CHAPTER 4

RESULTS

4. RESULTS:

4.1 Bacterial isolates:

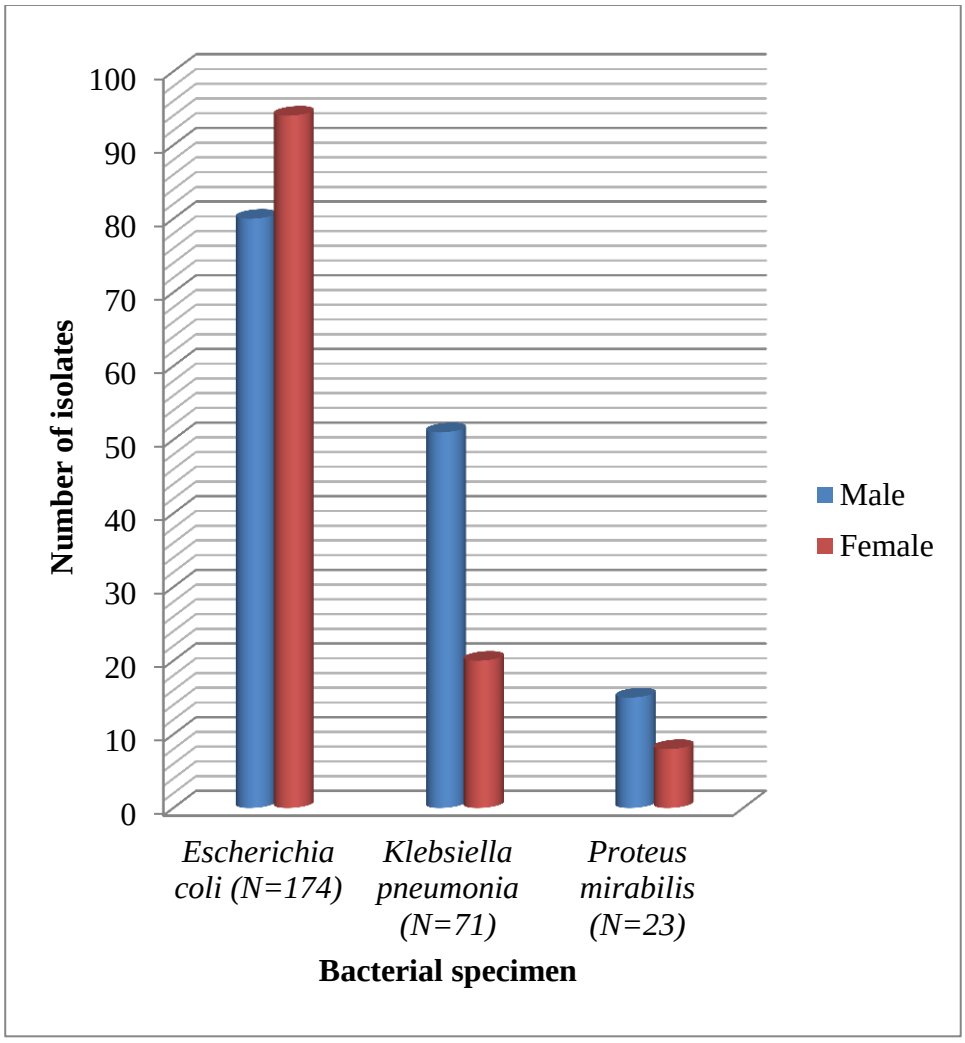
A total number of 268 isolates were obtained from patients admitted to different wards and attended OPDs of Silchar Medical College and Hospital, Silchar and patient's population were distributed among different age groups. The following (Chart 1) represents the number of clinical isolates of bacteria obtained from different gender. *Escherichia coli* was found to be most predominant followed by *Klebsiella pneumonia* and *Proteus mirabilis* (Table 11).

Table 11: Different isolates obtained in the study

Name of organisms	***Escherichia coli***	***Klebsiella pnuemoniae***	***Proteus mirabilis***
Isolated from indoor patients (n)=118	70	27	16
Isolated from outdoor patients (n)=150	104	44	7
Total =268	174	71	23

n= number of isolates

Chart 1: Patient's population based on gender.



4.2 Identification of bacterial isolates based on colony morphology:

Isolates were streaked on Mac-Conkey agar and their cultural characteristics were observed as mentioned (Table 12). Lactose fermenting pink colonies was observed for *Escherichia coli* (Figure 26) whereas for *Klebsiella pneumoniae* colonies were lactose fermenting and mucoid.

Table 12: Cultural characteristics:

Organisms	**Colony morphology on MacConkey Agar and CLED Agar**	**Motility**
Escherichia coli	Pink,non-mucoid,lactose fermenting on MacConkey Agar	Motile
Klebsiella pneumonia	Pink, and mucoid colony Lactose fermenting MacConkey Agar	Non-Motile
Proteus mirabilis	Colourless , non-lactose fermenting on CLED agar	Motile

Figure 26: Colony Morphology of *E.coli* on MacConkey agar plate.

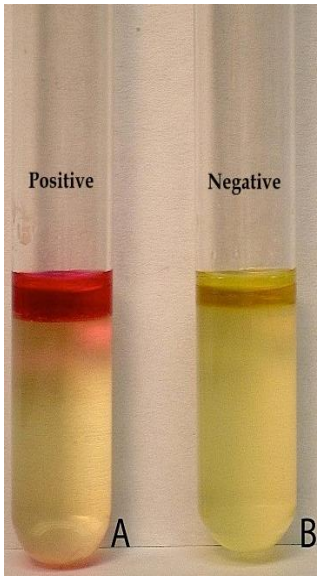
4.3 Biochemical Characterization:

Further catalase and oxidase test IMViC (indole, methyl red, VogesProskauer and citrate), urease, triple sugar iron test and sugar fermentation test were performed to identification of *Enterobacteriaceae* family (Figure 27).Other miscellaneous tests were also performed viz: Amino acid decarboxylase and dehydrolase (Moller's method), phenyl alanine deaminase, sugar fermentation, nitrate reduction, oxidative fermentative test. Organisms were identified as per biochemical reactions observed (Table 13, Figure 27).

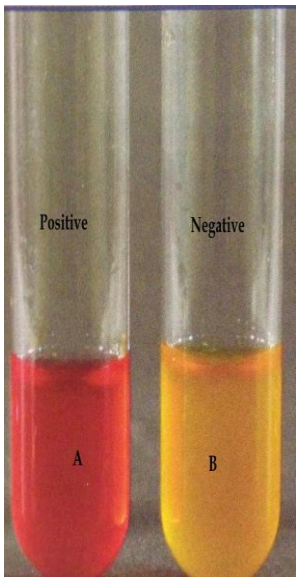
Table 13: Biochemical tests:

Organism	*Escherichia coli*	*Klebsiella pneumoniae*	*Proteus mirabilis*
Catalase	+	+	+
Oxidase	-	-	–
Indole	+	-	–
MR	+	-	+
VP	–	+	–
Citrate	-	+	+
Urease	–	+	+
TSI	**A/A**	**A/A**	**K/A, H_2S**
Moellers Decarboxylase – Arginine	+	–	–
Moellers Decarboxylase – lysine	–	+	–
Moellers Decarboxylase – ornithine	+	–	+
Deaminase test	–	+	+
Sugar fermentation – Glucose	+	+	+
Sugar fermentation – lactose	+	+	–
Sugar fermentation – sucrose	–	+	–
Sugar fermentation – manitol	+	+	–
Nitrate reduction	+	+	+
O/F huge and leifson test – oxidative	-	-	-
O/F huge and leifson test – fermentative	+	+	+

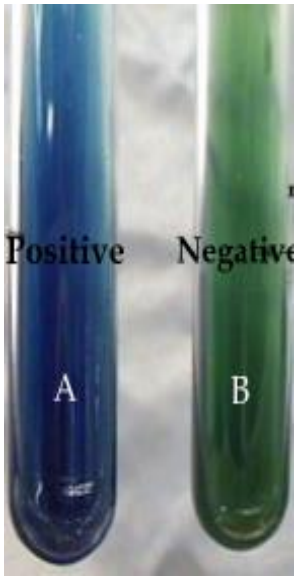
A/A= acidic slant /acidic butt. **K/A=** alkaline slant /acidic butt, with H_2S production.



27a

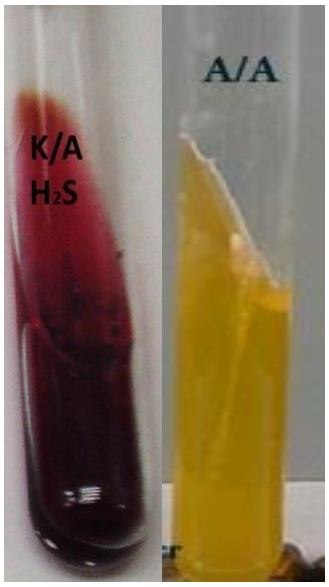


27b

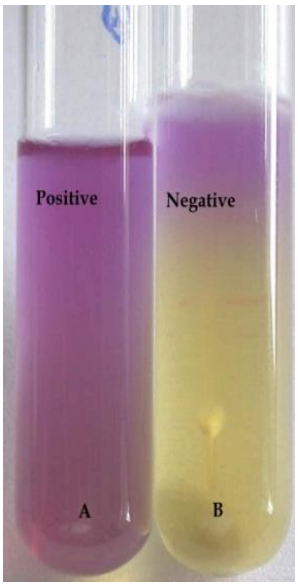


27c

27d



27e



27f

Figure 27: Biochemical characterization; 27a: Indole test, 27b: Methyl red, 27c: Citrate test, 27d: Urease test, 27e: Triple sugar iron test and 27f: (Decaboxylase test.)

