CHAPTER V
DISCUSSION

Antiseptics have been used extensively in food-animal production, Halquinol has been established as a commonly used antiseptic in feed for the treatment of bacterial infections, particularly *E. coli* in diarrheagenic pigs. As seen in antibiotics, bacteria my develop resistance to antiseptics and concerns that improper use of antiseptics may promote cross-resistance to antibiotics has been increasing. Of particular concern is that the wide use of Halquinol could lead to resistance in *E. coli* and result in cross-resistance to clinically-important antibiotics. In this case, Halquinol use may provide the coselection pressure for both types of resistance.

The susceptibility test showed that the Halquinol MIC of non-Halquinol-exposed and Halquinol-exposed *E.coli* isolates ranged 4-64 µg/ml and 4-256 µg/ml, respectively. The Halquinol MICs of *E.coli* in both groups were wider than that of *E.coli* reported in a previous study (Tripipat et al., 2005). The later was conducting in 29 *E.coli* isolated from pigs in Thailand. The difference may be attributed to the variations between the MIC determination techniques used. The previous study was performed with much less number of *E.coli* and history of Halquinol exposure of the isolates tested was not declared. The Halquinol MIC_{90} of from group I and II in this study were comparable but higher than that of *E.coli* formerly reported (Tripipat et al., 2005). Again, this occurrence could be resulted from the different numbers of *E.coli* used.

As most of *E.coli* in group I had Halquinol MIC of 32-64 µg/ml, those in group II formed large population at MIC range 4-32 µg/ml. The latter also showed frequency peak of MIC 128 µg/ml. Regardless of the peak, susceptibilities to Halquinol of *E.coli* from both group were similar. The present-MIC frequency peak may indicate that *E.coli* in group II had develop a limited degree of less susceptibility to Halquinol. However, this cannot be an absolute conclusion since the MIC of the peak was not time 4 times different from the highest MIC of *E.coli* in group I. Further studies of Halquinol susceptibility in a larger collection of *E.coli* are suggested.

All of the *E.coli* isolates in this study were highly resistant to antibiotics tested. Most of them were resistant to tetracycline and ampicillin, which is corresponded to medicines formulated in feed used in pig production. Up to 41 resistance patterns were identified in this study and similar high resistance profiles were recorded by NIAH from 1994 to 1996 (Pathanasophon et al., 1998). The NIAH reported that 91% to 99% of *E.coli* isolates from swine were resistant to oxytetracycline, tetracycline, streptomycin, trimethoprim and sulfamethoxazole and 60%-80% were resistant to ampicillin, neomycin, chloramphenicol and kanamycin (Pathanasophon et al., 1998).

Resistance rate to ciprofloxacin was found up to 52.39%, which was higher than a previous study where resistance was 26.8% (Jiwakanon, et al.,2008). As ciprofloxacin is rarely used in pig production, enrofloxacin a drug in the same fluoroquinolone group has been widely used. Therefore, high ciprofloxacin resistance rate observed is more likely due to the widespread use enrofloxacin in pig industry.

Interestingly, chloramphenicol has been banned for use in food animals for many years but the isolates resistant to this antibiotic are still identified at high rate. This may be due to the presence of chloramphenicol resistance genes on the same genetic elements with other resistant determinants as previously observed in *E. coli* (Antunes et al., 2007). In this case, use of other antibiotics may also coselect for chloramphenicol-resistance-encoding genes that are located on the genetic elements.

Resistance rates to all antibiotics (except trimethoprim) of non Halquinol-exposed *E.coli* isolates were higher than that of Halquinol-exposed *E.coli* isolates. The observation indicated that Halquinol may not be involved in development of antibiotic-resistance in the *E.coli* isolates. A previous study showed that *E.coli* isolates from pigs fed with feed-containing Halquinol at the concentration of 120 ppm for 6 weeks did not develop high MIC to Halquinol and antibiotics (Cosgrove et al., 1981). Taken together, these results suggest that use of Halquinol does not contribute to antibiotic resistance in *E.coli* and other factors affecting antibiotic resistance of the *E. coli* isolates in this study do exist i.e. antibiotic use in the farm. This is also support by the findings in exposure experiment. After exposure to Halquinol at sublethal, no spontaneous- Halquinol resistant colonies were observed. Colonies obtained from Halquinol exposure exhibited

the similar MIC to Halquinol and antibiotics to that of pre-exposed parents. These results confirmed that *in vitro* exposure to Halquinol did not select for *E.coli* that were less susceptible to Halquinol and did not promote cross-resistance to antibiotics. In general, antiseptics at the in use concentrations would kill most of bacteria. However, antiseptics including Halquinol are regularly used in farms. Pigs have been fed with Halquinol for a period of time and the substance will be diluted to sublethal concentrations downstream of its application. Bacteria could be exposed to subinhibitory concentrations of Halquinol and develop resistant to the antiseptic.

