The Contribution of Active Efflux in Reduced Susceptibilities to Copper Sulfate and Zinc Chloride in *Escherichia coli* Isolates from Swine

Chailai Koowatananukul¹ Nisit Chansong² Rungtip Chuanchuen¹*

Abstract

A hundred-eighty *Escherichia coli* isolates from swine were tested for minimal inhibitory concentrations (MICs) of 9 antimicrobials and two heavy metals copper sulfate and zinc chloride. All the *E. coli* isolates were resistant to at least one antibiotic and 175 (97.2%) isolates were multi-drug resistant. Forty antibiotic resistance patterns were identified, of which the most common pattern was AMP-CHP-CIP-ERY-GEN-STR-SUL-TET-TRI (13.3%). All the isolates formed one large population of susceptibility to copper sulfate and zinc chloride at the concentrations of 6.4-12.8 mM and 3.2-6.4 mM, respectively. Exposure to copper sulfate and zinc chloride at sublethal concentration did not change the susceptibility. Addition of carbonyl cyanide m-chlorophenylhydrazone (CCCP) and reserpine caused at least 4-fold reduction of MIC value of copper sulfate and zinc chloride in 66 (36.7%) isolates and 142 (78.9%) isolates, respectively. The results highlighted the contribution of active efflux systems driven by proton motive force and/or ATP in reduced susceptibilities to copper sulfate and zinc chloride in *E. coli* from swine.

Keywords: Active efflux systems, copper sulfate, *Escherichia coli*, swine, zinc chloride

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Original Article

Introduction

In swine production, most animal feeds are supplemented with copper sulfate and/or zinc chloride in different concentrations. The use is most pronounced in piglets receiving a concentration of 175 ppm and in slaughter pigs fed at a concentration of 35 ppm (Aarestrup and Hasman, 2004). The growth-promoting effects may be involved in inhibition of the growth of some bacterial population in the digestive tract that allows more nutrients available for the intestinal flora. This will improve the nutrient utilization, enhance average daily weight and promote health in the animals. Copper sulfate and zinc chloride have been used as growth-promoting feed supplements for pigs for over 40 years (Edmonds, et al., 1985; Williams, et al., 1993). As seen in antibiotics and disinfectants, bacteria may acquire resistance to copper sulfate and zinc chloride (Hasman and Aarestrup, 2002; Hasman and Aarestrup, 2005). Of particular concern is that these resistant bacteria may also develop cross-resistance to other antibiotics, especially when such resistance is mediated by active efflux. Efflux pumps play a major role in development of resistance to various antimicrobial agents in bacteria, in particular multidrug efflux systems that mediate resistance to structurally-unrelated antimicrobial agents and also cross-resistance among the compounds. However, there are only limited data currently available on the susceptibility to the metals used as growth promoters including copper sulfate and zinc chloride in the commensal flora Escherichia coli. Data on the contribution of efflux systems to the resistance among clinical isolates is much less.

The aims of the present study were to determine the susceptibilities to copper sulfate and zinc chloride in E. coli isolated from pigs and to explore the possible involvement of active efflux systems in reduced susceptibility to these metals. The susceptibilities to antimicrobial agents were determined to further investigate the epidemiology of antibiotic resistance in E. coli in Thailand.

Materials and Methods

Bacterial strains: A total of 180 E. coli isolates were obtained from swine and their farm environment including feed, water and feces. All the samples were collected by farm veterinarians and submitted to Veterinary Diagnostic Laboratory, Faculty of Veterinary Science, Chulalongkorn University for isolation and identification. All the E. coli strains were isolated using the protocol described in Bacteriological Analytical Manual and tested for their biochemical characteristics.
using methods described previously (Carter and Cole, 1990). One colony was collected from each positive sample.

