* (facultative gram-negative rod-shaped bacteria belonging to the *Enterobacteriaceae* family) is reported as the main causative agent of hospital-acquired UTIs followed by *Klebsiella sp.*, *Proteus sp.*, *Pseudomonas aeruginosa*, *Enterobacter sp.*, *Acinetobacter sp.*, *Citrobacter sp.* and gram-positive bacteria such as *Staphylococcus aureus* and *S. saprophyticus.*[5-10] More than 85% of community-acquired urinary tract infections are also caused by uropathogenic *Escherichia coli*(*E. coli*).[11] It has been estimated that every year, 150 million cases of UTI occur on a global basis and cost about 6 billion dollars for national health resources.[12]

Although the development of antibiotics led to the optimism that infectious diseases can be controlled and prevented, infectious diseases still are the second leading cause of death worldwide and the third leading cause of death in developed countries due to antimicrobial resistance. Antimicrobial drug resistance (AMR) becomes a global phenomenon in both developed and developing countries. Due to prolonged use and misuse of antibiotics pathogens

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especially *E. coli* develop resistance against multiple drugs by various methods including enzymatic inactivation of antibiotics, alteration of the antibiotic target site, and reduced permeability or uptake. AMR is a major clinical and public health problem that diminishes the efficacy of antimicrobial drugs resulting in an increment in healthcare-related costs as well as illness and death rates.[13-15]

Many different typing methods have been developed which are very useful in describing the epidemiology of pathogenic strains. The earlier methods were based on phenotypic typing while modern methods have been based on genotypes of bacteria.[16,17] PCR (Polymerase Chain Reaction), RFLP, SSCP, and sequencing are the molecular methods routinely used for typing and classification of *E. coli.* Genotyping methods are based on direct analysis of the genetic structures of strains under study. Restriction fragment length polymorphism analysis (RFLP), Random amplified fragment analysis (RAPD), Pulsed-field gel electrophoresis (PFGE), Ribo-typing, Single-stranded length polymorphism (SSCP), enterobacterial repetitive intergenic consensus sequence PCR (ERIC-PCR) and multilocus sequence typing (MLST) are several genotyping methods commonly used for the characterization of *E. coli.*[18-20] RFLP technique is also widely used for the detection of polymorphism among strains based on the number and location of restriction sites which results in a different DNA banding pattern. This method has been widely used in the typing of different pathogens such as *Salmonella*, *Campylobacter species,* and *E. coli.*[21-25] PCR-RFLP is also widely used in the determination of population structure as well as in various characteristics such as the detection of antibiotic resistance.[26,27]

The emerging trend of multidrug resistance among uropathogenic*E. coli* further compromised the efficacy of antibiotics and decreased remedial options in services associated with healthcare. This trend also eventually leads to an increase in medical costs, mortality, and morbidity rates.[28] It is also reported that different factors such as geographical region, type of healthcare service, and period of antibiotic evaluation of uropathogenic bacteria are responsible for variations in susceptibility profiles of uropathogenic*E. coli.*[29] Therefore, for effective treatment of UTI, it is crucial to gain knowledge about antibiotic susceptibility testing profiles of pathogenic bacteria associated with infections.[30,31] Thus, the present study has been proposed to determine antibiotic resistance and molecular epidemiology of multidrug-resistant uropathogenic *E. coli* (UPEC) circulating within a particular region under study using molecular marker technique *viz.* RFLP. Different statistical tools such as AMOVA analysis, Structural analysis, and Principal Coordinate analysis (PCoA) were used for the analysis of genetic data. AMOVA analysis was used to estimate the strength of genetic differentiation among multidrug-resistant UPEC isolates by calculating several genetic statistical parameters. To further test this population structure, the clustering method was implemented in the admixed model, STRUCTURE program[32] which calculated the estimate of the likelihood of data (LnP (D)) without prior information about populations.

2. Experimental section

2.1 Sample collection

A total of seventy urine samples of patients having UTI infections were collected from the Microbiology Laboratory of Maharishi Markandeshwar Institute of Medical Sciences and Research (MMISR) in a sterile container. Samples were inoculated onto cysteine lactose electrolyte deficient media followed by incubation at 37 °C for 24 hrs.

2.2 Identification of E. coli isolates

Screening of all isolates was done for identification and isolation of *E. coli* isolates on selective media viz. Mac Conkey agar and Eosin Methylene blue agar (EMB) media. Further confirmation of *E. coli* isolates was done by using different biochemical tests viz. indole test, methyl-red test, citrate test, Voges-Proskauer test, triple sugar iron test, urease test, and mannitol motility test. Cultures of all *E. coli* isolates were maintained in glycerol stock and stored at -20 °C.

2.3 Antibiotic susceptibility testing

The minimum inhibitory concentration (MIC) was determined by the agar dilution method on Mueller-Hinton agar to assay the antibiotic susceptibility pattern of *E. coli* isolates at varying concentrations of different antibiotics (tetracycline, doxycycline, ampicillin, amoxicillin, erythromycin, azithromycin, amikacin, gentamycin, ciprofloxacin and levofloxacin) used in this study.[33] The MICs of each isolate were determined according to interpretive standards defined by the Clinical and Laboratory Standards Institute.[34] The antibiotics were obtained from Cipla Ltd, Mumbai.

2.4 Isolation of genomic DNA and amplification of housekeeping genes and 16S rRNA gene

DNA isolation was accomplished using the Hi*PurA*™ bacterial genomic DNA purification kit following the manufacturer's instructions. The eluate was then stored at -20 °C. Amplification of seven housekeeping genes (*adk*, *gyrB*, *icd*, *mdh*, *purA*, *recA,* and *fumC)* and *16S rRNA* gene was done by PCR using gene-specific primers (Sigma) (Table 1). PCR reaction mixture for 50 µl reaction was as follows: 25µl 2X PCR Taq Mixture, 2 µl forward primer (10 pmol), 2 µl reverse primer (10 pmol), 2 µl DNA, and 19 µl nuclease-free water. The components of the reaction mixture were processed in a thermal cycler (Applied Biosystems) which was programmed for 35 cycles in the following conditions (Initial denaturation-94 °C for 5 minutes, denaturation- 94 °C for 1 minute, annealing-30 seconds, extension- 72 °C for 1 minute and final extension-72 °C for 10 minutes). The PCR amplicons were stored at -20 °C for further analysis.

