CHAPTER IV
RESULTS

1. Susceptibilities to Halquinol and antimicrobial

The Halquinol MICs of *E. coli* isolates from group I (non Halquinol-exposed isolates) and group II (Halquinol-exposed isolates) are shown in Table 6. The MIC value *E. coli* ranged between 4-64 μg/ml and 4-256 μg/ml for group I and II, respectively. Most of *E. coli* (69.1%) in group I had Halquinol MIC of 32-64 μg/ml whereas most of the strains (50.7%) in group II exhibited Halquinol MIC of 16-32 μg/ml. Distribution of Halquinol MICs of the group II *E. coli* was looked like a bi-modal curve. The strain in this group formed a large population at MIC range 4-32 μg/ml and then a frequency peak at MIC of 128 μg/ml. The Halquinol MIC_{90} of non Halquinol-exposed and Halquinol-exposed *E. coli* isolates were 64 and 128 μg/ml, respectively.

Table 6: Distribution of Halquinol MICs of non Halquinol-exposed and Halquinol-exposed *E. coli* isolates from pigs (*n*=355)

E. coli isolates	Total number	Number (%) of the isolates with MICs (µg/ml)						
		4	8	16	32	64	128	256
Group I								
non-exposed[a)]	152	13 (8.6)	9 (5.9)	25 (16.4)	62 (40.8)	43 (28.3)	0	0
Group II								
exposed[b)]	203	41 (20.2)	26 (12.8)	58 (28.5)	45 (22.2)	6 (3)	24 (11.8)	3 (1.5)
Total	355	54 (15.3)	35 (9.9)	83 (23.4)	107 (30.1)	49 (13.8)	24 (6.7)	3 (0.8)

[a)] non Halquinol-exposed *E. coli* strains [b)] Halquinol-exposed *E. coli* strains

Antimicrobial resistance rates of all *E.coli* isolates are shown in Figure 6. Three hundred-fifty two (99.15%) isolates were resistant to at least one antibiotic. Most of the strains (96.34) were resistant to tetracycline. Resistance to ciprofloxacin was found at the lowest frequency (52.39%). Ninety-eight percentage of the *E.coli* were multidrug-resistant. Five *E.coli* isolates (1.41%) were susceptible to all antibiotics tested.