4.4 Antibiotic susceptibility:

All the isolated (n=268) organisms were subjected to an initial screening for antibiogram profiling. Highest resistance towards Co-trimoxazole was observed in *Klebsiella pneumoniae* (95.77%) followed by *Proteus mirabilis* (91.30%) and *Escherichia coli* (88.5%). Forty eight isolates found resistance towards all the tested antibiotics (Table 14, Figure 28).

Table 14: Antibiotic susceptibility testing

Organism	No. of Resistant strains/total no. of Resistance strains (%)										
	Co-trimoxazole		Gentamicin		Ciprofloxacin		Cefepime		Ampicillin		Five of antibiotics
	n	%	n	%	n	%	n	%	n	%	
***Escherichia coli* (N) =174**	154	88.5%	155	89.1%	93	53.4%	83	47.7%	146	83.9%	36
***Klebsiella pneumoniae* (N) = 71**	68	95.8%	49	28.2%	51	71.8%	58	81.7%	68	95.8%	9
***Proteus Mirabiliss* (N) =23**	21	91.3%	14	60.9%	16	69.6%	15	65.2%	20	87%	3
Total	243	90.7%	218	81.3%	160	57.7%	156	58.2%	234	87.3%	
Total = 268											

N = Total no of isolates, n = Number of resistance isolates, % = Rate of resistance isolates.

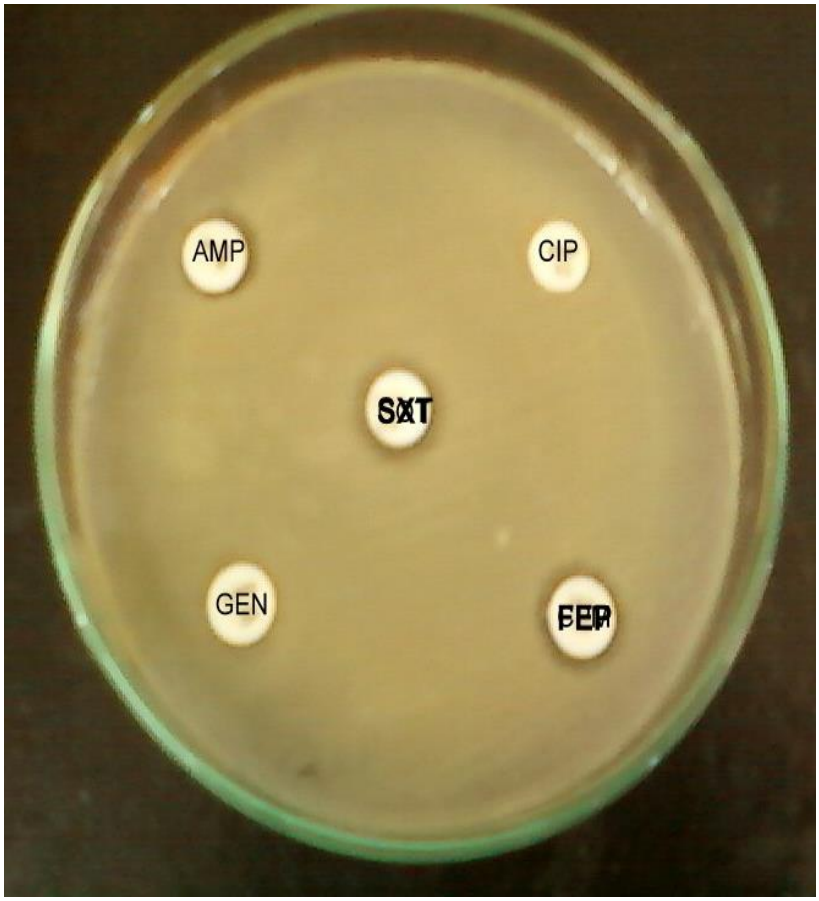


Figure 28: (Susceptibility profiling of corresponding antibiotics; showing resistance against all antibiotics). **SXT= Co-trimoxazole, FEP= Cefepime, AMP= Ampicillin, CIP= Ciprofloxacin**, **GEN= Gentamicin.**

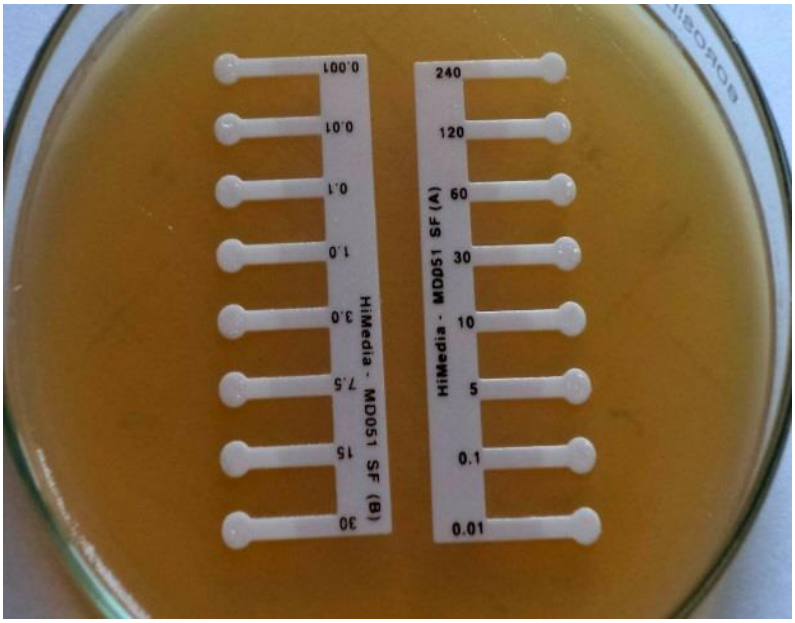
4.5 Minimum Inhibitory concentration

The test **was** done by using Hi-**comb strips (Hi-Media, Mumbai)** of Trimethoprim (TMP) and Sulphafurazole (SUL) (Figure 29a and 29b). The MICs of Trimethoprim varied between 4 µg/ml and >240 µg/ml among Co-trimoxazole resistant isolates (N=243) whereas all the isolates subjected to Sulphafurazole were shown resistance range above 240 µg/ml (Table 15).

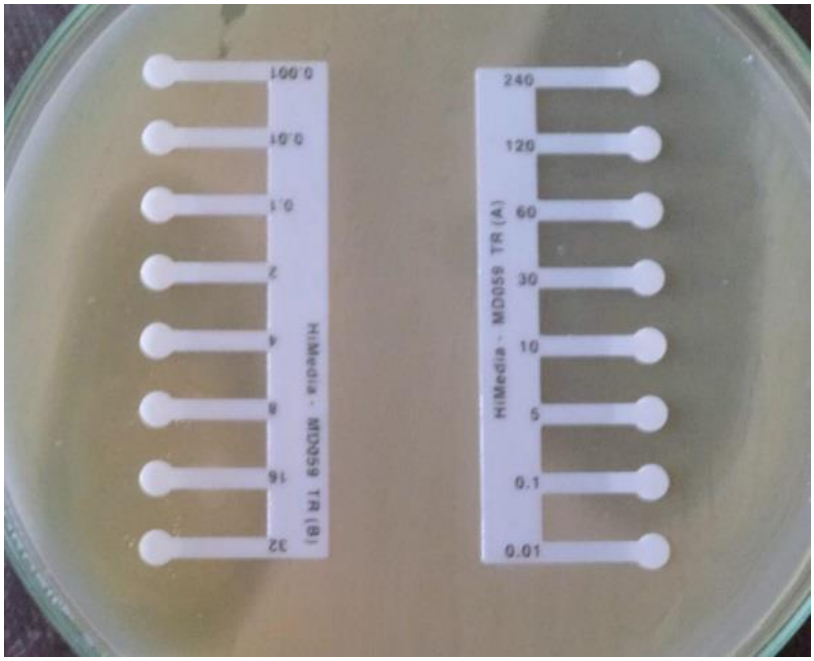
Table 15: Minimum Inhibition concentration

Arrangement of antibiogram combination number	**SUL (µg/ml)**	**TMP (µg/ml)**	**Number of isolates**
1	240<	4	2
2	240<	8	1
3	240<	16	1
4	240<	32	2
5	240<	>240	237

SUL = Sulphapurazole , **TMP** = Trimethoprim



29 a



29 b

Figure 29: (Minimum Inhibitory Concentration performed with Hi-Comb MIC strips). 29a; (MIC against Sulphafurazole) 29b; (MIC against Trimethoprim)

4.6 Genotypic characterization:

4.6.1 Screening of Class I and class II Integron among co-trimoxazole resistant isolates:

A total number of 149 isolates were found to harbour class I integron, whereas class II integron carriage was observed in 15 isolates. In 32 isolates, both class I and class II integrons were observed (Table 16 and Figure 30).