**Antimicrobial susceptibility test:** the minimum inhibitory concentrations (MIC) were determined by a two-fold agar dilution technique according to the Clinical and Laboratory Standards Institute (CLSI, formerly NCCLS) (NCCLS, 2002). The antimicrobial agents included two heavy metals i.e. copper sulfate and zinc chloride, ampicillin (AMP), chloramphenicol (CHP), gentamicin (GEN), ciprofloxacin (CIP), erythromycin (ERY) tetracycline (TET), trimethoprim (TRI), streptomycin (STR), sulphamethoxazole (SUL). All antimicrobials, except copper sulfate and zinc chloride, were purchased from Sigma (Detroit, USA). Copper sulfate and zinc chloride were obtained from UNILAB (New South Wales, Australia).

**Exposure experiments:** The E. coli isolates with low MICs to copper sulfate (n= 5) or zinc chloride (n= 2) were exposed to gradually-increasing concentrations of copper sulfate and zinc chloride as previously described with some modifications (Gradel, et al., 2005). For the stepwise exposure, a 100-µl overnight inoculum was inoculated into 4 ml LB broth containing copper sulfate or zinc chloride at a concentration of half the MIC. This procedure was repeated daily in LB broth with increasing concentrations of copper sulfate or zinc chloride by a factor of 1.5 until no visible growth was observed. Five colonies were randomly-picked from the final passage and tested for their MICs for copper sulfate and zinc chloride.

**Activity of the active efflux system inhibitor:** The MICs for carbonyl cyanide m-chlorophenylhydrazone (CCCP) and reserpine were firstly determined in 10 representatives of the E. coli isolates and the MIC values were 400 µM and 100 µg/ml for CCCP and reserpine, respectively. Therefore, susceptibilities to copper sulfate and zinc chloride were determined in the presence and absence of 50 µM CCCP or 20 g/ml reserpine. Experiments were repeated on two separate occasions. Controls were cells that were grown in the presence of CCCP or reserpine without copper sulfate and zinc chloride to ensure that CCCP and reserpine did not have inhibitory effect to the cell growth.

**Results**

**Antimicrobial susceptibilities:** The MICs of antimicrobials and heavy metals copper sulfate and zinc chloride were determined against all the E. coli strains (Figure 1). All the isolates were resistant to erythromycin. At least eighty percent of the E. coli isolates exhibited resistance to tetracycline and ampicillin. As all the isolates tested were resistant to at least one antibiotic, 175 isolates (97.2%) were multidrug resistant (resistant to 3 or more separate classes of antibiotics). Forty resistance patterns were identified (Table 1), of which the most common resistance phenotypes found were the AMP-CHP-CIP-ERY-GEN-STR-SUL-TET-TRI phenotype (13.33%).

The E. coli isolates formed one large population of susceptibilities to copper sulfate (6.4-12.8 mM) and zinc chloride (3.2-6.4 mM). Most E. coli (92.2%) had an MIC of 6.4 mM to copper sulfate while 14 (7.8%) had the MIC of 12.8 mM. Most the isolates (98.9%) exhibited an MIC of 6.4 mM to zinc chloride and only 2 isolates (1.1%) showed the MIC at 3.2 mM.