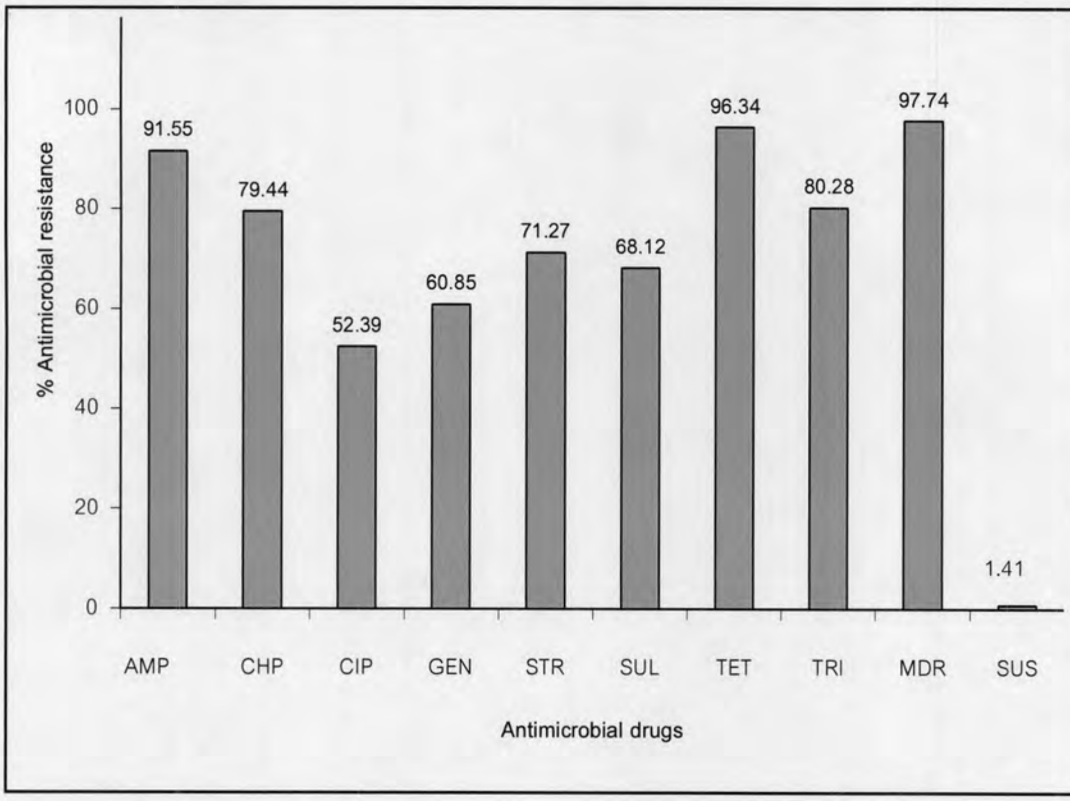

Figure 7: Frequency of resistance to 8 antibiotics of all the *E. coli* isolates. MICs were determined by two-fold agar dilution technique according to Clinical and Laboratory Standards Institute (CLSI, formerly NCCLS) (NCCLS, 1998). Multidrug resistance (MDR) was defined as isolates being resistant to 3 or more separate classes of antibiotics. Abbreviations: AMP, ampicillin; CHP, chloramphenicol; CIP, ciprofloxacin; GEN, gentamicin; STR, streptomycin; SUL, sulfamethoxazole; TET, tetracycline; TRI, trimethoprim; MDR, multidrug resistance; SUS, susceptible to all antibiotics

Antimicrobial resistance pattern was also analyzed (Table 7). All of the isolates can be grouped into 41 resistance patterns. The most common resistance pattern was AMP-CHP-CIP-GEN-TET-STR-SUL (16.05%).

Table 7. Antibiotic resistance patterns of *E. coli* isolates (*n*=355)

Antibiotic resistance pattern	No. of isolates (%)
AMP-CHP-TET	8(2.25)
AMP-CIP-STR	1(0.28)
AMP-TET-STR	1(0.28)
AMP-CHP-CIP-TET	2(0.56)
AMP-CHP-TET-TRI	6(1.69)
AMP-GEN-TET-SUL	1(0.28)
AMP-TET-STR-SUL	5(1.41)
AMP-CHP-CIP-TET-TRI	7(1.97)
AMP-CHP-GEN-TET-STR	3(0.84)
AMP-CHP-GEN-TRI-STR	1(0.28)
AMP-CHP-TRT-TRI-SUL	7(1.97)
AMP-CIP-GEN-TET-STR	4(1.12)
AMP-GEN-TET-STR-SUL	17(4.78)
AMP-GEN-TET-TRI-STR	3(0.84)
AMP-GEN-TET-TRI-SUL	1(0.28)
AMP-TET-TRI-STR-SUL	1(0.28)
AMP-CHP-CI-GEN-TRI-STR	3(0.84)
AMP-CHP-CIP-TET-TRI-STR	1(0.28)
AMP-CHP-CIP-TET-TRI-SUL	4(1.12)
AMP-CHP-GEN-TET-STR-SUL	10(2.81)
AMP-CHP-GEN-TET-TRI-STR	12(3.38)
AMP-CHP-TET-TRI-STR-SUL	26(7.32)
AMP-CIP-GEN-TET-STR-SUL	2(0.56)
AMP-CIP-GEN-TET-TRI-STR	2(0.56)
AMP-CIP-GEN-TET-TRI-SUL	2(0.56)
AMP-GEN-TET-TRI-STR-SUL	7(1.97)
AMP-CHP-CIP-GEN-TET-STR-SUL	9(2.53)

Antibiotic resistance pattern	No. of isolates (%)
AMP-CHP-CIP-GEN-TET-TRI-STR	35(9.85)
AMP-CHP-CIP-GEN-TET-TRI-STR	2(0.56)
AMP-CHP-CIP-TET-TRI-STR-SUL	33(9.29)
AMP-CHP-GEN-TET–TRI-STR-SUL	35(9.85)
AMP-CIP-GEN-TET-TRI-STR-SUL	17(4.78)
AMP-CHP-CIP-GEN-TET-STR-SUL	57(16.05)
CIP	1(0.28)
CIP-GEN	2(0.56)
CHP-TET-TRI	11(3.09)
CHP-CIP-TET-TRI	1(0.28)
CHP-TET-TRI-STR	7(1.97)
CHP-GEN-TET-TRI-STR	1(0.28)
AMP-TET-TRI-STR-SUL	1(0.28)
CHP-CIP-GEN-TET-TRI-STR	1(0.28)
Susceptibility to all antibiotics	5(1.41)
Total	355

