Enterotoxigenic *Escherichia coli* Antibio-Resistance Induced by Copper

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ABSTRACT

Cupric sulfate is widely used as growth promoter for swine productions, in spite of its ecological risks. The purpose of this paper was to evaluate the in vitro effect of copper as inducer of antibio-resistance in Enterotoxigenic. *E. coli*. A-1 (O149: K91: K88ac) and E68-I (O141: K85: K88ab) reference strains were grown in Mueller-Hinto agar, according to the concentration gradient technique with weekly increasing values, from 5 to 50 mg/mL of $CuSO_4$. Both bacteria were cultured for three weeks; the controls were cultured parallely, in the same medium, without the inducer. Then, antibiograms were made to evaluate sensitivity against gentamicin, kanamycin, tetracycline, chloramphenicol, carbenicilin, and nalidixic acid. The results from the strains challenged with salt showed halos compatible to resistant phenotypes, in contrast to the controls (all sensitive). The results evidence the capacity of copper to induce *in vitro* bacterial antibio-resistance, which must be considered by producers when copper salts are used as growth promoters. In addition to ecological damages, antimicrobials lose effectiveness in human and animal therapeutics.

Key words: *antimicrobials; environment; enteropathogens; growth promoters; swine; tolerance*

INTRODUCTION

The Adjoin Director General of the United Nations Food and Agriculture Organization (FAO), Helena Semedo, said in Amsterdam that excessive and inadequate use of antibiotics and other antimicrobial agents is a threat to public health and endangers food safety in the world. She also expressed that the situation is so alarming that the progress made in human and animal health could go a century back. She concluded that her organization was committed to tackle this problem based on the One Health approach, that links humans, animals, and the environment (FAO, 2016).

Cupric sulfate used as enhancer of growth in animal diets dates back to the mid Twentieth Century. Its effectiveness has been associated, among others, to the metal´s antagonistic action against Enterotoxigenic *E. coli* (ETEC) and *Salmonella*, just to mention two examples. Despite the results achieved, right from the start there were fears about the toxicity of the action (Apley, Bush, Morrison, Singler and Snelson, 2012). More recently, it was evidenced that it had ecological implications, since the stools from the treated animals had copper concentrations 14 times than the animals who did not consume it, causing a negative effect on the soil. According to predictions, as long as this practice continues, within 10-50 years, the concentrations of the metal in the soil will surpass the permissible levels (Seiler and Berendonk, 2012).

Colibacillosis is one of the main causes of mortality and economic losses in swine systems (Barreto, Rodríguez, Bertot and Delgado, 2015). *E. coli* has long been characterized by an amazing adaptability to diverse environments, including those with high concentrations of heavy metals (Barreto and Rodríguez, 2009). Due to its toxicity, copper is the main antimicrobial component of many products, but it is not an exception. In vitro reference strains and isolates compatible with ETEC from pigs with colibacillosis at increasing concentrations of $CuSO_4$ have proved the previous. That resistance was associated to biofilm production, which, in addition to the above mentioned, calls for a more efficacious strategy to deal with ineffectiveness (Barreto, Rodríguez and Barreto, 2016).

The development of tolerance mechanisms for heavy metals leads to an added quality in successful gram-positive bacteria: parallel resistance to a range of antibiotics, though they are not present in

the environment (Agga, Scott, Mamachawadi, Nagaraja, Vinasco, Bait *et al.*, 2014). No reports have been made in Gram-negative bacteria (Amachawadi, Scott, Alvarado, Mainini, Vinasco, Drouillard and Nagaraja, 2013).

The aim of this study was to evaluate the in vitro effect of copper as inducer of antibio-resistance in Enterotoxigenic *E. coli.*

MATERIALS AND METHODS

Strains

Two Enterotoxigenic *E. coli* reference strains were used: *Escherichia coli* A-1 (O149: K91: K88ac), and *Escherichia coli* E68-I (O141: K85: K88ab). The strains were stored in Mueller-Hinton broth glycerinate (30 %), at -8 ºC, until use.

Induction of antibio-resistance with $CUSO_4$

To induce in vitro antibio-resistance to $CuSO_4$, standard Petri dish were filled with Mueller-Hinton agar, according with the concentrations gradient (Karadjov, 1985), containing 5-25; 10-35; and 15-50 mg/mL of the salt in the first, second, and third weeks, respectively. After culturing (from the lowest to the highest concentration), incubation was made at 37 ºC (24-48 h in the first cultures; 24 h from late the first week to the end of the experiment), until growth was observed. Then, subcultures were made with samples from the highest copper concentration area. Parallely, A-1 and E68-I were placed in Mueller-Hinton agar, without coppers salts, used as the experiment control. At the end, antibiograms were performed to both groups, according to Kirby-Bauer Disk Diffusion Susceptibility Test Control (Hudzicki, 2009); determination was made using three replicas. The diameter reported for each inhibition halo was the average of measurements in two perpendicular directions, using a ruler calibrated in millimeters.

RESULTS AND DISCUSSION

All the halos achieved in the antibiograms of copper exposed strains, corresponded to resistance diameters (Hudzicki, 2009); the control halos did not behave the same way (Table).

Both *E. coli* A-1, and its antagonist E68-I have a pathogenic history that goes back to the beginning of the second half of the Twentieth Century, when they were isolated from pig with colibacillosis that caused their death. Since then, and after characterization as reference strains, they have been kept in the laboratory (Barreto, 1988); hence, they are very sensitive to a wide range of antimicrobials, including tetracycline, and chloramphenicol, as can be observed in the diameter of the halos displayed (Table), ideal for this type of study (Barreto *et al.*, 2006). Overall, they comprise six antibiotics, of which four have been used for a long time in human and veterinary therapeutics (gentamicin, kanamycin, tetracycline, chloramphenicol), whereas carbenicilin and nalidixic acid have been used in humans (MINSAP, 2006).