Table 16: Screening result of integron carriage within Enterobacteriaceae

Clinical specimen	***Int*1**	***Int2***	***Int*1 and *int*2**
Escherichia coli = 174	98	11	23
Klebsiella pneumoniae =71	44	2	4
***Proteus* mirabilis** =23	7	2	5
Total =	149	15	32

4.6.2 Detection of Sulphonamide resistance gene by multiplex PCR:

Among sulphonamide resistant strains *sul1* gene was found in 46 isolates, followed by *sul2* in 30 and *sul3* in one isolate respectively. Eighty four isolates were found to co-harbour multiple *sul* genes. (Table 17 and Figure 31).

Table 17: Screening result of Sulphonamide determinants with Enterobactericeae.

Clinical specimen	***Sul*1**	***Sul*2**	***Sul*3**	***Sul*1 and *Sul*2**	***Sul* 1, *Sul*2 and *Sul* 3**
Escherichia coli =174	25	17	1	58	3
Klebsiella pneumoniae = 71	17	12	0	14	0
Proteus mirabilis=23	4	1	0	12	1
Total =	46	30	1	84	4

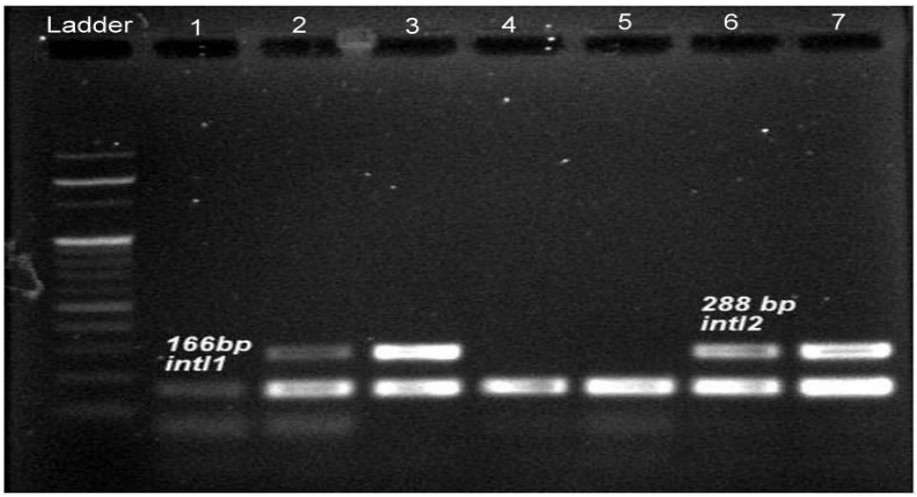


Figure 30: Characterization of class I and class II integron among bacterial isolates; lane 1, lane 4, lane 5 showing only class I integron and lane 2, lane 3, lane 6 and 7 showing amplification of both class I and class II integrons. Ladder, 100bp DNA ladder.

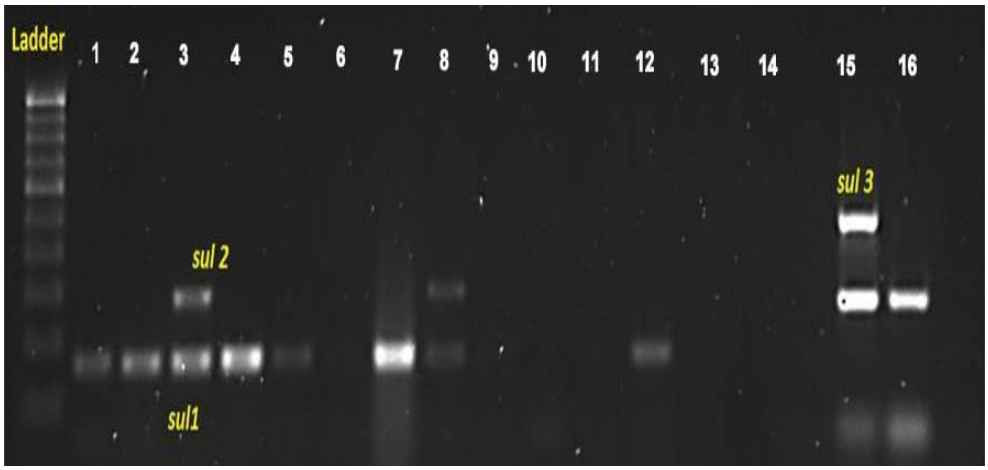


Figure 31: Detection of *sul* gene; lane 1, lane 2, lane 4, lane 5, lane7 and lane 12 amplifies only *sul 1*; lane 3, lane 8 amplify both *sul 1* and *sul 2* gene; lane 16 showing only *sul 2* gene and lane 15 showing both *sul2* and *sul 3* gene. 100 bp DNA ladder.

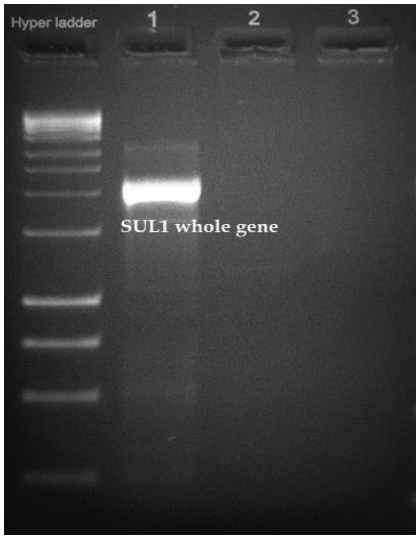
4.7 Cloning of *sul* gene:

Transformants were confirmed for the presence of *sul* genes by PCR (Figure 32a, 32b and 32c). The transformats were further subjected to MIC determination against trimethoprim and sulphafurazole. Cloning of all individual genes from each isolates was attempted where the MIC value for *sul2* and *sul3* against sulphonamide were in resistant range for both parent strains and their clones. However, for *sul1* gene variable MIC value was noticed for clones, where half of the clones showed the MIC range below break point (Table 18) (Figure 33 and 34).

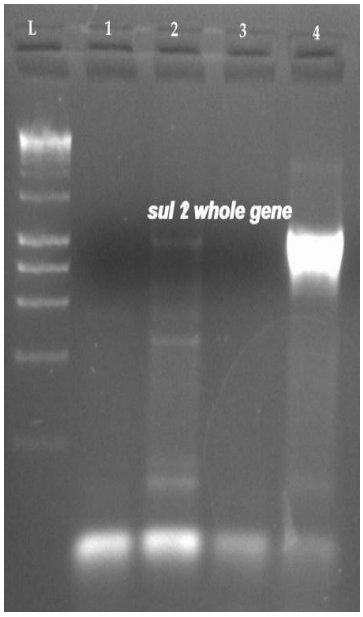
Table 18: MICs of wild types and cloned *sul* gene.

	Strains	***Sul1* (µg/ml)**	***Sul2* (µg/ml)**	***Sul3* (µg/ml)**
Sulphafurazole	Wild type	32 - >240	240<	240<
Trimethoprim	Clone type (*E.coli* JM107)	MIC_{50} ,	240<	240<

MIC_{50} = MIC range below 32(µg/ml).



32a



32b **32c**

Figure 32: Amplification of whole cloned *sul* gene among bacterial isolates; 32a, 32b and 32c showing the amplification whole *sul1*, *sul2* and *sul3* gene in lane1, lane 2 and lane 3 respectively. L, Hyperladder.