**Table 1 Antimicrobial resistance patterns in E. coli (n= 180)**

<table>
<thead>
<tr>
<th>Resistance pattern</th>
<th>No. of isolates (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ERY</td>
<td>5 (2.78)</td>
</tr>
<tr>
<td>AMP-CHP-ERY-TET</td>
<td>8 (4.44)</td>
</tr>
<tr>
<td>CHP-ERY-TET-TRI</td>
<td>11 (6.11)</td>
</tr>
<tr>
<td>AMP-CHP-ERY-TET-TRI</td>
<td>6 (3.33)</td>
</tr>
<tr>
<td>AMP-ERY-STR-SUL-TET</td>
<td>5 (2.78)</td>
</tr>
<tr>
<td>CHP-ERY-STR-TET-TRI</td>
<td>6 (3.33)</td>
</tr>
<tr>
<td>AMP-CHP-ERY-GEN-STR-TET</td>
<td>3 (1.66)</td>
</tr>
<tr>
<td>AMP-CIP-ERY-GEN-STR-TET</td>
<td>4 (2.22)</td>
</tr>
<tr>
<td>AMP-CHP-ERY-GEN-SUL-TET</td>
<td>9 (5.00)</td>
</tr>
<tr>
<td>AMP-CHP-ERY-GEN-SUL-SUL-TET</td>
<td>6 (3.33)</td>
</tr>
<tr>
<td>AMP-CHP-ERY-GEN-STR-TET-TRI</td>
<td>6 (3.33)</td>
</tr>
<tr>
<td>AMP-CHP-ERY-STR-SUL-STR-TET-TRI</td>
<td>15 (8.33)</td>
</tr>
<tr>
<td>AMP-CHP-ERY-GEN-STR-STR-STR-TET-TRI</td>
<td>4 (2.22)</td>
</tr>
<tr>
<td>AMP-CHP-ERY-GEN-STR-SUL-STR-TET-STR-TET-TRI</td>
<td>11 (6.11)</td>
</tr>
<tr>
<td>AMP-CIP-ERY-GEN-SUL-SUL-TET-SUL-TET-TRI</td>
<td>6 (3.33)</td>
</tr>
</tbody>
</table>

Only the resistance patterns with 3 or more E. coli isolates are shown.

**Adaptation to copper sulfate and zinc chloride:** Five E. coli isolates with copper sulfate MIC of 6.4 and two strains with zinc chloride MIC of 3.2 mM were selected for exposure to gradual increasingly higher concentration of both heavy metals. None of the E. coli strains grew beyond the second passage. The growth of E. coli isolates exposed to copper sulfate ended at a concentration of 14.4 µg/ml while that of those exposed to zinc chloride ceased at 7.2 µg/ml.

**Inhibitory effect of CCCP and reserpine:** Since all the E. coli strains exhibited generally few variations in MICs to copper sulfate and zinc chloride, all of them were tested for their responses to CCCP and reserpine (Table 2). The addition of CCCP reduced at least 4

![Figure 1](image-url)
folds of the copper sulfate MIC in 12 isolates (6.7%) but the addition of reserpine did not change the copper sulfate MIC in any isolates. Twenty-eight E. coli isolates (15.6%) had the reduced zinc chloride MIC at least 4 fold in the presence of CCCP and up to 132-isolates (73.3%) exhibited at least 4 fold decline in the zinc chloride MIC when reserpine was added.

Table 2 The effects of CCCP and reserpine on copper sulfate and zinc chloride susceptibilities in E. coli (n=180)

<table>
<thead>
<tr>
<th>Heavy metal</th>
<th>No. of isolates (%)</th>
<th>CCCP</th>
<th>reserpine</th>
<th>CCCP/reserpine</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Copper sulfate</td>
<td>12(6.7)</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2. Zinc chloride</td>
<td>28(15.6)</td>
<td>132(73.3)</td>
<td>20(11.1)</td>
<td>-</td>
</tr>
<tr>
<td>3. Copper sulfate/zinc chloride</td>
<td>6(3.3)</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

* Numbers of the isolates expressing at least 4 fold-reduction of MICs.

Among these isolates, 20 isolates (11.1%) had at least 4-fold reduction in zinc chloride MIC in the presence of either CCCP or reserpine. Of all the isolates tested, 6 isolates (3.3%) had at least 4 fold decline in MICs of copper sulfate and zinc chloride when CCCP was added.

Discussion

The E. coli strains in the present study exhibited high resistance to antimicrobial agents, in agreement with previous studies (Phongpaichit, et al., 2007; Lundin, et al., 2008). While more than eighty percent of the E. coli isolates were resistance to ampicillin, erythromycin and tetracycline, most strains were resistant to multiple drugs. Such high resistance is likely due to the extensive long-term use of antibiotics in swine production. It should be noted that the chloramphenicol E. coli isolates were detected at high rate (79.9%) even though the use of this antibiotic has been withdrawn in food animal production (Abouzeed, et al., 2000; Bischoff, et al., 2005; Gebreyes and Thakur, 2005). This may be associated with coselection and/or cross resistance with other antibiotics (Chuanchuen, et al., 2008). However, the mechanism (s) underlying the phenomenon was not pursued in this study.