The presence of trimethoprim resistance among *E.coli* in group II was significantly higher that that among *E.coli* in group I. As mode of action of Halquinol is to inactivate RNA polymerase, thus inhibiting both RNA and protein synthesis of the bacteria (Fraser and Creanor, 1975), mechanism of resistance has not been identified. Trimethoprim inhibits bacterial growth through inhibition of dihydrofolate reductase (DHFR) resulting in depletion of tetrahydroforate coenzyme-required for purine and pyrimidine biosynthesis. Bacteria exhibit resistance to trimethoprim by overproduction of DHPR, production of trimethoprim-DHFR, decreased cell permeability and expression of active efflux. The common-resistance mechanism between trimethoprim and Halquinol has not been identified. From current knowledge regarding resistance mechanisms in bacteria, it is possible that multidrug efflux system may connect trimethoprim and Halquinol resistance. If this is the case, it will be involved in resistance to other antibiotics as well. This is because multidrug efflux system could extrude variety of substrates that are not structurally related (Kumar and Schweizer, 2006). The Multidrug efflux pump that has been well characterized in *E. coli* is AcrAB-TolC. This system has been shown to extrude various antibiotics and dyes (Nikaido et al, 1998; Okusu et al, 1996; Zhao et al, 2005) but its role in Halquinol resistance has never been reported. However, in this study, expression of multidrug efflux systems and their contribution to resistance observed was not determined. Even though such significant high resistance rate was observed, it cannot conclude that Halquinol exposure promote high-trimethoprim resistance. *In vitro*, exposure study did not demonstrate any cross-resistance as well. However, it may be agued that *in vitro* and *in vivo* situations are

different in term of resistance development. Therefore, further studies are necessary to elucidate the resistance mechanism underlying trimethoprim-resistance among the *E.coli* isolates in this study.

Some antibiotics e.g. rifampicin are inhibitors of RNA polymerase enzyme, which is similar to the function of Halquinol. The *E. coli* isolates acquired resistance to rifampicin through certain mutations i.e. base pair substitutions, deletions and insertions in the β-subunit of RNA polymerase (Russell and Chopra, 1996). Since there were some of the *E. coli* isolates in group II exhibited the high Halquinol MIC (up to 256 μg/ml) and it is not clear if these cells have developed tolerance to Halquinol or not, it may worth to examine mutations in the RNA-polymerase encoding gene in these isolates.

In conjugation experiment, no colonies were observed in the selective agar, indicating that Halquinol-tolerance encoding gene was not horizontally transferred. Therefore, the correspondent gene was not located on conjugative plasmid. However, Halquinol-resistance encoding gene may be located on very small or very large plasmid that may not transfer *in vitro*. In addition, there may be differences between *in vivo* and *in vitro* transfer of antimicrobial resistance genes. Further studies are required for testing of Halquinol-resistance transfer in *in vivo* conditions.

Conclusion and suggestion

From the findings of this study it can be concluded that

1. Susceptibilities to Halquinol of non Halquinol-exposed and Halquinol-exposed *E. coli* isolates isolated from swine were similar, no difference of MICs of *E.coli* between the both groups.
2. *E. coli* isolates from both groups showed high frequency of antibiotic-resistance and multidrug resistance rates among the antibiotics
3. *In vitro* exposure to Halquinol did not yield the *E.coli* isolates that were less susceptible to Halquinol and antibiotics. This indicated that there was no cross-resistance occurred.

Taken together, the results supported that Halquinol may not promote cross-resistance to clinically-important antibiotic. However, further studies are suggested and could be as follows:

1. To date, there are only limited of data on Halquinol susceptibility. The susceptibility test should be conducted with a larger collection of *E. coli* and other foodborne bacterial pathogens e.g. *Salmonella enterica*. Routine monitoring for Halquinol susceptibility should be performed.
2. Even though Halquinol has been widely used in pig production, no breakpoints for classifying susceptible and resistance exist. Therefore, experiments should be taken to identify the appropriate breakpoint for this substance.
3. Halquinol-resistance encoding gene has never been identified. Characterization of genetic basis of Halquinol susceptibility should be performed. This will also help to elucidate the possible common resistance mechanisms between Halquinol and antibiotics.
4. Horizontal-transfer of Halquinol susceptibility should be tested *in vivo* in that dynamics of resistance transfer between *in vitro* and *in vivo* are different.
5. Risk analysis of development and resistance of Halquinol in *E. coli* and other pathogenic bacteria is another special topic of interest.