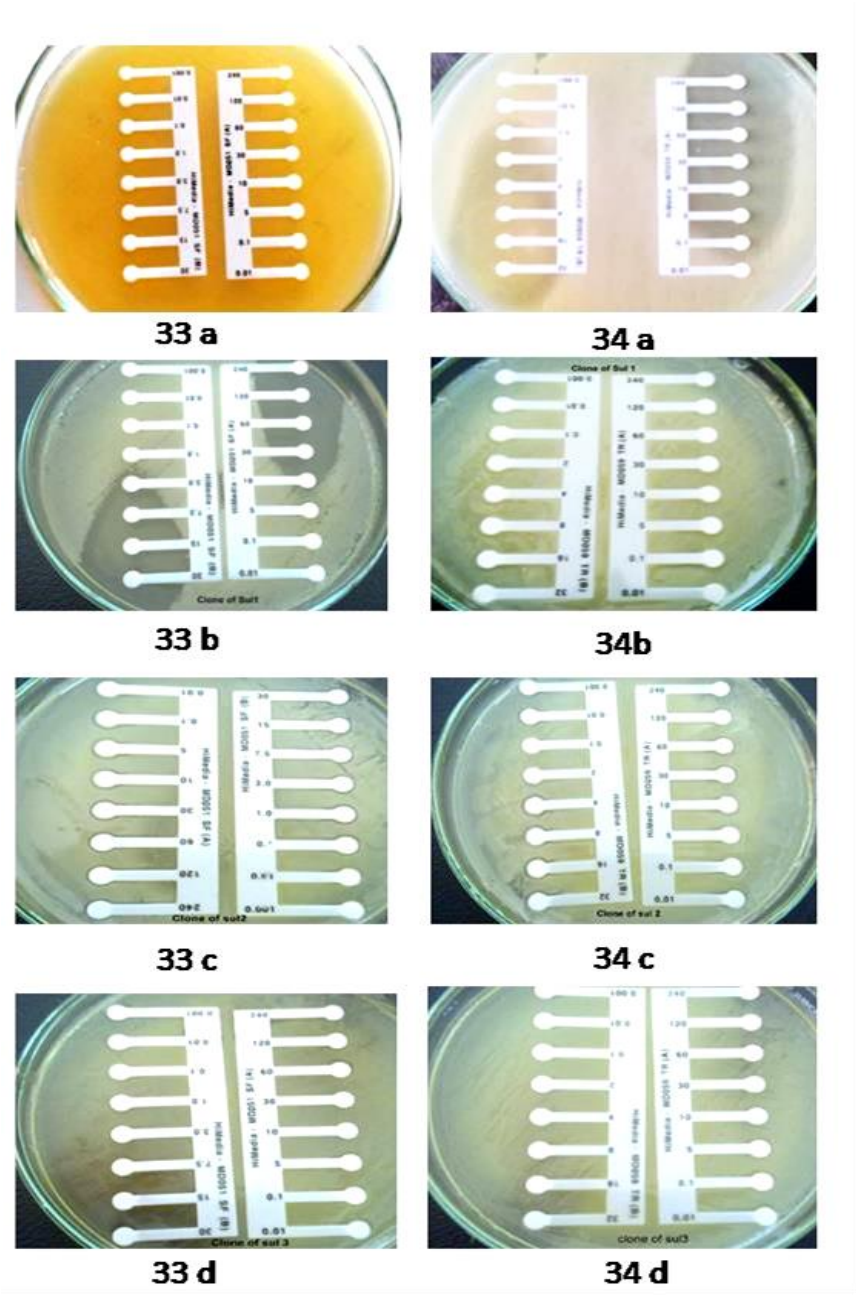


Figure 33 and 34: MIC panel of *sul* whole gene: **33a and 34a** showing the wild type strains against TMP and SUL respectively, **33b and 34b** showing the cloned *sul1* strains against TMP and SUL respectively, **33c and 34c** were showing the cloned *sul2* strains against TMP and SUL respectively, **33d and 34d** showing the cloned *sul3* strains against TMP and SUL respectively.

SUL= Sulphafurazole, TMP = Trimethoprim

4.8 Amplification of gene cassettes:

Total one hundred and seven isolates were screened out to amplify 59 base element (59-be) among them sixty three isolates were amplified above 600 bp (Figure 35). Twenty four isolates were amplified below 600 bp. For fifty four isolates no visible amplification could be observed with our target primers (Table 19). Sequencing results revealed the five different types of arrangement and mostly associated with *dfr* resistance gene (*dfrA12- orfF-aadA2*, *met-orfD-aadA6*, *dfrA1-met-aadA*, *dfrA12-aadA2-qacΔ1-sul*, *dfrA17-aadA5*). However, 59-be PCR also amplified some *orf* region and putative methylase transferase gene (*met*).

Table 19: Results of 59 base element PCR assay.

Isolates	**Number of cassette positive isolates/ total number of tested isolates**	**Size of integron gene cassette (bp)**
Escherichia coli = 174	61/174	200,300,500,700, 800,900,1000 , 1500,2500
Klebsiella pneumoniae =71	32/71	230,400.700,900
***Proteus* mirabilis** =23	14/23	200,400,700,800, 900,1000,1300

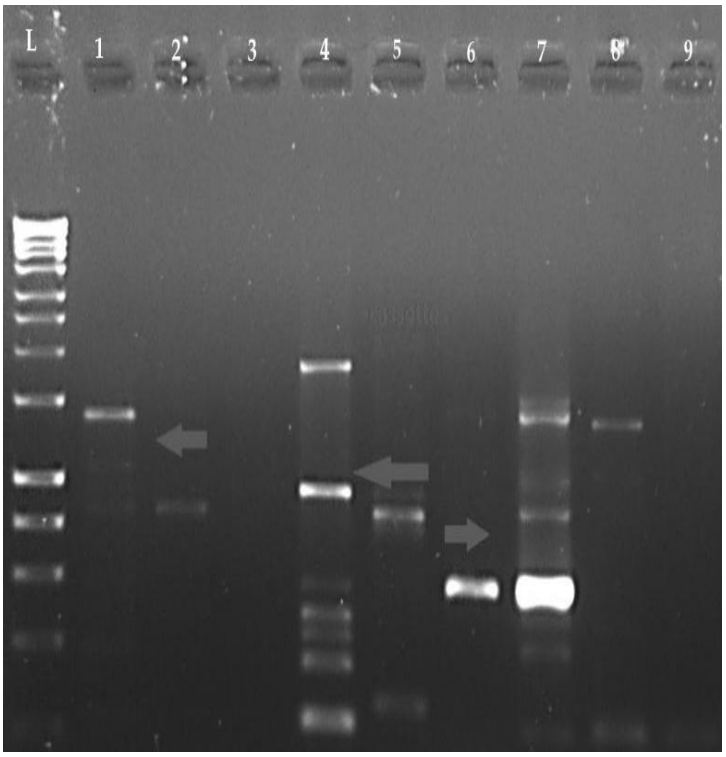


Figure 35: Amplification of gene cassette by 59 base elements; lane 2, lane 5 and lane 6 showing the amplification below 800 bp, lane 1, lane 4 and lane 7 showing the amplification ˜1.5 kb, lane 4 showing amplification at 2kb. L-Hyperladder.

4.9 Amplification of whole class 1 integron:

While amplifying the whole class I integron gene cassette as per the PCR array of Levesque et al, ninety five isolates showed distinct amplification. The variable size of class I integrons was between 800 bp-2.5 kb (Figure 36). Sequencing results of amplified products ranging 1.5 kb -2 kb (n=50) revealed five different array of variable regions and four different types of trimethoprim resistance determinants as gene cassette i.e; *dfrA*1, *dfrA*12, *dfrA*17, *dfrA*30 (Figure 37).

In arrangement I, is arranged as *dfrA17* and *aadA5* (confers streptomycin and spectinomycin resistance) cassette array, and found to be the most prevalent types (n=44). The amplified product with size 1.5 kb showed arrangement II of *dfrA30*, *aadA*5 in the downstream of the class I integron in one isolates, whereas arrangement III showed the presence of *orfF* in between two resistant determinants *dfrA*12 and *aadA*2 and this arrangement was observed in three isolates. In arrangement IV the association of *dfrA*1 and *aadA*1 gene was observed and this association could be observed only in one isolate. The V arrangement showed presence of *aacA*7 (confering aminoglycoside resistance) and *aadA*6 with two chronological 3'-conserved sequence which indicates the association of more than one transposable mechanism.

When tested for susceptibility of each arrangement that was cloned within *E.coli* JM107. Based on the gene cassette array, clones of five arrangements were further subjected to antibiotic susceptibility against co-trimoxazole (25 µg), gentamicin (10 µg), amikacin (30 µg), sulphafurazole (300

µg), trimethoprim (5 µg), streptomycin (10 µg) and kanamycin (30 µg). While testing the susceptibility patterns of clones of all respective arrangements, it was observed that the clones of the different arrangements showed variable resistance pattern (Figure 38). It was observed that in arrangement I and II the clones were resistance towards co-trimoxazole, trimethoprim and kanamycin whereas, found to be susceptible towards sulphafurazole, gentamicin, streptomycin and amikacin. In arrangement III and IV resistance was observed towards trimethoprim, cotrimoxazole and amikacin and whereas susceptible against rest of the tested aminoglycoside drugs. In arrangement V resistance against streptomycin was observed with amikacin and sulphafurazole (Table 20).