2.5 RFLP analysis of PCR amplicons of genes (adk, gyrB, icd, mdh, purA, recA, fumC, and 16S rRNA)

The amplified products of the above-mentioned genes were digested with different restriction enzymes. The reaction mixture components for restriction digestion were used following the manufacturer's instructions (amplified product-5μl, restriction enzyme-1 unit, buffer-1μl, nuclease-free water-3.5 μl, and total volume-10 μl). The reaction components were mixed into PCR tubes for digestion and placed on a thermal cycler with the following reaction conditions (Table 2). All digested products were stored at 4 °C till further use. The digested products were run on 2% agarose gel for 1 h at 75 volts. A 100bp DNA ladder was used as a molecular marker.

2.6 Data collection and analysis

The RFLP banding pattern of each of the isolates of housekeeping genes and 16S rRNA gene were scored manually as present (1) or absent (0) to form a binary matrix. The light bands were omitted from scoring as they were not reproducible. Cluster analysis was performed by bootstrapping and neighbor-joining methods using the DARWIN program. The genetic population structure of all 50 isolates was inferred using STRUCTURE software (Bayesian clustering software) which assigns individuals into population group (K) by using RFLP loci data. POPGENE version 1.32 was used to calculate the genetic diversity parameters within each population: observed number of alleles (Na), the effective number of alleles (Ne), Nei's gene diversity (H), number of polymorphic loci (NPL), Shannon's information index (I), and percentage of polymorphic loci (PPL) for all 50 isolates. The total genetic diversity (Ht) for all loci of housekeeping genes RFLP was also observed for each isolate within three major groups (based on the response of each isolate towards antibiotics used in this study) using POPGENE software.

Table 1. Primers and reaction conditions are used for the PCR amplification of genes.

Name	Melting temperature (Tm°)	Sequence (5'-3')	Annealing Temperature (°C)
*adk*F	74.5	ATTCTGCTTGGCGCTCCGGG	54
*adk*R	63.8	CCGTCAACTTTCGCGTATTT	
*fumC*F	73.3	TCACAGGTCGCCAGCGCTTC	55
*fumC*R	61.4	GTACGCAGCGAAAAAGATTC	
*gyrB*F	78.1	TCGGCGACACGGATGACGGC	69
*gyrB*R	71.8	ATCAGGCCTTCACGCGCATC	
*icd*F	70.9	ATGGAAAGTAAAGTAGTTGTTCCGGCACA	54
*icd*R	65.5	GGACGCAGCAGGATCTGTT	
*mdh*F	59.3	TCTGAGCCATATCCCTACTG	53
*mdh*R	60.8	CGATAGATTTACGCTCTTCCA	
*purA*F	66.5	CGCGCTGATGAAAGAGATGA	52
*purA*R	66.0	CATACGGTAAGCCACGCAGA	
*recA*F	69.6	CGCATTCGCTTTACCCTGACC	53
*recA*R	71.6	TCGTCGAAATCTACGGACCGGA	
8F	58.5	AGAGTTTGATYMTGGCTCAG	54
1495R	57.5	CTACGGCTACCTTGTTACG	

Table 2. Conditions for restriction digestion of housekeeping genes amplicon.

S. No	Gene	Restriction enzyme	Incubation temperature (°C)	Incubation time (minutes)
1	*Adk*	FokI	37	90
2	*gyrB*	BglII	37	90
3	*Icd*	BstNI	60	90
4	*mdh*	TspRI	65	90
5	*purA*	BsaAI	37	90
6	*recA*	EcoRI	37	90
7	*fumC*	EcoRI	37	90
8	*16S rRNA*	MseI	37	15
9	*16S rRNA*	EcoRI	37	90
10	*16S rRNA*	AluI	37	30
11	*16S rRNA*	HaeIII	37	15

AMOVA (analysis of molecular variance) was performed through GenAlex 6.503 to observe variance components and percentage of variation among isolates and groups (based on the antibiotic pattern of each isolate). The dendrogram based on the unweighted pair group method of the arithmetic mean (UPGMA) was constructed using DARWIN. The Principal Coordinates Analysis (PCA) was also performed using RFLP data of each isolate.

3. Results and discussion

3.1 Antibiotic susceptibility testing

Fifty isolates (71%) out of seventy urine samples were confirmed as *E. coli*on on the basis of biochemical and morphological characteristics. *E. coli* was found to be the predominant uropathogen in this study**.** The MICs of all fifty *E. coli* strains have been depicted in Table 3. Based on the MIC range of the tested antibiotics, the *E. coli* isolates were divided into three groups: Group 1- susceptible (≤4 mg/L) and low resistant (≥4 mg/L-32 mg/L), group 2- medium resistant (64 mg/L-256mg/L) and group 3- high resistant (≥512 mg/L) (Table 4). In the present investigation, a remarkably high resistance level was observed for azithromycin (54%), erythromycin (84%), amoxicillin (52%), and ampicillin (86%) in *E. coli* isolate. The majority of the isolates (85%) exhibited MIC ≥512 mg/L for ampicillin and erythromycin. Only 20% of the isolates depicted MIC ≥512 mg/L for tetracycline, ciprofloxacin, amikacin, and gentamycin. In the present study, all *E. coli* isolates (100%) were resistant (low resistant, medium resistant, and high resistant) to azithromycin, erythromycin, and ciprofloxacin. The majority of isolates were found to be resistant against tetracycline (94%), levofloxacin (92%), ampicillin (88%), gentamycin (60%), amikacin (70%), amoxicillin (66%) and doxycycline (58%). The findings of the present study were comparable to different studies carried out worldwide.