Abbreviations: AMP, ampicillin; CHP, chloramphenicol; CIP, ciprofloxacin; GEN, gentamicin; STR, streptomycin; SUL, sulfamethoxazole; TET, tetracycline; TRI, trimethoprim

When comparisons between antibiotic resistance of Halquinol-exposed *E. coli* isolates and that of non- Halquinol-exposed strains were performed, it was found that anitibiotic-resistance rates (except trimethoprim) of the non-Halquinol exposed group was statistical significant ($p<0.05$; Fisher Exact Test) higher than that of the Halquinol-exposed group. Multidrug resistant rate of non-Halquinol exposed and Halquinol-exposed *E. coli* isolates were observed at 100% and 96.06%, respectively (Figure 7).

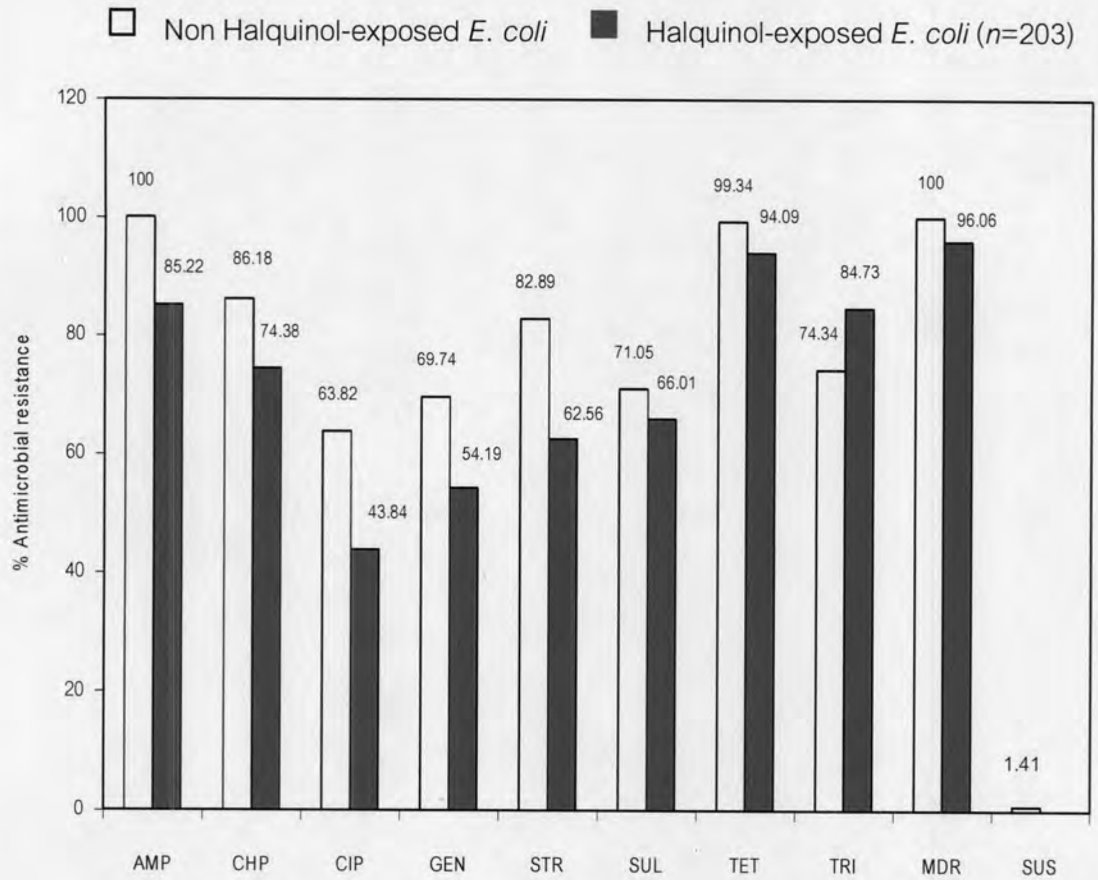

Figure 8: Frequency of resistance to 8 antibiotics of The *E. coli* isolates in group I and II. MICs were determined by two-fold agar dilution technique according to Clinical and Laboratory Standards Institute (CLSI, formerly NCCLS) (NCCLS, 1998). Multidrug resistance was defined as isolates being resistant to 3 or more separate classes of antibiotics. Abbreviations: AMP, ampicillin; CHP, chloramphenicol; CIP, ciprofloxacin; GEN, gentamicin; STR, streptomycin; SUL, sulfamethoxazole; TET, tetracycline; TRI, trimethoprim; MDR, multidrug resistance; SUS, susceptible to all antibiotics

The MIC_{90} of all antibiotics and Halquinol were shown in Table 8. The MIC_{90} value of all antibiotics except chloramphenicol and ciprofloxacin calculated for non Halquinol and Halquinol-exposed *E. coli* isolates and all the isolates was not different more than 4 folds. The chloramphenicol MIC_{90} of Halquinol-exposed *E. coli* isolates was 4 folds greater than that of non Halquinol-exposed strains. In contrast, the ciprofloxacin

MIC_{90} of Halquinol-exposed group was 4 folds lower than that of non-Halquinol exposed group.