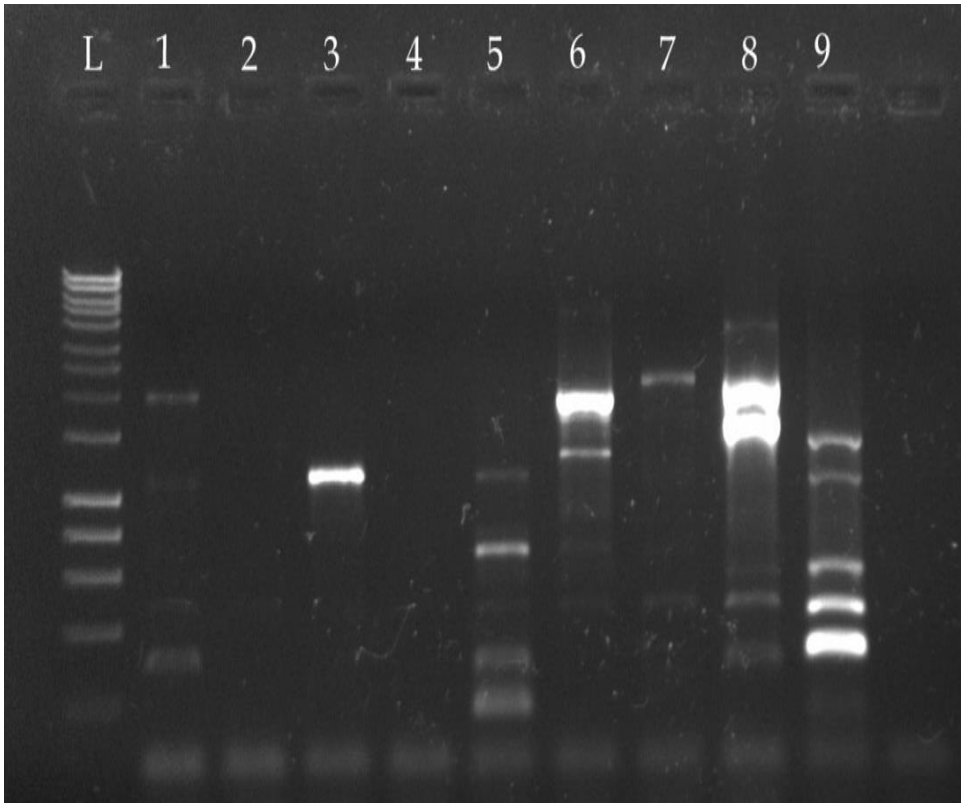


Figure 36: Amplification of whole class I integron; lane 1, 6, 7 and 8 showing amplification with 1.5 k and other ranges from 800 bp, L= Hyperladder**.**

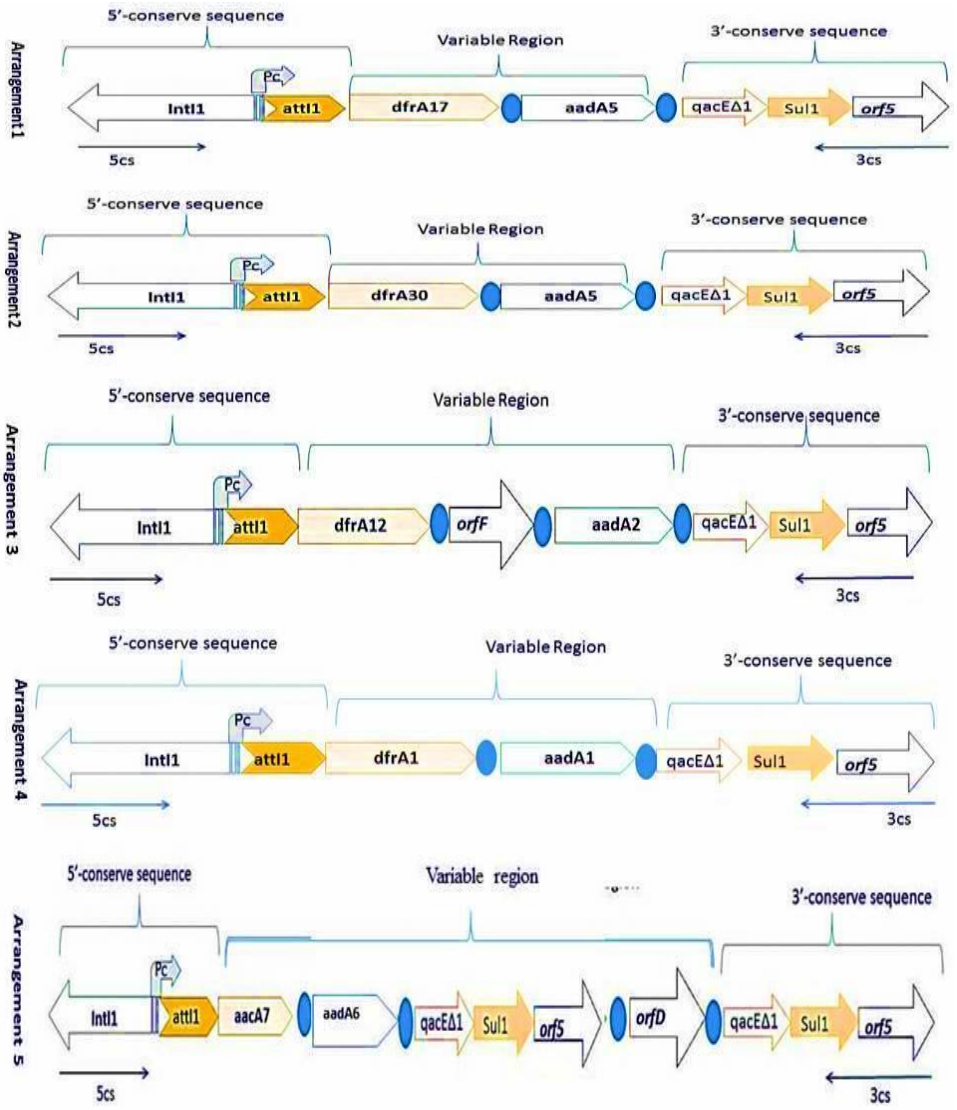


Figure 37: Schematic representation of variable region in class I integron, gene cassette are shown as a boxes, arrow represents the way of transcription and blue circle represents the *attC* site.

Arrangement 1: showing the chronological arrangement of class I integron and cassette carrying *dfrA*17 and *aadA*5.
Arrangment 2: showing the class I integron gene cassette along with *dfrA*30 and *aadA*5.
Arrangement 3: showing the trimethoprim determinant *dfrA*12 followed by *orfF*, hypothetical protein and *aadA*2.
Arrangement 4: showing class 1 integron associated with *dfrA*1 and *aadA*1
Arrangement 5: showing the variable region with *aacA*7 and *aad*

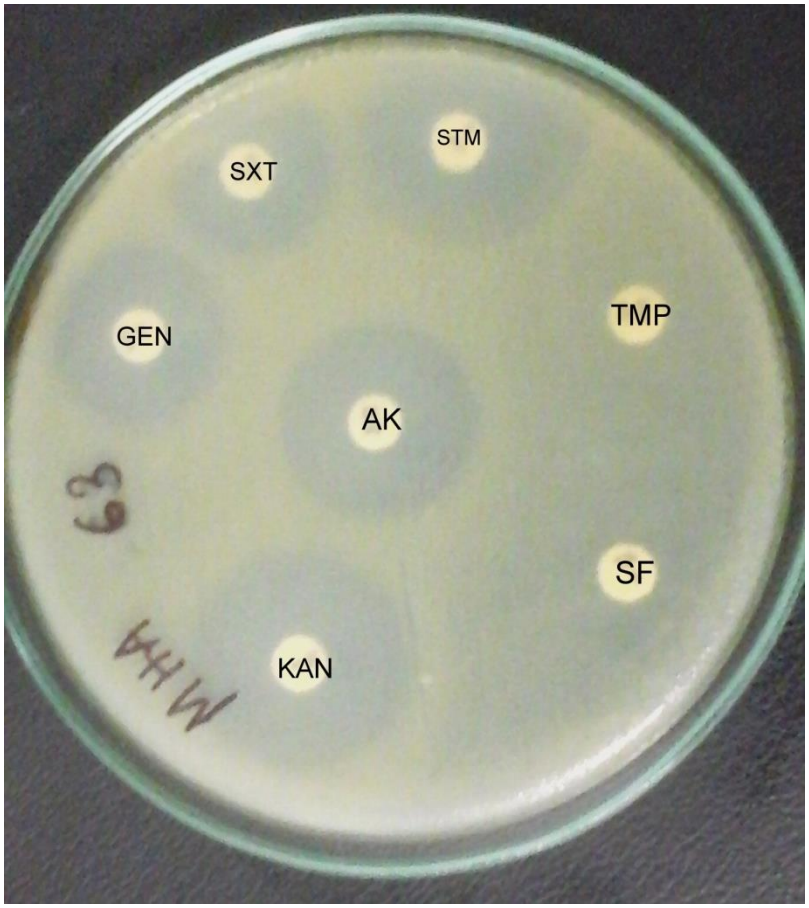


Figure 38: Susceptibility profiling of clones of all respective arrangements.

STM=streptomycin**, AK=** amikacin, **KAN=** kanamycin, **TMP=** trimethoprim**,**

SF= sulphafurazole**, GEN=**gentamicin and **SXT**= co-trimoxazole.

Table 20: Resistance profile of whole cloned class I integron

Arrangement	Resistance profile						
	STM	**AK**	**KAN**	**TMP**	**SF**	**GEN**	**SXT**
I	S	S	R	R	S	S	R
II	S	S	R	R	S	S	R
III	S	R	S	R	S	S	R
IV	S	R	S	R	S	S	R
V	R	R	S	S	S	S	S

STM=streptomycin**, AK=** amikacin, **KAN=** kanamycin, **TMP=** trimethoprim**, SF=** sulphafurazole**, GEN=**gentamicin, **SXT**= co-trimoxazole **and R=** resistance, **S=** susceptible.