Table 3. MICs of different antibiotics against *E. coli* isolates.

S. No	MICs (mg/L)									
	Tetracycline		Macrolides		Quinolones		Beta-lactams		Aminoglycosides	
	TET	DO	AZM	ERY	CIP	LVX	AMX	AMP	AK	GEN
S1	256	64	64	1024	128	16	>1024	>1024	64	512
S2	256	32	64	512	256	32	>1024	>1024	64	512
S3	256	16	16	256	128	128	1024	512	>1024	>1024
S4	256	4	16	512	32	64	8	>1024	32	512
S5	256	4	128	512	32	16	8	512	64	8
S6	64	4	1024	1024	1024	32	>1024	1024	64	1024
S7	64	4	64	1024	128	32	1024	1024	16	8
S8	1024	4	16	512	32	64	8	>1024	64	2
S9	256	4	16	512	64	32	>1024	>1024	16	2
S10	256	64	256	1024	512	64	>1024	>1024	>1024	4
S11	512	128	1024	1024	1024	32	>1024	>1024	64	2
S12	1024	32	128	512	256	64	>1024	>1024	64	256
S13	1024	32	>1024	1024	>1024	64	>1024	>1024	>1024	>1024
S14	256	16	1024	512	256	32	1024	1024	>1024	>1024
S15	1024	32	16	256	128	32	>1024	>1024	512	128
S16	1024	16	1024	512	128	32	1024	1024	>1024	>1024
S17	256	16	>1024	1024	16	32	>1024	512	64	256
S18	256	16	1024	1024	256	64	>1024	>1024	>1024	128
S19	256	4	256	1024	32	16	1024	1024	16	128
S20	256	64	128	>1024	1024	64	1024	1024	>1024	4
S21	256	16	64	512	128	32	1024	512	16	8
S22	256	32	16	512	128	32	1024	1024	16	256
S23	256	32	128	256	512	64	>1024	>1024	>1024	1024
S24	256	4	16	512	16	8	1024	1024	16	4
S25	256	2	16	256	64	16	8	512	64	2
S26	256	64	1024	>1024	>1024	64	>512	>1024	64	256
S27	512	32	128	1024	512	64	>1024	>1024	>1024	>1024
S28	256	128	16	512	16	32	1024	1024	16	128
S29	256	32	128	512	1024	32	8	512	16	4
S30	256	16	1024	512	16	32	8	1024	16	2
S31	256	8	1024	512	16	32	8	1024	16	2
S32	128	2	1024	512	16	16	8	1024	16	4

S. No	MICs (mg/L)									
	Tetracycline		Macrolides		Quinolones		Beta-lactams		Aminoglycosides	
	TET	DO	AZM	ERY	CIP	LVX	AMX	AMP	AK	GEN
S33	128	4	1024	512	16	16	32	8	32	2
S34	128	2	1024	512	16	32	8	1024	16	4
S35	256	4	>1024	512	16	32	8	8	16	2
S36	256	8	1024	512	16	32	8	8	16	4
S37	256	16	1024	512	16	16	32	8	16	2
S38	256	16	>1024	1024	256	32	32	8	32	8
S39	1024	4	1024	1024	32	2	32	>1024	32	8
S40	1024	4	1024	256	32	2	32	>1024	32	16
S41	1024	4	1024	128	16	2	8	32	64	8
S42	128	4	>1024	1024	16	128	8	8	64	16
S43	256	32	1024	1024	256	8	8	>1024	64	16
S44	128	8	1024	256	32	128	16	512	64	16
S45	128	4	1024	512	256	8	8	512	64	16
S46	4	16	1024	256	32	128	8	>1024	64	2
S47	4	8	1024	1024	32	128	16	>1024	32	16
S48	4	4	>1024	1024	16	2	8	>1024	32	2
S49	256	64	256	1024	512	64	1024	>1024	64	4
S50	128	4	16	512	256	16	1024	1024	1024	512

** TET-Tetracycline, DO-Doxycycline, AZT-Azithromycin, ERY-Erythromycin, CIP-Ciprofloxacin, LVX-Levofloxacin, AMX-Amoxicillin, AMP-Ampicillin, AK-Amikacin, and GEN-Gentamycin*

Table 4. Grouping of fifty isolates based on antibiotic susceptibility pattern against tested antibiotics.

Antibiotic pattern	*E. coli* isolates
Group 1 (Sensitive/low resistance)	S4, S5, S7, S8, S9, S24, S25, S30, S31, S33, S34, S35, S36, S37, S38, S39, S40, S29, S41, S42, S32, S43, S44, S45, S46, S47, S48
Group 2 (Moderate resistance)	S1, S2, S3, S12, S15, S17, S18, S21, S22 S23, S28, S49, S19
Group 3 (High resistance)	S6, S10, S11, S13, S14, S16, S20, S26, S27, S50

Dash *et al.*[35] conveyed a similar resistance level for erythromycin, doxycycline, and ampicillin but a high percentage of resistance for gentamycin (70%) and a low percentage of resistance for amikacin (40%) and ciprofloxacin (70%) in their study compared to the present study. Mandal *et al.*[36] depicted a similar resistance percentage for gentamycin (59.6%) in their study. Resistance was comparatively high for amikacin (70%) from other studies conducted worldwide.[35-37] The percentage of resistance for ampicillin observed in the present study was quite similar to other worldwide and local studies.[38-41]The variations observed in the resistance pattern in uropathogenic *E. coli* for different antimicrobial drugs in different regions may be due to the extensive and inappropriate use of particular antimicrobial agents in that particular region.