Table 8. The MIC_{90} of all antibiotics and Halquinol in non-Halquinol exposed (*n*=152) and Halquinol exposed *E. coli* isolates (*n*=203).

Antimicrobial	MIC_{90} (µg/ml)		
	non-exposed[a)]	Exposed[b)]	Total
ampicillin	1024	1024	1024
chloramphenicol	256	1024	1024
ciprofloxacin	256	64	128
gentamicin	256	512	512
halquinol	64	128	128
streptomycin	1024	1024	1024
sulfamethoxazole	1024	1024	1024
tetracycline	256	512	512
trimethoprim	1024	1024	1024

[a)] non-Halquinol exposed *E. coli* isolates   [b)] Halquinol-eposed *E. coli* isolates

2. Halquinol-spontaneous resistance mutants and cross-resistance to antibiotics

When the selected *E. coli* isolates i.e. EC 338 and EC 339 and *E. coli* K_{12} MG1655 rif^r were exposed to gradually increasing concentrations of Halquinol, none of them grew beyond the first passage and its growth stopped at a concentration of 12 µg/ml (Table 9). From confirmation of the continuity of the pre-and post-Halquinol exposed isolates by rep-PCR profiling, each pair of pre-and post-Halquinol exposed *E. coli* isolates yield the identical electrophoresis patterns (Figure 8).

Table 9: The Halquinol MICs of the *E. coli* strain pre-and post-Halquinol exposure

E. coli strain	MIC (μg/ml)	
	pre-Halquinol exposure	post-Halquinol exposure
EC 338	16	12
EC 339	16	12
MG1655 rif^r	16	12

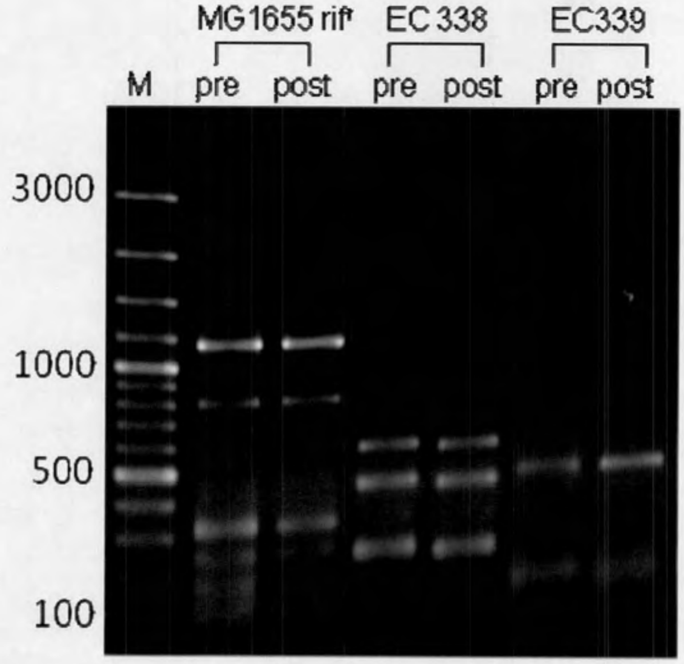

Figure 9: rep-PCR profiling of *E. coli* isolates pre-and post exposed to Halquinol. Lane 1, 3 and 5 *E. coli* K_{12} MG1655 rif^r, EC 338 and EC 339 before exposed to Halquinol and Lane 2, 4 and 6 *E. coli* K_{12} MG1655 rif^r , EC 338 and EC 339 after exposed to Halquinol

The Halquinol and antibiotic MICs of the *E. coli* parents and the Halquinol-exposed strains were compared (Table 10). The MIC value of Halquinol and all antibiotics of each *E. coli* pair was not different.