4.10 Amplification, sequencing and mapping of class 2 integron gene cassettes and analysis of their clones:

Thirty seven isolates were found to harbour class II integron. In order to study the genetic context of the variable regions, 2-2.5 kb amplified products were obtained with four isolates, no amplification could be observed. Sequencing results revealed four different types of cassette arrays. Amplicon with sizes of 2 kb-2.3 kb were found in 11 stains (Figure 39), and were designated as arrangement I with *dfrA1*, *Sat2* and *aadA1*. Arrangement II, array of *ybeA*, *ybfA*, *ybfB* and *ybgA* were present in the downstream of the integron II and two was observed only in one strain in arrangement III *dfrA12* was followed by *Sat2* and *aadA1* and this particular arrangement was observed in one isolate. The arrangement IV was unusual and was only observed in two isolates of *Protues mirabilis* where *linF* was flanked by *dfrA1* and *aadA1* in upstream and downstream region respectively with an amplified product size 2.5 kb (Figure 40).

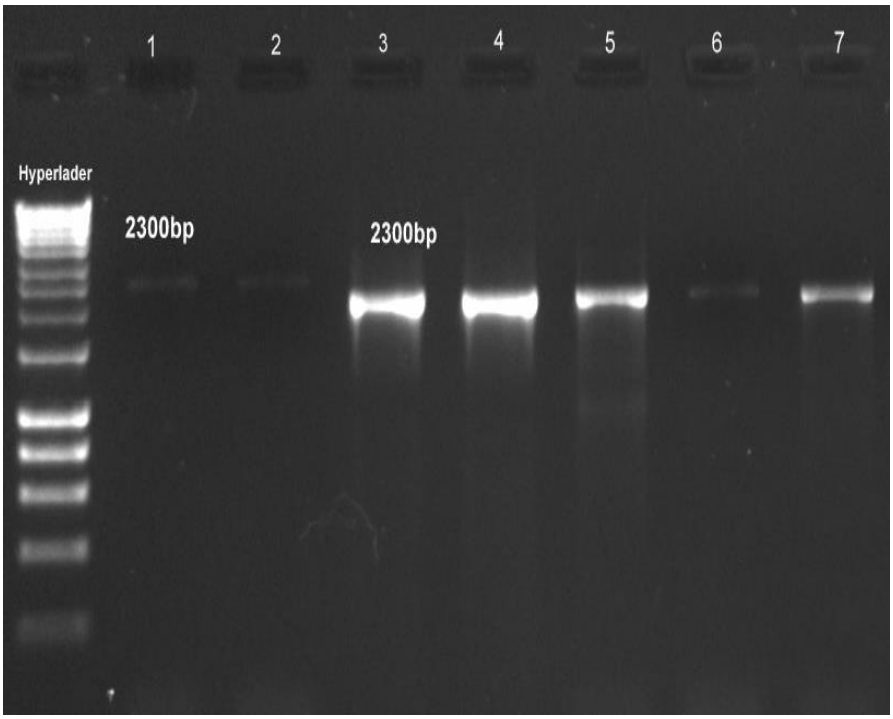


Figure 39: Characterization of class II integron; Lane 1 to 7 showing amplifies class II integrons with amplified size approx. 2300 bp. L, Hyperladder.

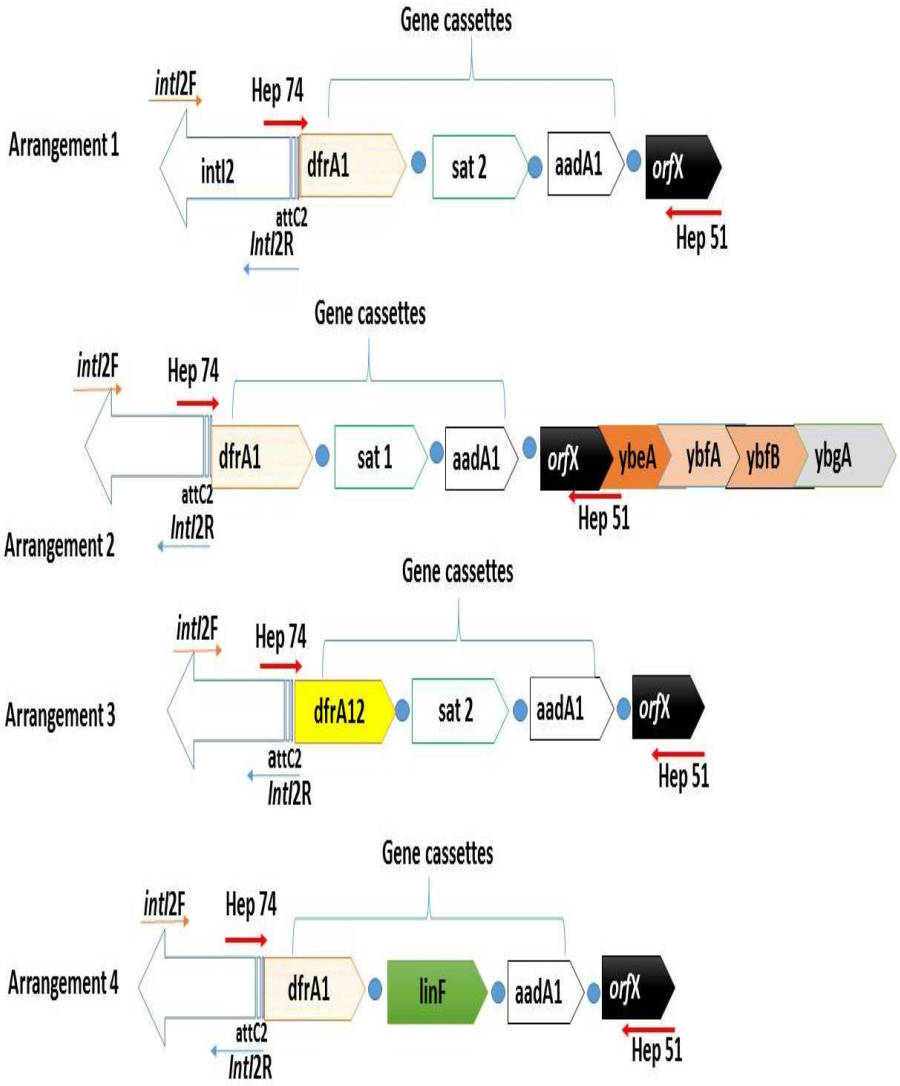


Figure 40: Representation of arrays of class II integron found among the bacterial population, gene cassettes are shown as boxes, with arrows indicating the orientation of transcription and circle represents the 59 base elements:

Arrangement 1 showing: chronological arrangement of class II integron gene cassette.
Arrangement 2: showing gene cassette arrangement with *ybfA-ybfB-ybgA*.
Arrangement 3: showing gene cassette pattern along with *dfrA12*.
Arrangement 4: showing cassette arrangement associated with *linF* instead of *sat2*.

4.10.2 Susceptibility results of clones of class II integron:

Based on the gene cassette array, clones of four arrangements were further subjected to antibiotic susceptibility against gentamicin (10 µg), netilmicin (30 µg), amikacin (30 µg), sulphafurazole (300 µg), trimethoprim (5 µg), kanamycin (30 µg) and ciprofloxacin (5 µg) [Hi-Media, Mumbai, India]. While testing the susceptibility patterns of clones of all respective arrangements, it was observed that the clones of the different arrangements showed variable resistance pattern (Figure 41). It was observed that in arrangement I and II the clones were resistance towards trimethoprim and kanamycin whereas, found to be susceptible towards netilimicin, gentamicin and amikacin. In arrangement III and IV resistance was observed towards trimethoprim and amikacin and whereas against rest of the tested aminoglycoside drugs (Table 21).

Table 21: Resistance profiles of cloned class II integron.

Arrangement	Resistance profile						
	NET	**GEN**	**CIP**	**SF**	**TMP**	**AK**	**KAN**
I	S	S	R	R	R	S	R
II	S	S	S	S	R	S	R
III	S	R	R	R	R	R	S
IV	S	R	S	S	R	R	S

NET= netilmicin, **GEN=** gentamicin, **CIP=** ciprofloxacin, **SF=** sulphafurazole, **TMP=** trimethoprim, **AK=** amikacin**, KAN=** kanamycin and **R=** resistance, **S=** susceptible.