The capacity of certain heavy metals, like copper to expand and keep the pool of bacteria resistant to antibiotics, like tetracycline, has been reported. The implications of this phenomenon in gram-positive enteric bacteria has been studied in several animal species, in relation to this metal salts, as growth promoters (Amachawadi *et al.*, 2013). Unfortunately, the same interest has not been observed in the gram-negative bacteria, though enteropathogens like ETEC and *Salmonella* are included, according to Agga *et al.* (2014), who concluded that the role of copper supplementation to weaned piglets, and resistance to metal and the antibiotics studied, (2014), demands further research, concerning *E. coli*. In their proposal, no positive correlation between Cu input (125 mg/kg of feed) and resistance to tetracycline and other antibiotics in the *E. coli* isolates from the stools of these animals.

However, high doses of copper (100-250 ppm) used in swine production for over sixty years; as well as exposure to environmental accumulation, have contributed gram-negative adaptation, regardless of their high toxicity (Araestrup and Hasman, 2004).

It is worth remembering that these results are the outcome of two in vitro reference strains (sensitive to previously tested antibiotics), at highly superior metal concentrations to the ones studied by Agga *et al.*, 2014). Tolerance to heavy metals increases as their contact with bacteria prolongs, since stress response and resistance genes are gradually activated (Harrison, Ceri and Turner, 2007). It has been concluded that when they cumulate at critical concentrations in natural environments, they may unleash in the micro biota, especially in bacteria. Along with ion tolerance, resistance to antibiotics is produced (Seiler and Berendonk, 2012).

This double resistance is associated to diverse mechanisms, two of which can explain the results achieved: a) complex formation, linked to biofilm production increases, mucilage where the greatest antimicrobial fixation takes place (Harrison *et al.*, 2007; Barreto and Rodríguez, 2010). *E. coli* A-1 began to excrete abundant exopolysaccharides (EPS), around the growth line in the third week of Cu^{2+} exposure, (Fig.) (Barreto *et al.*, 2016). b) Efflux mechanisms, commonly observed in gram-negative bacteria (as in this study), which allow for metal and antibiotic extrusion from the cytoplasm, through the inner and outer membranes, to the surrounding environment (Seiler and Berendok, 2012). The likelihood of this choice is very high, theoretically, in this case, though lacking confirmation.

These results corroborate the previous analysis. Additionally, they pose a warning, regarding the inclusion of $CuSO_4$ as an additive in swine diets, provided that the proportions suggested to farmers are highly variable (Zhao, Allee, Gellermann, Ma, Gracía, Parker *et al.*, 2014). In that proposal, six antibiotics decreased effectiveness due to copper activity, against *E. coli*, including nalidixic acid, one of the most frequently used antibiotics against enteropathogens like ETEC and *Shigella*, in cases of acute human diarrhea (Cisneros, Barreto, Guevara and Rodríguez, 2008). It corroborates FAO´s appeals to implement production systems that guarantee One Health.

CONCLUSIONS

Exposure of *in vitro E. coli* to $CuSO_4$ through the gradient concentration method, induces bacterial resistance to gentamicin, kanamycin, tetracycline, chloramphenicol, carbenicilin and nalidixic acid, another element to be considered in swine production that uses this salt as a growth promoter.

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Table. Effect of $CuSO_4$ on *E. coli* (A-1 and E68-1) sensitivity

Strains	Gentamicin		Kanamycin		Tetracycline		Chloramphenicol		Carbenicilin		Nalidixic acid	
E68-I	18		19		20		20		23		20	
E68-I*	11		10		14		12		2		13	
A-1	19		18		19		19		23		19	
A-1*	10		7		12		10		0		11	
Antibiotic sensitivity criteria according to Kirby and Bauer (Hudzicki, 2009)												
	R	S	R	S	R	S	R	S	R	S	R	S
	12	15	13	18	14	19	12	18	17	23	13	19

E68= *E. coli* reference strain E68-I; A-1= *E. coli* reference strain A-1; *Strains treated with $CuSO_4$; Ac= acid; R= resistant; S= sensitive

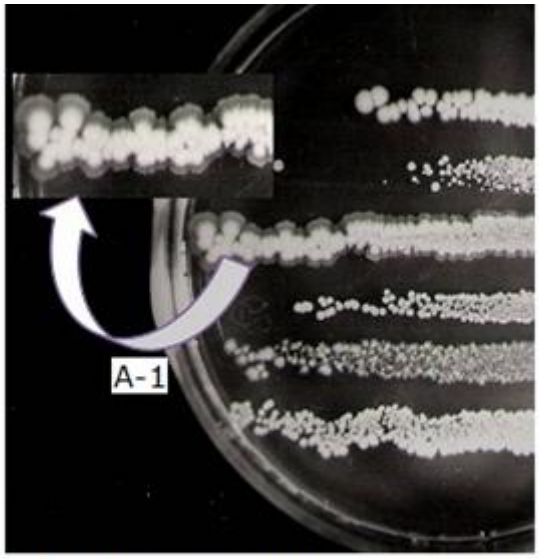

Fig. **Exopolysaccharide formation around A-1 growth. The mucoid area increases as copper concentration increases**