CHAPTER III
MATERIALS AND METHODS

The experiment was divided into 2 phases: phase 1 test, of susceptibilities to Halquinol and antibiotics and phase 2, examination of cross-resistance between Halquinol and antibiotics. The conceptual framework is shown in Figure 5.

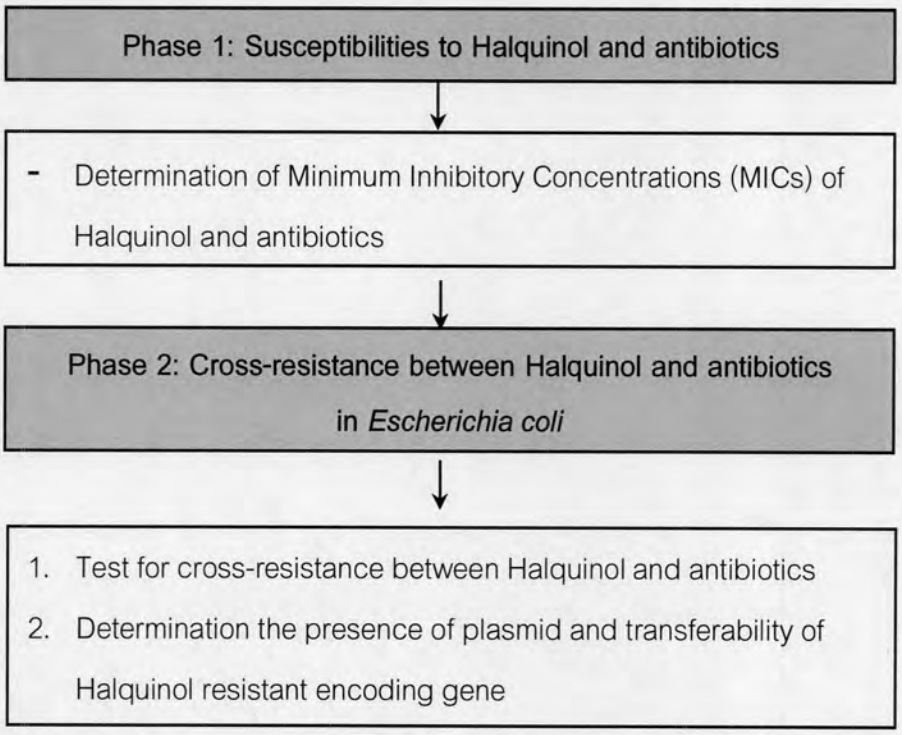


Figure 5: The conceptual framework in this study

Escherichia coli isolates

A total of 355 *E. coli* isolates were included in this study. All the isolates were divided into 2 groups: group I, the *E. coli* isolates that were from pigs never exposed to Halquinol (n= 152) and group II, the isolates that were from pigs administered Halquinol (n= 203). At farms, most of pigs were fed with Halquinol-containing feed at different dosage (Table 1) for 7-10 days. The detail of *E. coli* isolates in each group is summarized and showed in Table 1.

All of the *E. coli* isolates were from the strain collection of Veterinary Diagnostic Laboratory (VDL), Faculty of Veterinary Science, Chulalongkorn University. They were isolated from the faecal samples collected from pigs in Ratchaburi, Chonburi, Buriram, Nakhonratchasrima and Udon Thani. Sixty of the faecal samples were collected per one farm per one province. Approximately 2-5 grams of faecal material per sample was collected using Curity® Bioswab and kept in Stuart media (Kendall-Gammatron Ltd., Thailand) by farm veterinary practitioners. Faecal samples were delivered on ice box directly to the VDL for isolation of *E. coli*.

The *E. coli* strains were isolated using the standard method (Quinn et al., 1994) and confirmed biochemically (Carter and Cole, 1990). The isolates were purified to get single colonies. One colony from each positive sample was collected. All the *E. coli* isolates were stored as 20% glycerol stocks at -80°C. All the *E. coli* isolates were shipped to Department of Veterinary of public health, Faculty of Veterinary Science, Chulalongkorn University for further studies.

Table 1: Number of *E.coli* isolates from rectal swab samples from pigs that have and have not administered Halquinol in different provinces during 2007- 2008.