Both copper and zinc are essential trace elements for all animals that have been formulated into trace-mineral premixes and there were few studies reporting resistance in bacteria (Hasman and Aarestrup, 2002; Hasman and Aarestrup, 2005). The MICs of copper sulfate and zinc chloride in the E. coli isolates in this study were slightly different from those previously reported in the pig isolates (Aarestrup and Hasman, 2004). This difference could be attributed to differences in laboratory techniques. The E. coli isolates in our collection formed one large population of MICs to copper sulfate and zinc chloride, suggesting that none of these strains have developed resistance to neither copper sulfate nor zinc chloride or they have developed only a limited degree of resistance. This result was similar to those in a previous study (Aarestrup and Hasman, 2004). The MICs to copper sulfate and zinc chloride detected in this study may reflect intrinsic susceptibility of E. coli. However, it cannot be warranted that no acquired resistance occurred among the E. coli strains. Further studies are required for elucidation of this puzzle.

In the exposure studies, none of the E. coli isolates tested grew beyond 2 passages, indicating that the isolates did not develop resistance to copper sulfate and zinc chloride. Since in vitro environment is different from in vivo conditions, laboratory data may not always represent what happens under real life conditions. Therefore, it cannot assure that resistance to copper sulfate and zinc chloride will never take place in clinical conditions where these two metals have been extensively used.

Active efflux pumps have been shown to play a major role in acquisition of antimicrobial resistance in bacteria. Both CCCP and reserpine are considered efflux pump inhibitors that impede different types of efflux systems. CCCP, an energy uncoupler, stalls members of the resistance nodulation division (RND) family using proton motive force (pmf). Reserpine blocks the function of the RND efflux systems and also those driven by ATP i.e. the major facilitator (MFS) family and the ATP-binding cassette (ABC) family (Ricci and Piddock, 2003; Braoudaki and Hilton, 2005). In this study, the contribution of efflux systems in reduced susceptibility to copper sulfate and zinc chloride among the clinical E. coli isolates was investigated. Only CCCP could lessen the MIC of copper sulfate up to 4 folds, suggesting that reduced susceptibility to this metal was mediated by the pmf-driven systems. Some isolates exhibited higher susceptibilities to zinc chloride in the presence of either CCCP or reserpine, indicating that the reduced susceptibility to zinc chloride in these strains are mediated by more than one active efflux systems using different energy sources and either by RND, MFS or ABC types of active efflux systems. Six E. coli isolates simultaneously exhibited at least 4-fold reduction in MICs to copper sulfate and zinc chloride in the presence of CCCP. This suggested that the common efflux pump extruding both heavy metals may exist. However, such reduction may be due to simultaneously interfering of two or more efflux systems. Several divalent-metal efflux pumps has been previously documented in E. coli, including the CzxC system mediating resistance to zinc, cobalt and cadmium (Nies, 1995), the CusCFBA systems extruding copper and silver (Franke, et al., 2003) and the CopA efflux expelling copper (Rensing, et al., 2000). Further investigations are warranted to investigate the mechanism(s) underlying reduced susceptibility to copper sulfate and zinc chloride in
the E. coli isolates in this study.

In summary, the data from the present study showed that the E. coli isolates from pigs have not developed or only a limited degree of development of resistance to copper sulfate and zinc chloride that are commonly used as feed additives. In vitro exposure to gradually increasing concentrations of both elements did not reduce susceptibility of E. coli. However, the impact of such exposure in clinical setting needs further evaluation. The reduced susceptibilities to copper sulfate and zinc chloride could be intrinsic or acquired and could be mediated by the expression of one or more active efflux systems.

Acknowledgement

This work was funded by Chulalongkorn University-Veterinary Science Research Fund RG6/2552. We also thank Novatis (Thailand) Ltd., Thailand for sample collection and the bacterial strain providing.

References