3.2 Molecular analysis using RFLP markers

The genomic DNA was isolated from multidrug-resistant *E. coli* isolates and observed on 0.8% Agarose gel electrophoresis. PCR amplification of seven housekeeping genes *viz. gyrB*, *mdh*, *purA*, *icd*, *recA, adk,* and *fumC* and *16S rRNA* gene was done for the molecular characterization of multi-drug resistant *E. coli* isolates. The amplified product of *adk*(583bp), *gyrB*(911bp), *icd* (878bp), *mdh*(708bp), *purA*(816bp), *recA* (780bp), *fumC*(806bp), and *16S rRNA* (1.5 kb) genes was visualized on Agarose gel electrophoresis (Figs. 1(a-h)). RFLP analysis was performed to study polymorphism among collected *E. coli* isolates. RFLP analysis of PCR products using six restriction enzymes for the restriction digestion of seven housekeeping genes *viz. BsaAI* (*purA* gene), *EcoRI* (*recA*and *fumC*), *FokI* (*adk*), BglII (*gyrB*), *BstNI* (*icd*), and *TspRI* (*mdh*) led to the characterization of three (*purA*, *recA* and *adk* gene), two (*fumC*, *mdh* and *icd* gene) distinguishable RFLP patterns suggesting remarkable polymorphism among UPEC isolates. All *E. coli* isolates of *gyrB* gene amplicon showed the same banding pattern with two bands of size 349bp and 551bp except six isolates that remained uncut after digestion with *BglII* restriction enzyme showed less polymorphism.

Similarly, RFLP analysis of 16S rRNA amplified products using four different restriction enzymes *viz. MseI, EcoRI, AluI, and HaeIII* showed distinguishable banding patterns among

UPEC isolates revealing remarkable polymorphism (Figs. 2(a-g), Fig. S(a-d)). Combined dendrogram analysis of 16S rRNA and housekeeping genes based on bootstrapping and unweighted neighbor-joining method grouped all fifty *E. coli* isolates into three major clusters, which were further divided into sub-clusters: cluster I (25 isolates), cluster II (18 isolates)

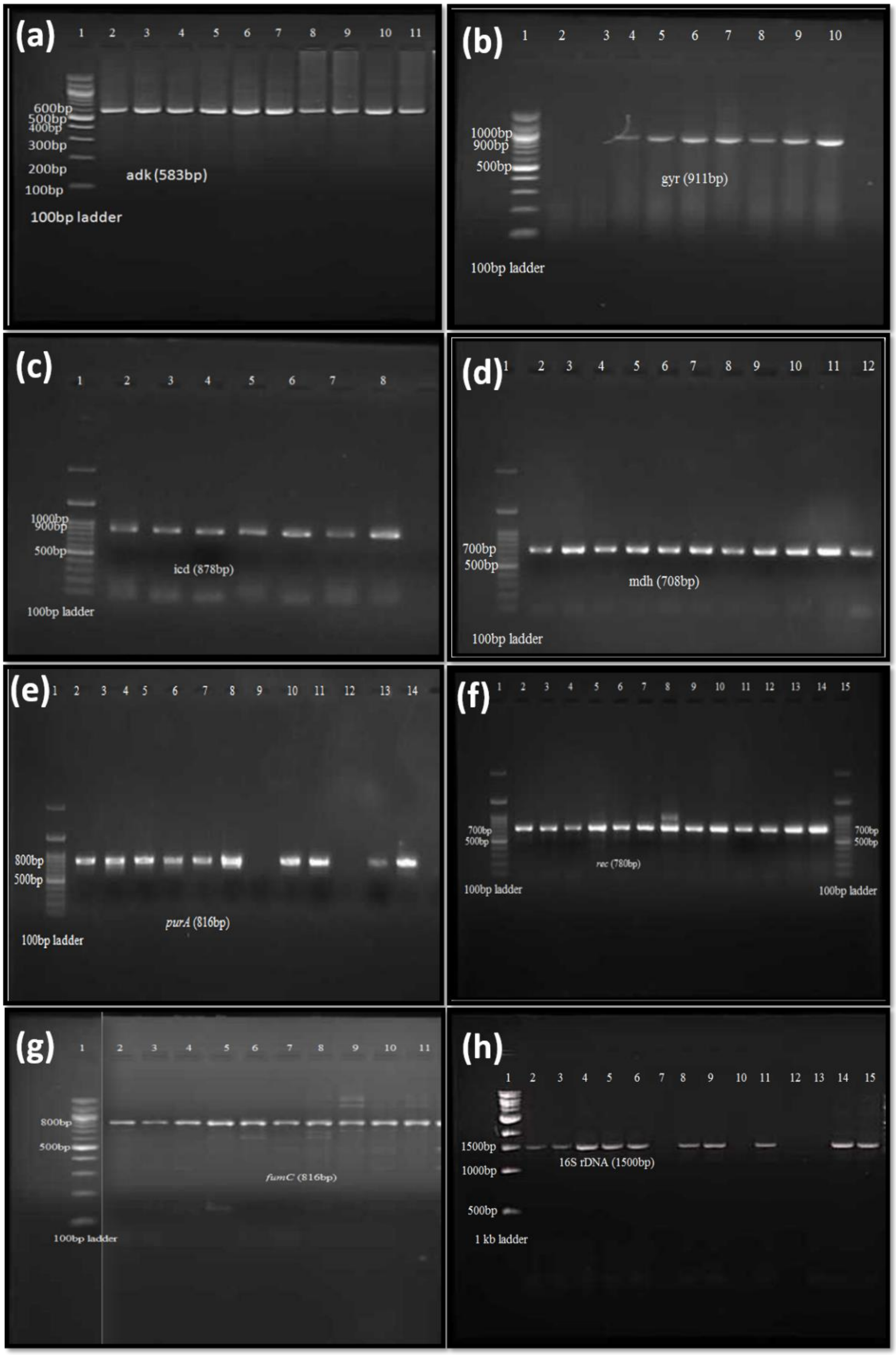

Fig. 1(a-h) A respective gel picture showing amplified products of *adk* (583bp), *gyrB* (911bp), *icd* (878bp), *mdh* (708bp), *purA* (816bp), *recA* (780bp), *fumC* (806bp) and *16S rRNA* (1500 bp) genes respectively (lane 1- 100 bp DNA ladder).