Dosage (ppm) of Halquinol in feed	No. of isolates	Source
Group I non Halquinol-exposed *E.coli* (*n*= 152)		
0	92	Ratchaburi
0	60	Udon Thani
Group II Halquinol-exposed *E.coli* (*n*= 203)		
180	39	Nakhon Ratchasima
180	121	Chon Buri
240	43	Buriram
Total	355	5

Phase 1 Susceptibility testing for Halquinol and antibiotics

Antibiotic and Halquinol susceptibilities were examined by determination of Minimum Inhibitory Concentration (MIC) using a two-fold agar dilution technique according to the Clinical and Laboratory Standards Institute (CLSI, formerly NCCLS) (NCCLS, 2002). The antibiotic agents tested were ampicillin (AMP), gentamicin; (GEN), streptomycin (STR), ciprofloxacin (CIP), chloramphenicol (CHP), sulfamethoxazole (SUL), tetracycline (TET), trimethoprim (TRI). All antibiotics were purchased from Sigma (Poole, UK).

Halquinol (HAL) performs to British Pharmacopoeia 80, Lot no. HLA-1387, was obtained from Vetcare Organics (Bangalore, India). Its purity was 98.0076% and formulated in greenish powder. It is soluble in 50 part of chloroform. Its solubility in ethanol and ether was at ratio of 4 g/l and 7.7 g/l, respectively. The optimum temperature for Halquinol storage is 22-32°C.

Antibiotics were dissolved in appropriate diluents and distilled water. The diluents and antibiotic concentrations used are shown in Table 2.

The *E. coli* isolates were grown overnight at 37 °C in Muller-Hinton agar (MHA, Difco, MD, USA). Single colonies were picked to suspend in 0.85% NaCl solution (NSS) to 0.5 McFarland. Then, the suspension was ten fold diluted to 10^{-1} in NSS. The suspension was inoculated onto the MHA containing suitable concentrations of antibiotics using a multi-point inoculator. The MHA plates were incubated at 37°C for 18-24 hours. The MIC was defined as the lowest concentration of an antimicrobial in MHA plate, which inhibited growth appearing of the test isolates.

Table 2 : Solvents and concentrations of antibiotics used in this study

Antibiotics	Solvent	Concentration range (µg/ml)
ampicillin	distilled water	1, 2, 4, 8, 16, 32, 64, 128, 256, 512
chloramphenicol	95% ethanol	1, 2, 4, 8, 16, 32, 64, 128, 256, 512
ciprofloxacin	0.1 M NaOH and distilled water	0.125, 0.25, 0.50, 1, 2, 4, 8, 16, 32, 64, 128, 256
halquinol	dimethyl sulfoxide	1, 2, 4, 8, 16, 32, 64, 128, 256, 512
gentamicin	distilled water	0.25, 0.50, 1, 2, 4, 8, 16, 32, 64, 128, 256
streptomycin	distilled water	1, 2, 4, 8, 16, 32, 64, 128, 256
sulfamethoxazole	0.1 M NaOH and distilled water	1, 2, 4, 8, 16, 32, 64, 128, 256, 512, 1024
tetracycline	70% ethanol	1, 2, 4, 8, 16, 32, 64, 128, 256, 512
trimethoprim	dimethyl acetamide	1, 2, 4, 8, 16, 32, 64, 128, 256, 512

Break points that are concentrations used to define isolates as susceptible or resistant are those established by CLSI and shown in Table 3. Breakpoints for Halquinol have not been available yet. Multidrug resistance (MDR) was defined as isolates being resistant to 3 or more separate classes of antibiotics (Hsu et al., 2006). *E. coli* ATCC 25922 was used as quality control organisms.

Table 3 : Breakpoints used in this study

Antibiotics	Breakpoint (μg/ml)
ampicillin	32
chloramphenicol	32
ciprofloxacin	4
gentamicin	8
streptomycin	32
sulfamethoxazole	512
tetracycline	16
trimethoprim	16

Phase 2: Cross-resistance between Halquinol and antibiotics in *E. coli*

The experiments in this phase contained 2 parts, including exposure experiment for testing of Halquinol cross-resistance and conjugation experiment for testing of transfer of Halquinol resistance-encoding gene(s).

2.1 Test for cross-resistance between Halquinol and antibiotics by exposure experiment

The strategy was to examine if the *E. coli* isolates that had low Halquinol MIC and were susceptible to all antibiotics could develop tolerance to Halquinol at sub-lethal concentration and if the *E. coli* isolates that could tolerate to Halquinol were able to develop cross-resistance to other antibiotics.