Table 10: Comparison of the Halquinol and antibiotics MICs of the *E. coli* parents and the exposed strains

E. coli strain	MIC (μg/ml)								
	HAL	AMP	CHP	CIP	GEN	STR	SUL	TET	TRI
Pre-exposure									
EC 338	16	1	8	0.125	0.25	2	1	2	1
EC 339	16	1	8	0.125	0.25	2	1	2	1
MG1655 rif^r	16	8	8	0.125	0.25	2	1	2	1
Post-exposure									
EC 338	16	2	8	0.50	0.50	2	2	4	1
EC 339	16	2	8	0.50	0.50	2	2	4	1
MG1655 rif^r	16	8	8	0.125	0.50	2	1	4	1

Abbreviations: HAL, Halquinol; AMP, ampicillin; CHP, chloramphenicol; CIP, ciprofloxacin; GEN, gentamicin; STR, streptomycin; SUL, sulfamethoxazole; TET, tetracycline; TRI, trimethoprim

3. Test for transferability of Halquinol resistance encoding gene(s)

3.1 Presence of plasmids

All nineteen *E. coli* donors that were used in conjugation experiment containing plasmids that could be classified into 3 patterns (Figure 10). The plasmid-profile is summarized Table 11.

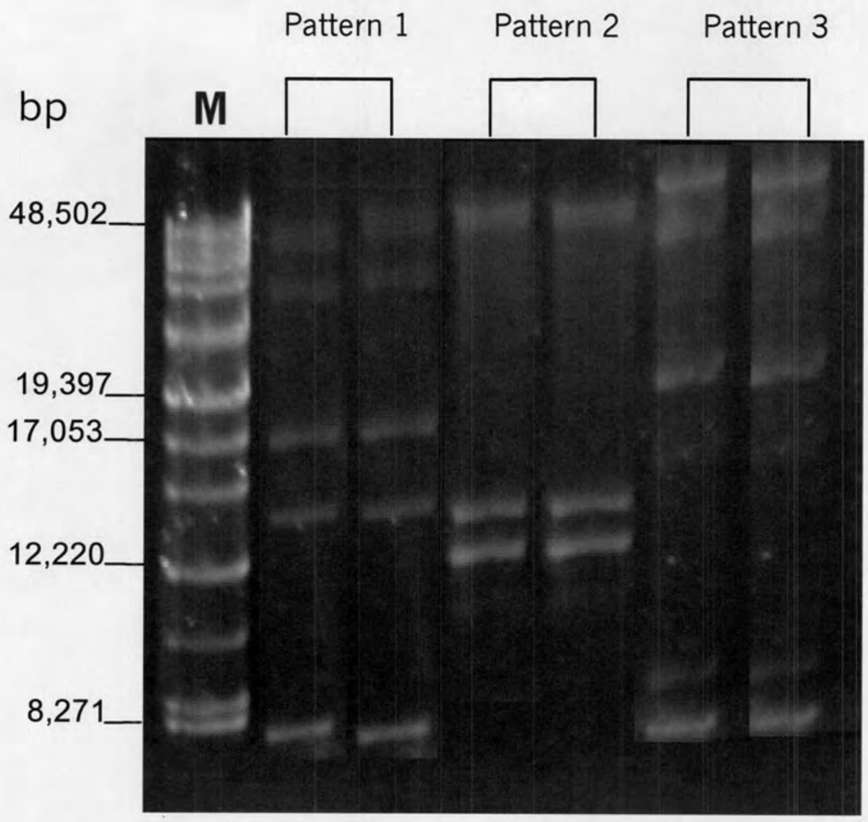

Figure 10: Plasmid profiles of the *E.coli* isolates with high Halquinol MIC from group II

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Table 11: Plasmid profiles of *E.coli* strains with high Halquinol MIC from group II

Plasmid profiles	Number of strain	Plasmid pattern *E.coli* strain
1	8	EC 338, EC 339, EC 561, EC 562, EC 571, EC 573, EC 921, EC 922
2	9	EC 121, EC 122, EC 123, EC 141, EC 142, EC 143, EC 181, EC 182, EC 183
3	2	EC 201, EC 202
Total	19	

3.2. Test for transfer of Halquinol resistance encoding gene(s)

Of 28 *E. coli* isolates tested, nineteen of the *E.coli* isolates tested (67.8%) were found to contain plasmid DNA. After biparental mating, none of the donor-recipient combinations yielded colonies on LB containing Halquinol and rifampicin. The results were reproducible in the repeated experiment. This indicated that there was no horizontal transfer of Halquinol-resistance encoding genes. The same results were obtained in the repeated-experiments.