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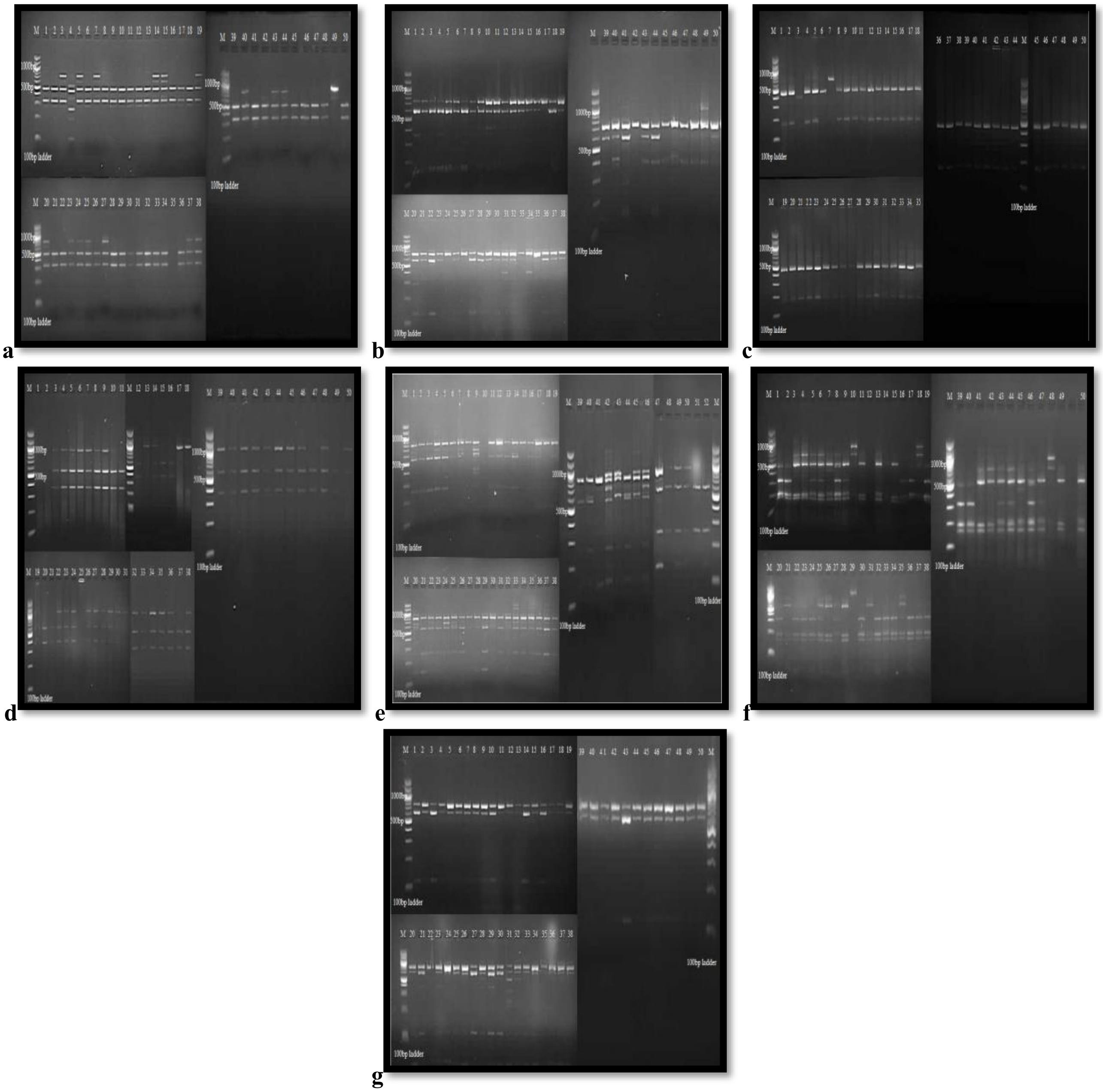

Fig. 2 (a-g) RFLP analysis of housekeeping genes of *E. coli* isolates: a) *purA* amplicon digested with BsaAI, b) *recA* amplicon digested with EcoRI, c) *adk* amplicon digested with FokI, d) *gyrB* amplicon digested with BglII, *e) fumC* amplicon digested with EcoRI, *f) mdh* amplicon digested with TspRI, *g)icd* amplicon digested with BstNI.

and cluster III (7 isolates). Among all tested antibiotics against isolates, a remarkable correlation between antibiotic susceptibility and dendrogram analysis was observed against the amoxicillin antibiotic. All moderate and high resistant isolates were clustered into cluster I except the S26 isolate which may be due to gene flow. Cluster II and cluster III contained all sensitive and low resistant isolates (Fig. 3). An unbiased clustering of *E. coli* isolates based on STRUCTURE software without prior knowledge about the populations clustered all fifty isolates into three major groups. Using the admixed model, STRUCTURE calculated the estimate of the likelihood of the data [LnP(D)] was maximum when K = 3. For K> 3, LnP(D) increased slightly but more or less plateaued (Fig. 4), *i.e.*, delta K reached its maximum value when K = 3 (Fig. 5(a)), suggesting that all the populations were into one of the three clusters with a lot of interference. Fig. 5(b) depicts that the STRUCTURE software assumed a total of three clusters (populations) based on the allele frequency of the isolates as highlighted by three different colors. The observed results of the bar plot are in agreement with the results of the neighbor-joining method, indicating three populations described as Cluster I, Cluster II and cluster III (Fig. 3). The population genetic variability analysis of all fifty isolates in terms of some important genetic diversity parameters *i.e.* Na, Ne, H, I, Ht and PPL with respect to three different groups such as sensitive/low resistant, intermediate and high resistant revealed higher values indicating remarkable polymorphism among isolates (Table 5).