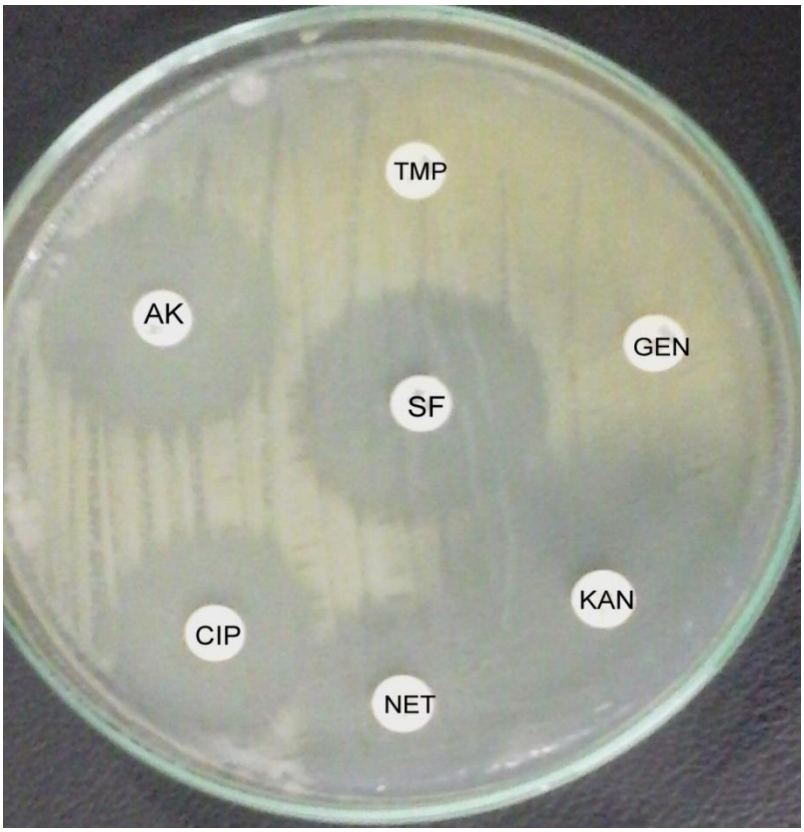
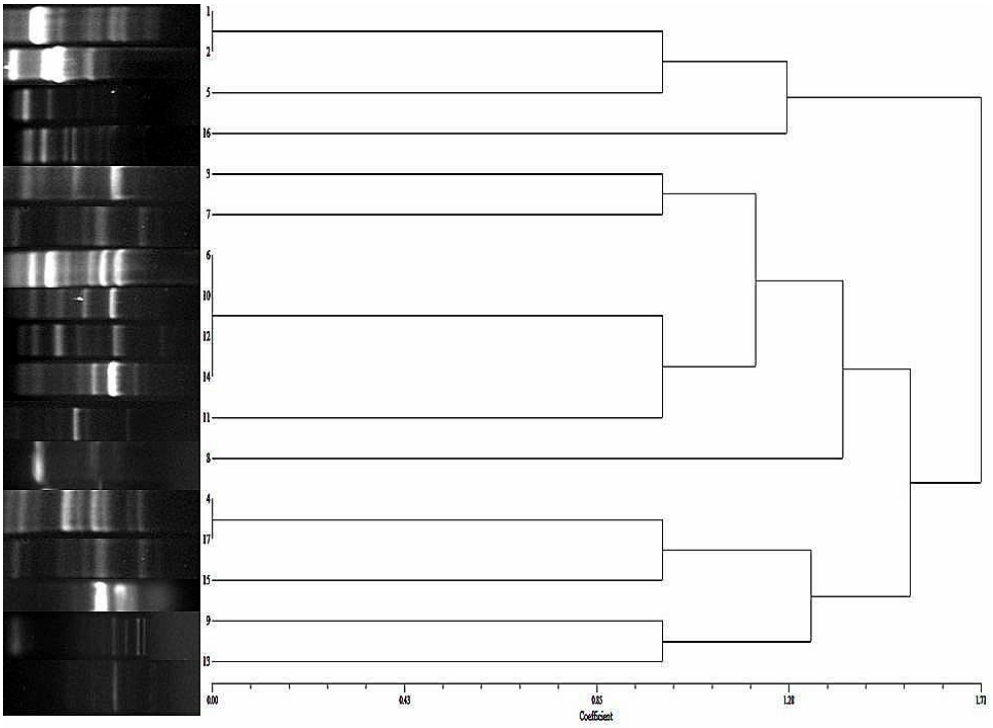


Figure 41: Susceptibility profiling of clones of all respective arrangements.

AK= amikacin, **KAN** = kanamycin, **TMP**= trimethoprim, **SF**= sulphafurazole, **CIP**= ciprofloxacin, **GEN**= gentamicin, and **NET**= netilimicin,

4.11 DNA fingerprinting of study isolates:

As per ERIC–PCR, 19 different haplotypes of *Escherichia.coli*, 17 types of *Klebsiella peumoniae* and 10 types of *Proteus mirabilis* was observed (Figure 42a, 42b and 42c)


1
2
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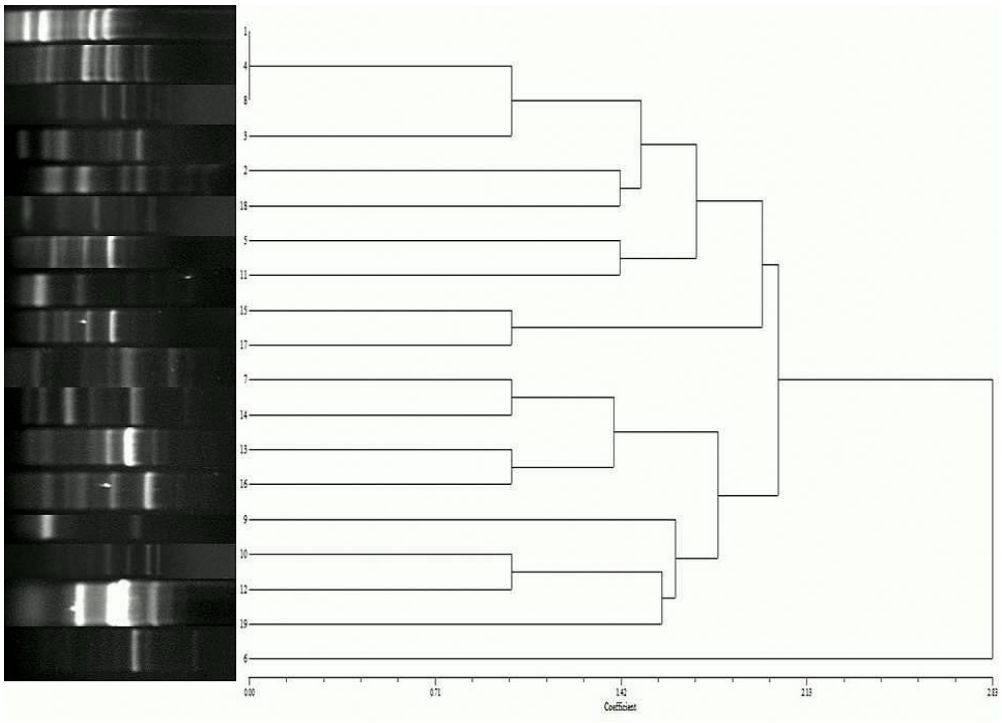
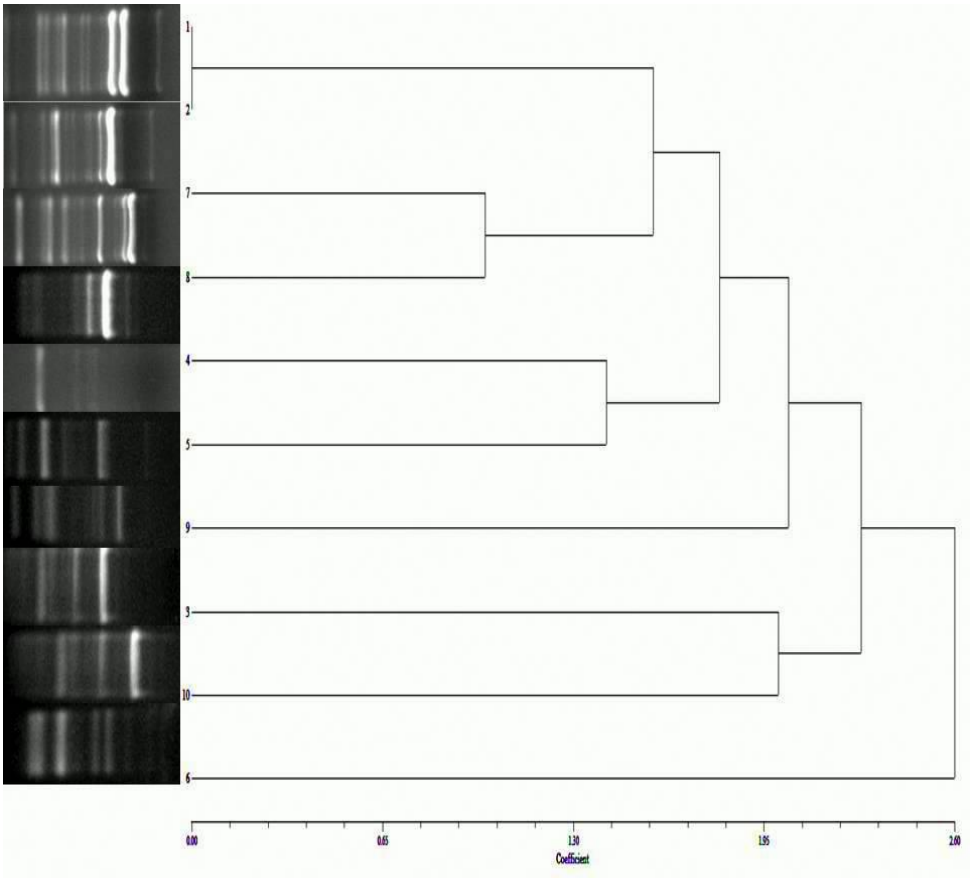
13
0.00
0.43
0.85
1.28
1.71
Coefficient

42a


1
4
8
3
2
18
5
11
15
17
7
14
13
16
9
10
12
19
6
0.00
0.71
1.42
2.13
2.83
Coefficient

42b



42c

Figure 42: Clonal types of clinical isolates: 42a; showing clonal types of *Escherichia coli;* 42b, showing clonal types of *Klebsiella pneumoniae* and 42c, showing clonal types of *Proteus mirabilis*. by ERIC-PCR.