Two *E. coli* isolates from group II (Halquinol exposed *E.* coli) i.e. EC 338 and EC 339 and *E. coli* K_{12} MG1655 rif^r were selected for the experiment. As *E. coli* K_{12} strain MG1655 is an *E. coli* wild-type, MG1655 rif^r is a spontaneous-derivative of MG1655. The latter was previously made for using as a recipient strain in biparental mating (Khemtong, 2007). It was used as a reference strain in the present study. All of them had low MIC to Halquinol and were susceptible to ampicillin, streptomycin, chloramphenicol, tetracycline, gentamicin, ciprofloxacin, trimethroprim and sulfamethoxazole (Table 4).

Table 4: The MICs of Halquinol and antibiotics of the *E.coli* strains used in exposure experiment

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

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

These *E. coli* isolates were exposed to gradually increasing concentrations of Halquinol as previously described (Braoudaki and Hilton, 2005). All *E. coli* isolates were streaked on LB agar plates to get single colonies. After an overnight incubation at 37°C, an 80-µl inoculum was inoculated into 4 ml Luria-Bertani (LB, Difco, BD Diagnostic Systems, Sparks, MD) containing Halquinol at a concentration of half the MIC (8 µg/ml). This procedure took place everyday in LB broth with increasing concentrations of Halquinol by a factor of 1.5 until no growth was observed.

At each passage, the inoculums were cultured onto LB agar without Halquinol and saved as stocks. Colonies from the final passage were randomly picked and determined MICs for Halquinol, ampicillin, streptomycin, chloramphenicol, tetracycline, gentamicin, ciprofloxacin, trimethroprim and sulfamethoxazole. If colonies with high Halquinol MIC were obtained, the colonies would be sub-cultured on non-selective LB medium without antimicrobials for 10 consecutive days. Then, the MIC determinations would be repeated.

The genetic continuity of colonies between pre- and post-Halquinol exposed *E. coli* strains were confirmed by repetitive sequence based polymerase chain reaction

(rep-PCR) as previously described (Amonsin et al., 1997). The oligonucleotide primer pair used was ERIC IR-5'-ATGTAAGCTCCTGGGGATTCAC and ERIC II-5'-AAGTAAGTGACTGGGGTGAGCG (Fermentas International Inc., Canada).

PCR amplifications were performed using whole cell DNA templates. The DNA templates were prepared by the boiling procedure as previously described (Berg et al., 1955). Colonies were resuspended in 50 µl distilled–water and boiled in boiling eater for 10 minutes. The cell suspension was centrifuged at 12,000xg for 1 minute. The clear supernatant was removed to a new eppendrof tube and stored at -20°

All PCR assays were carried out in a final volume of 25 µl using PCR Master Mix (Fermentas International Inc., Canada), according to the manufacturer's instructions. Each PCR reaction consisted of 12.5 µl of Master Mix, 6.5 µl of nuclease free water, 1.0 µl of each primer at 10 µM and 4 µl of DNA template. PCR amplifications were conducted on a thermal cycler (Thermo Electron Corporation/PCR Sprint, USA), with an initial denaturation at 94°C for 5 minutes, followed by 35 PCR cycles of denaturation at 94°C for 30 seconds, annealing at 45°C for 3 minutes, and extension at 72°C for 2 minutes, with a final extension step at 72°C for 7 minutes. A 5-µl volume of PCR product was electrophoresed in 2 % agarose gel electrophoresis and photographed under UV light with Gel Documentation System (Vilbur Lourmat, La Vallee Cedes, France). Experiments were separated in two separate occasions.

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

Antimicrobial-resistance encoding genes can be located on conjugative plasmids. These plasmids can be horizontally transferred to other bacteria. The objective of this part was to investigate if Halquinol-resistance encoding gene(s) were located on conjugative plasmid(s).

2.2.1 Determination of the presence of plasmids

Plasmid DNA were extracted from 28 *E. coli* isolates with high MIC to Halquinol (Table 5) using NucleoSpin® Plasmid (Macherey Nagel, German) following the company instruction. Plasmid DNA was run in agarose electrophoresis gel and

visualized by UV-transluminator. The isolates that had plasmids were used for testing of their transferability in conjugation experiment.