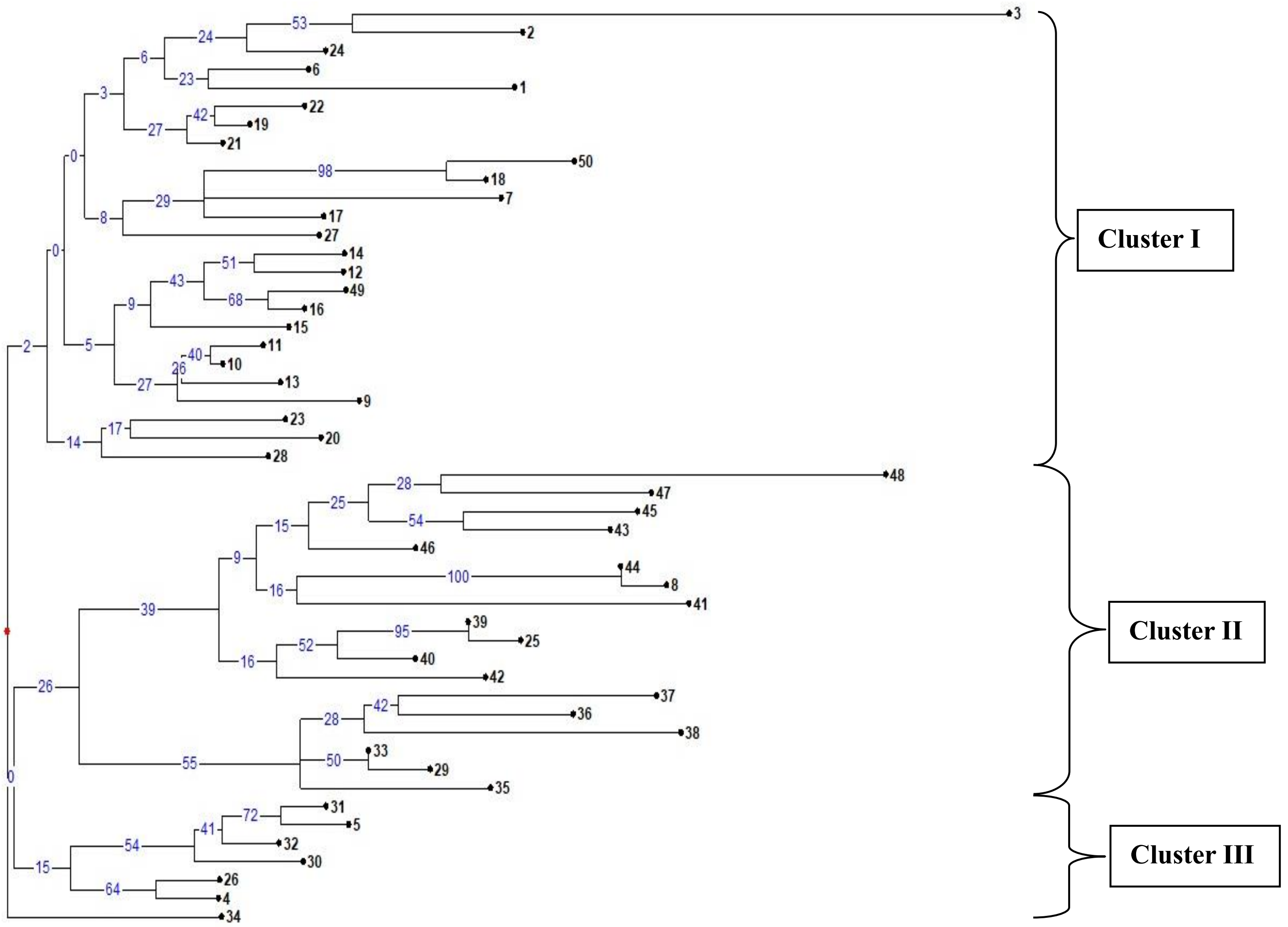

Fig. 3 Neighbor-joining tree representing clustering of *E. coli* isolates along with supported bootstrap values based on RFLP profiling showing three clusters: cluster I include 25 *E. coli* isolates (Isolates number- 3, 2, 24, 6, 1, 22, 19, 21, 50, 18, 7, 17, 27, 14, 12, 49, 16, 15, 11, 10, 13, 9, 23, 20, 28), cluster II includes 18 *E. coli* isolates (Isolates number- 48, 47, 45, 43, 46, 44, 8, 41, 39, 25, 40, 42, 37, 36, 38, 33, 29, 35) and cluster III includes 7 *E. coli* isolates (Isolates number- 31, 5, 32, 30, 26, 4, 34). Cluster I consists of all moderate and high resistant isolates except isolate number 26, and cluster II and cluster III consists of sensitive and low resistant isolates.

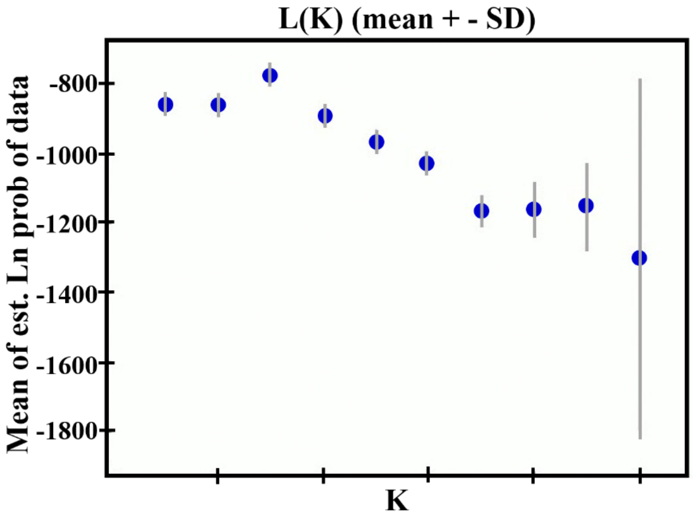

Fig. 4 Admixed model: STRUCTURE shows the relationship between the number of clusters (K) and the estimated likelihood of data (LnP (D) and finds [LnP(D)] maximum when K = 3. K denotes the population.