4.12 Plasmid Incompatibility typing of isolates with class I and class II integrons:

All the wild types were subjected to PCR based replicon typing and it was observed that F inc type was more prevalent (n=44), while I (n=21), and Y (n=8) inc types were also observed (Table 23) (Figure 43a-43d).

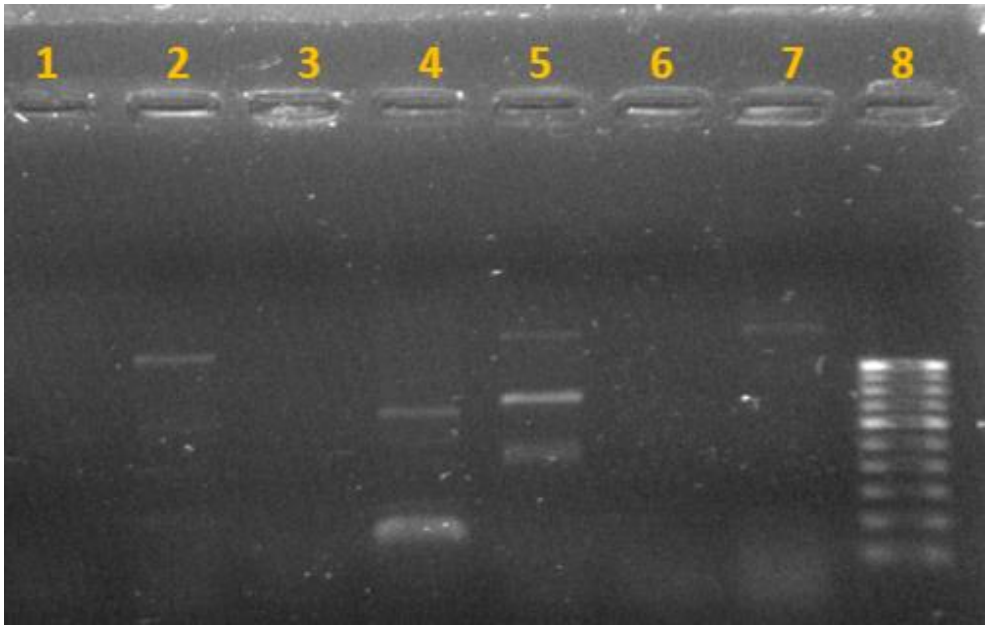


Figure 43a: PCR based replicon typing of wild types: Lane 2, 4 and 5 showing inc type, HI2, F and Y respectively. Lane 8, 100bp DNA ladder.

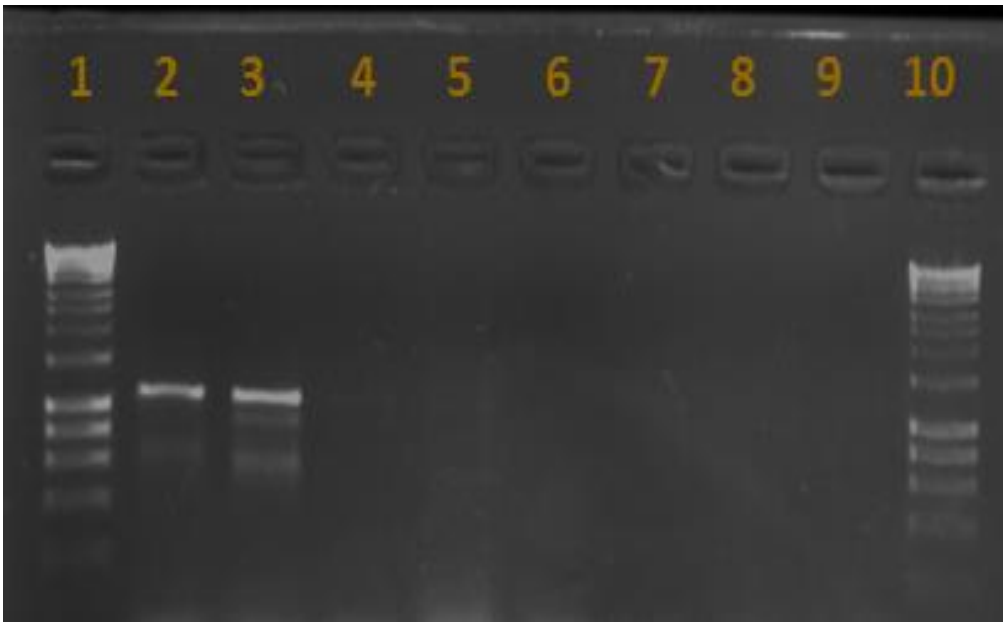


Figure 43b: PCR based replicon typing of wild types: Lane 2 and 3 showing inc type HI and lane 1, 10 100bp DNA Ladder.

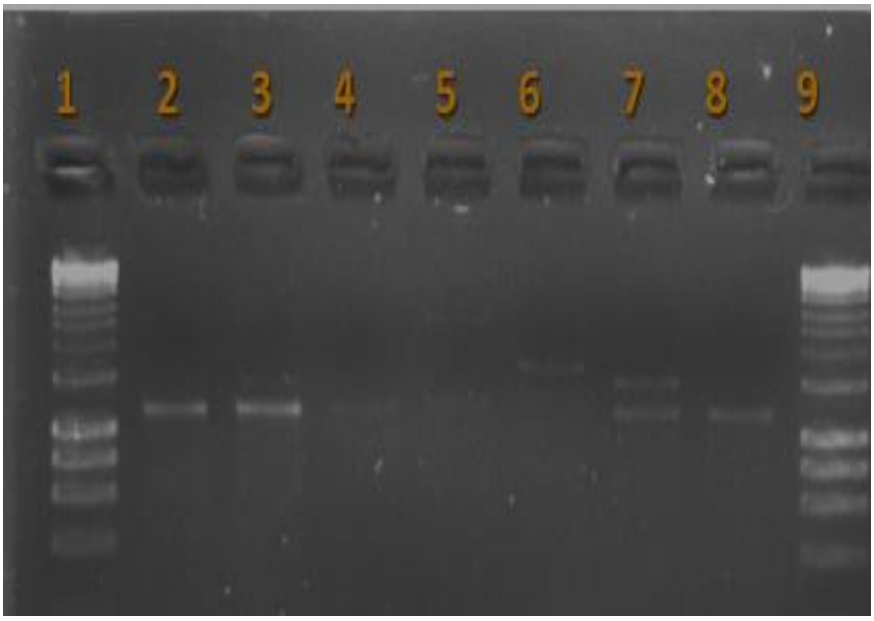


Figure 43c: PCR based replicon typing of wild types: Lane 2, 3, 7 and 8 showing inc type FIA and Y respectively and lane 1,9 100bp DNA Ladder.

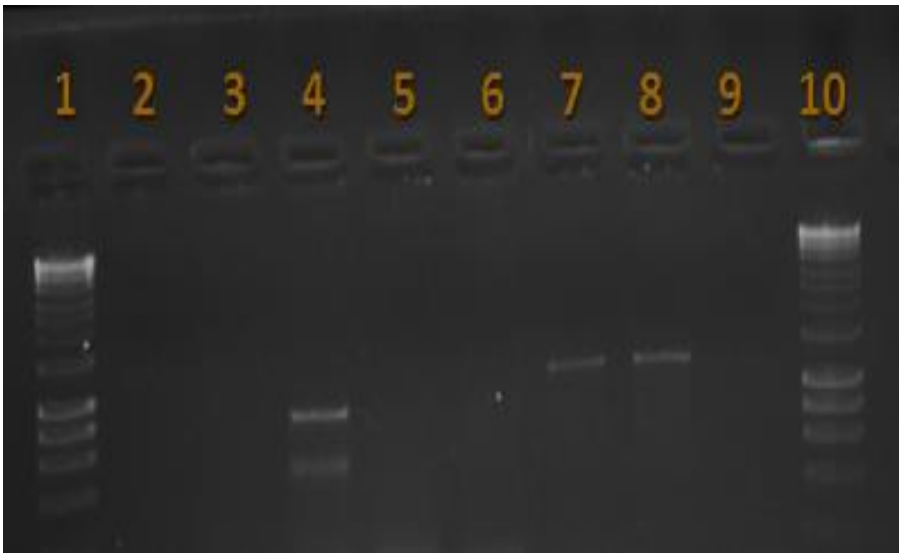


Figure 43d: PCR based replicon typing of wild types: Lane 4, 7 and 8 showing inc type FIA, HI1 and HI2 respectively and lane 1, 9 100bp DNA Ladder.

Table 22: Distribution of replicon type among wild strains.

Organism	Multiplex				
	F inc (n=44)	I inc (n=21)	Y inc (n=8)	HI1 (n=6)	HI2 (n=14)
Escherichia coli	21	12	4	2	6
Klebsiella pneumoniae	14	6	3	3	5
Proteus mirabilis	9	3	1	1	3