Table 5: The MICs of *E.coli* strain selected for plasmid extraction (*n*=28)

E.coli strain	Number of strain	MIC (µg/ml)
EC 561 [a], **EC 562**, EC563, **EC 571**, EC572, **EC 573**, EC581, EC 582, EC583	9	32
EC 841	1	64
EC O61, EC O62, EC O71, EC O72, **EC 121**, **EC122**, **EC 123**, **EC 141** **EC 142**, **EC 143**, **EC 181**, **EC 182**, **EC 183**, EC 204, **EC 338**, **EC 339**, **EC 921**, **EC 922**	18	128
EC 201, **EC 202**, EC 203	3	256
Total	28	

[a] The *E. coli* strains used for conjugation experiment are indicated in bold

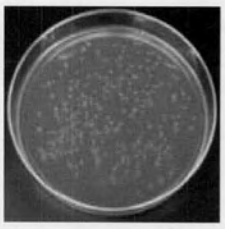
2.2.2 Conjugation experiment

Nineteen plasmid-containing *E. coli* isolates identified in 2.2.1 were tested for transferability of Halquinol resistance-encoding gene(s) by filter mating method as previously described (Chen et al., 2004, Gebreyes and Thakur, 2005). These *E. coli* isolates had high MIC to Halquinol as shown in Table 5. The spontaneous rifampicin-resistant derivative of *E. coli* K_{12} MG1655 rif^r that has no plasmid, was used as recipient. All nineteen donors and the recipient *E. coli* isolates were grown in 4 ml LB broth and incubated overnight at 37°C in shaking incubator. Eighty-µl aliquots of the overnight cultures of the donors and the recipients were separately added into 4 ml fresh LB broth and incubated at 37°C for 3-4 hours in shaking incubator until OD_{600} of 0.3–0.5 was reached. Each of 700 µl cultures of the donors and the recipients were gently mixed at

1:1 ratio in warm eppendrof tubes and then centrifuged at 8,000 rpm for 1 minute. The supernatant was completely pipetted out and the cell pellets were resuspended in 30 µl LB broth warmed at 37°C. The suspension was spreaded on the sterile membrane filter (0.45 µm pore size, Millipore, Massachusetts, USA) placed on LB agar plate without Halquinol and any antibiotics. Then, the inoculated filter was put in incubator overnight at 37°C. To remove the attached cells, the inoculated-filter membrane was carefully removed from the LB agar plate and placed into 1 ml of 0.85% NSS in a new eppendrof tube. The tube was vortexed to unattach the cells from the membrane filter and the filter was discarded. The suspension was centrifuged at 12,000 rpm for 1 minute and the supernatant was removed. A hundred-µl of fresh LB broth was added into the bacterial pellets. The conjugation mixture was spreaded on selective LB agar and incubated overnight at 37°C. The selective LB medium was LB agar supplemented with 32 µg/ml of rifampicin and 16 µg/ml of Halquinol for those donors; EC 561, EC 562, EC 571 and EC 573 and 64 µg/ml of Halquinol for those donors; EC 121, EC122, EC 123, EC 141 EC 142, EC 143, EC 181, EC 182, EC 183, EC 338, EC 339, EC 921, EC 922 EC 201 and EC 202. The MG1655rif^r was spreaded on LB agar supplemented with 32 µg/ml of rifampicin alone and combined with 16 µg/ml and 64 µg/ml of Halquinol. The nineteen donors were spread on LB agar supplemented with 32 µg/ml of rifampicin. The transconjugants that were MG1655rif^r with plasmid containing Halquinol-resistance genes and exhibiting high Halquinol MIC (>16 µg/ml). Transconjugants were confirmed by plasmid extraction using NucleoSpin® Plasmid (Macherey Nagel, German) and their identity to those in *E. coli* donors are confirmed by using of restriction endonuclease analysis (REA).

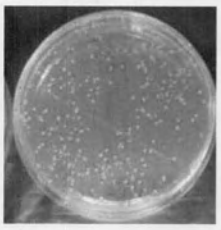
Statistical Analysis

The MIC_{90} value of both *E. coli* isolates group of Halquinol and each antibiotic was statistical compared with Wilcoxon rank-sum test. Fisher's exact test was used to compare resistance proportion between *E. coli* isolates group of Halquinol and each antibiotic.



E. coli donors

+



E. coli K_{12} MG1655rif^r recipients

Mating on membrane filter

Selected on LB agar containing rifampicin and Halquinol

Figure 6: Biparental mating. The *E. coli* donors were those with high MICs to Halquinol and antibiotics. All of them also carried plasmids. *E. coli* K_{12} MG1655 rif^r was a recipient. Transconjugants were selected on LB agar containing rifampicin and Halquinol at concentrations of 32 and 64 µg/ml, respectively.