The percentage of polymorphic loci (PPL) using POPGENE was found maximum in the sensitive/low resistant group (83.33%) followed by the intermediate resistant group (69.44%). The minimum percentage of polymorphic loci was found in the highly resistant group (33.33%). The variability in percentage hence indicates a significant amount of genetic diversity in *E. coli* isolates. The Nei's gene diversity and total genetic diversity were observed maximum in group 1 (0.2032) followed by group 2 (0.1650) and minimum in group 3 (0.1006). Shannon's information index observed in group 1, group 2, and group 3 were 0.3265, 0.2703, and 0.1570, respectively. The results for overall genetic variability across fifty isolates in terms of the observed number of alleles (Na), the effective number of alleles (Ne), Nei's genetic diversity (H), Shannon's information index (I), heterozygosity (Ht), homozygosity (Hs), genetic differentiation (Gst), gene flow (Nm), number of polymorphic loci (NPL) and percentage of polymorphic loci (PPL) were reported in Table 6.

Table 5. Genetic variation statistics of all isolates for all loci of housekeeping genes RFLP with respect to their response towards antibiotics.

Response Towards Antibiotics	Sample size	Na	Ne	H	I	Ht	PPL
Group 1 (Sensitive/Low)	27	1.8333 (0.3780)	1.3120 (0.2953)	0.2032 (0.1609)	0.3265 (0.2245)	0.2032 (0.0259)	83.33
Group2 (Intermediate)	13	1.6944 (0.4672)	1.2462 (0.2754)	0.1650 (0.1539)	0.2703 (0.2234)	0.1650 (0.0237)	69.44
Group 3 (High)	10	1.3333 (0.4781)	1.1578 (0.2716)	0.1006 (0.1590)	0.1570 (0.2386)	0.1006 (0.0253)	33.33

Na = Observed number of alleles; Ne = Effective number of alleles; H = Nei's gene diversity; I = Shannon's Information index; Ht = Total genetic diversity; PPL = percentage of polymorphic loci.

Table 6. Overall genetic variability across all 50 *E. coli* isolates based on RFLP.

Sample size	Na	Ne	H	I	Ht	Hs	Gst	Nm	NPL	PPL (%)
50	2.0000 (0.0000)	1.2734 (0.2893)	0.1821 (0.1503)	0.3047 (0.2000)	0.1821 (0.0226)	0.1727 (0.0199)	0.0513	9.2491	36	100.00

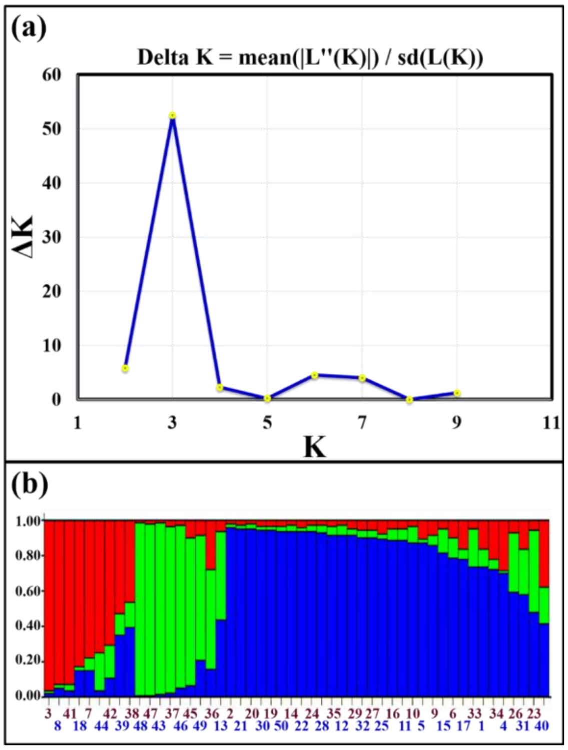

Fig. 5 (a) Relationship between K and delta K based on STRUCTURE analysis. The value of delta K observes maximum when K = 3. (b) STRUCTURE analysis of *E. coli* isolates based on RFLP data showing the grouping of isolates when K = 3.

The value of Gst for overall genetic variability was found to be low (0.0513) but the observed value of Nm was high (9.2491) indicating the significant genetic diversity in the collected *E. coli* isolates. The percentage of polymorphic loci was observed at 100% indicating the presence of a high degree of genetic variability among selected *E. coli* isolates. AMOVA analysis among isolates based on three major groups indicated the percentage of variation which was found to be 18.38% among groups and 81.62% within groups (Table 7). The percentage of variation was found to be low (18.38%) among groups which may be due to isolates under study belonging to a particular restricted geographical region.[42]

The two-dimensional PCA was also performed to accurately test the genetic relationship among all fifty isolates using EIGEN program analysis using AMOVA *via* GenALEx. The PCA was carried out with the collected RFLP binary data of each isolate depicted in Fig. 6. The populations or groups were coded in PCA as follows: the sensitive/low resistant isolates in blue color (pop 1), moderate resistant isolates in red color (pop 2) and the high resistant isolates in green color (pop 3). The PCA was carried out using the collected binary data of PCR-PFLP of 16S rRNA and housekeeping genes with GenAlex software. Principal Coordinates Analysis is a statistical method that converts data into distances between variables, and visualization into a map that can be used for understanding which variables are close to each other and which are different. The PCA analysis also showed an almost similar distribution of isolates observed in cluster analysis. In PCA analysis, all three populations were scattered in a plot that satisfied the percentage of variation (18.38%) among groups in AMOVA analysis.

