

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

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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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บทคัดย่อ

การมีส่วนร่วมของ Active efflux ในการลดความไวต่อคอปเปอร์ซัลเฟตและซิงค์คลอไรด์ใน *Escherichia coli* ที่แยกได้จากสุกร

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Escherichia coli ที่แยกได้จากสุกรจำนวน 180 Isolates ทำการหาค่า minimal inhibitory concentrations (MICs) ต่อยาปฏิชีวนะ 9 ชนิดและโลหะหนัก 2 ชนิด คือ Copper sulfate และ Zinc chloride ผลการศึกษาพบว่า *E. coli* ทุกตัวดื้อต่อยาปฏิชีวนะอย่างน้อยหนึ่งชนิดและจำนวน 175 Isolates (ร้อยละ 97.2) ดื้อต่อยาหลายชนิดพร้อมกัน จัดรูปแบบของการดื้อยาปฏิชีวนะได้ 40 รูปแบบ โดยรูปแบบที่พบมากที่สุดคือ AMP-CHP-CIP-ERY-GEN-STR- SUL-TET-TRI (ร้อยละ 13.33) ค่า MIC ต่ Copper sulfate และ Zinc chloride เกาะกลุ่มอยู่ที่ 6.4-12.8 mM และ 3.2-6.4 mM ตามลำดับ การสัมผัส Copper sulfate และ Zinc chloride ในระดับต่ำไม่ทำให้ความไวต่อโลหะหนักทั้ง 2 เปลี่ยนแปลง การเติม Carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) และ Reserpine มีผลให้ค่า MIC ต่ Copper sulfate และ Zinc chloride ลดลงมากกว่า 4 เท่าในเชื้อจำนวน 66 isolates (ร้อยละ 36.7) และ 142 isolates (ร้อยละ 78.9) ตามลำดับ ผลการวิจัยชี้ให้เห็นว่า ระบบ Active efflux ที่ใช้ Proton motive force และ/หรือ ATP เป็นพลังงาน มีส่วนร่วมในการลดความไวต่อ Copper sulfate และ Zinc chloride ใน *E. coli* ที่แยกได้จากสุกร

คำสำคัญ: ระบบแอคทีฟ อีฟลักซ์ คอปเปอร์ซัลเฟต เอสเคอริเคีย โคลี สุก ร ซิงค์คลอไรด์

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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- μ l 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 μ M and 100 μ g/ml for CCCP and reserpine, respectively. Therefore, susceptibilities to copper sulfate and zinc chloride were determined in the presence and absence of 50 μ M 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.

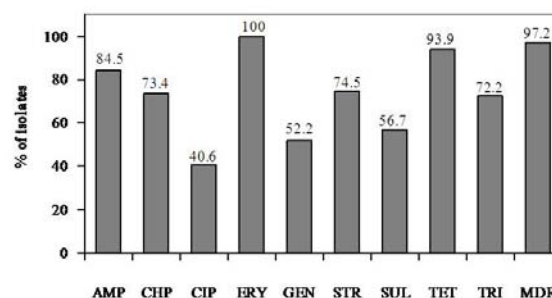


Figure 1 Resistance rates to antimicrobial agents in *E. coli* from swine (n= 180) AMP: ampicillin, CHP: chloramphenicol, CIP: ciprofloxacin, ERY: erythromycin, GEN: gentamycin, STR: streptomycin, SUL: sulfamethoxazole, TET: tetracycline, TRI: trimethoprim, MDR: multidrug resistance

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

Resistance pattern	No. of isolates (%)
ERY	5 (2.78)
AMP-CHP-ERY-TET	8 (4.44)
CHP-ERY-TET-TRI	11 (6.11)
AMP-CHP-ERY-TET-TRI	6 (3.33)
AMP-ERY-STR-SUL-TET	5 (2.78)
CHP-ERY-STR-TET-TRI	6 (3.33)
AMP-CHP-ERY-GEN-STR-TET	3 (1.66)
AMP-CIP-ERY-GEN-STR-TET	4 (2.22)
AMP-ERY-GEN-STR-SUL-TET	9 (5.00)
AMP-CHP-ERY-GEN-STR-SUL-TET	6 (3.33)
AMP-CHP-ERY-GEN-STR-TET-TRI	6 (3.33)
AMP-CHP-ERY-STR-SUL-TET-TRI	15 (8.33)
AMP-ERY-GEN-STR-SUL-TET-TRI	4 (2.22)
AMP-CHP-CIP-ERY-GEN-STR-TET-TRI	11 (6.11)
AMP-CHP-CIP-ERY-STR-SUL-TET-TRI	7 (3.89)
AMP-CHP-ERY-GEN-STR-SUL-TET-TRI	12 (6.67)
AMP-CIP-ERY-GEN-STR-SUL-TET-TRI	6 (3.33)
AMP-CHP-CIP-ERY-GEN-STR-SUL-TET-TRI	24 (13.33)

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 μ g/ml while that of those exposed to zinc chloride ceased at 7.2 μ g/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

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 folds 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.

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.

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

Heavy metal	No. of isolates ^a (%)		
	CCCP	reserpine	CCCP/reserpine
1. Copper sulfate	12(6.7)	-	-
2. Zinc chloride	28(15.6)	132(73.3)	20(11.1)
3. Copper sulfate/zinc chloride	6(3.3)	-	-

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

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 (73.9%) 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 motif 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 CzcABC 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.

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