Table 7. Analysis of molecular variance (AMOVA) obtained in fifty *E. coli* isolates using RFLP data concerning their response towards selected antibiotics.

Source of variation	Degree of Freedom	Variance component	Percentage of variation	P-value
Among groups	2.000	1.230	18.38	< 0.001
Within groups	47.000	5.461	81.62	< 0.001

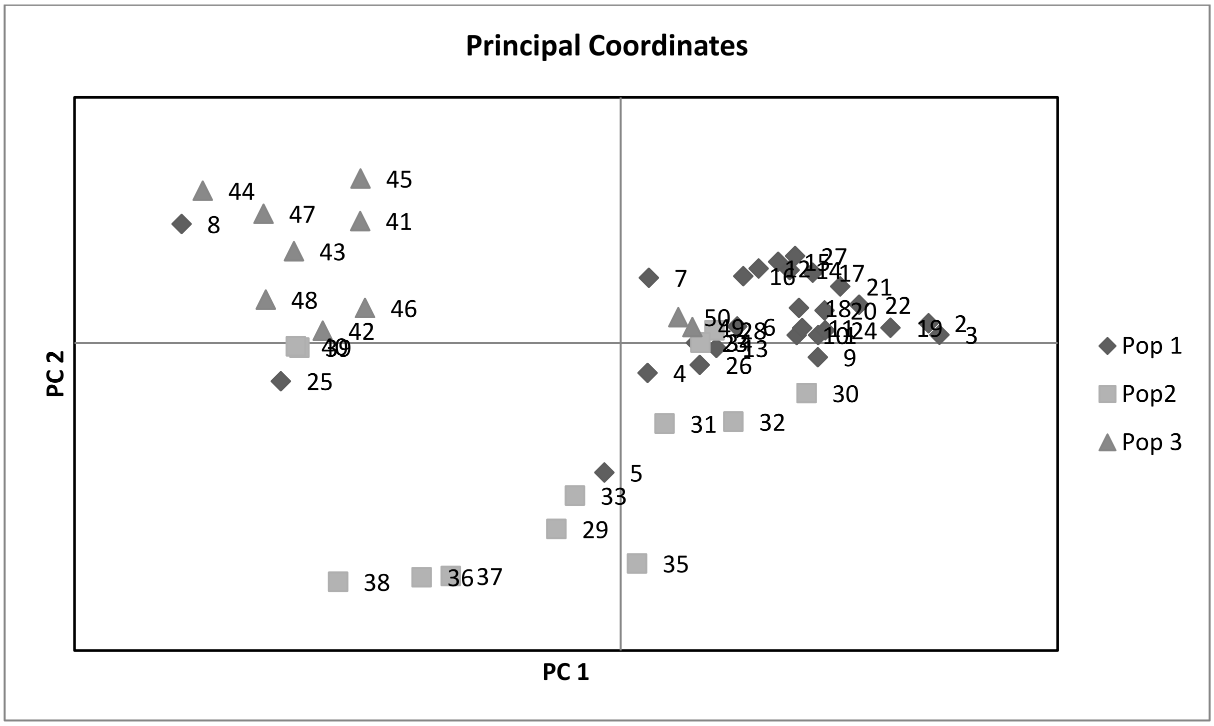

Fig. 6 Principal coordinates analysis of *E. coli* isolates in two-dimensions space-based using RFLP data, where Pop 1, 2, and 3 represent sensitive/low resistant, intermediate resistant, and high resistants groups, respectively.

4. Conclusion

In the present study, *E. coli* was found to be the most predominant uropathogen**.** Testing of *E. coli* isolates for susceptibility to tetracycline, doxycycline, ampicillin, amoxicillin, erythromycin, azithromycin, amikacin, gentamycin, ciprofloxacin, and levofloxacin revealed high resistance levels. Multidrug resistance was shown by all *E. coli* strains selected in this study emphasizing the need to educate the public about the appropriate use of antibiotics and for continuous surveillance of antimicrobial-resistant trends worldwide particularly MDR *E. coli* strains to cause UTIs. AMOVA analysis and population structure analysis revealed a significant level ($p<0.001$) of genetic and allelic diversity among *E. coli* isolates. In the present study, all the important genetic diversity parameters studied in population genetic analysis *i.e.* Nei's genetic diversity (H), Shannon's information index (I), and percentage of polymorphic loci (PPL) were observed maximum in group I (sensitive/low resistant group) which revealed the high polymorphism because of the presence of both sensitive *E. coli* strains and low resistant *E. coli* strains. With this study, we can conclude that molecular analysis using RFLP markers was extremely useful to study the genetic relationships of multidrug-resistant UPEC isolates. The results indicate the presence of high genetic polymorphism among isolates and clear differentiation between resistant and sensitive isolates based on dendrogram analysis. It was found that RFLP-PCR had good differentiation power for molecular typing of uropathogenic*E. coli* strains isolated from the patients in the study. The results can be used for developing a rapid detection method for the identification of multidrug resistance uropathogenic *E. coli.* For future studies, we suggest the use of more samples from different hospitals and the comparisons of the discrimination of the RFLP using other methods such as PFGE, MLST. We hope the results of our study will be useful for the epidemiological study of uropathogenic*E. coli* and upgrading public health. As shown by this study, the genetic variability in *E. coli* due to mutations is leading to multiple drugs resistant to generic drugs. So there is an immediate need for new alternatives for the treatment of UTIs with different targets and mechanisms of action. It is concluded that the results showed significant genetic differentiation among groups of uropathogenic *E. coli.* However, additional data such as gene sequences and analysis of samples from various geographical regions will be required for further insights into the evolutionary history of pathogens.

Conflict of interest

There are no conflicts to declare.

Supporting information

Applicable.

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