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In vitro Behavior of Enterotoxigenic *Escherichia coli* in Increasing Copper Concentrations

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ABSTRACT

Cupric sulfate is widely used as growth promoter for swine productions, in spite of its ecological risks. The aim of this paper was to study the in vitro behavior of Enterotoxigenic *Escherichia coli* in increasing concentrations of copper. To achieve that, two reference strains: A-1 (O149: K91: K88ac) and E68-I (O141: K85: K88ab), were used with other four strains isolated from herds with colibacillosis. The reference strains were grown in Mueller-Hinton agar, according to the concentration gradient technique with weekly increasing values, from 5 to 50 mg/mL of CuSO4. The experiment lasted three weeks. Though in the initial contacts two wild strains were the best adapted, at the end all developed high tolerance to the metal. For A-1, it may be determined by the formation of biofilm, which, apart from the said resistance, is an important factor of virulence in infectious bacterial processes. The results observed are another element to add to the dangers of using these salts as growth promoters in swine productions.

Key words: *antimicrobials, environment, growth promoters, swine, tolerance*

INTRODUCTION

Modern swine production systems have achieved efficiency levels only possible with the use of growth stimulators (Davies, 2011). Cupric sulfate used as growth enhancer in animal diets dates back to the mid Twentieth Century. Its effectiveness has been associated to the additive´s antagonistic action against pathogens like enterotoxigenic *E. coli* (ETEC) and Salmonella, just to mention two examples. Over the years, the only concern relied on the cation´s toxicity (Hastad *et al.*, 2001). Later, it ecological implications were observed, as the stools from the treated animals contained copper concentrations 14 times higher 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). Through the years, *E. coli* has proven a surprising ability to adapt to the most diverse environments, including those with increased concentrations of heavy metals (Barreto and Rodriguez, 2009). This is a worrisome growing phenomenon, especially when the strains, in addition to their cation resistance, usually display a wider arsenal of virulence factors. Hence, the potentials to develop diseases in susceptible hosts are notably increased (Barreto and Rodríguez, 2009).

The purpose of this research was to study the in vitro behavior of enterotoxigenic *Escherichia coli* in increasing concentrations of copper.

MATERIALS AND METHODS

Strains

Six enterotoxigenic *Escherichia coli* (ETEC) strains were used; two of them were reference strains: A-1 (O149: K: 91: K88ac), and E68-I (O141: K85: K88ab); and the other four were isolated from pigs with colibacillosis, before weaning identified as WS (wild strain) and numbered 1-4. Until their use, all the strains were stored in Mueller-Hinton broth, glycerinated (30 %), at -8 ºC .

ETEC behavior in increasing concentrations of $CuSO_4$

Standard Petri dishes were filled with Mueller-Hinton agar, according to 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 to the end of the experiment), until growth was observed. Then, subcultures were made with samples from the highest copper concentration area.

RESULTS AND DISCUSSION

The initial behavior of bacteria in the presence of Cu_2+ was variable: CS1 and CS2 wild strains had an adequate growth (especially the former) at first contact with the metal. Later, as the subcultures were repeated, all the bacteria moved closer to areas with maximum concentration of $CuCO_4$ in the dish. Growth around the culture line was more exuberant (Fig. 1).

Before characterization as reference strains, both *E. coli* and its homolog (E68-I) had been kept in the laboratory, away from any kind of antimicrobial contact. All wild strains (WS) came from colibacillosis infested herds. On farms where the isolates were performed for years, $CuSo_4$ was added at the suggested proportions to act as a growth promoter (Macías *et al.*, 2015).

The above mentioned may have influenced in terms of different growth intensities over the first week in contact with the metal studied. Throughout the evolution of the planet, bacteria were the first settlers, and they had to adapt to the presence of large numbers of cations in various natural media, after mutating. This natural selection only allowed for the survival of the most adaptable ones (Barreto and Rodríguez, 2006). The information for resistance acquired in such a way may lie in remaining plasmids, whereas the bacteria that carry them develop in media where the metal is present, but not when it is absent. For instance, by giving successive passes through conventional culture media for storage, they tend to lose such plasmids (Barreto, 1988a). It can be assumed for the reference strains; for CS3 and CS4, it may have been caused by the lack of previous contacts with copper. Meanwhile, the behavior of CS1, and CS2 (in a lesser degree), corresponds to bacteria with active mechanisms of metal resistance, which demonstrates its presence in that environment.

In the last one hundred years, industrialization and new technologies to increase agricultural, livestock raising and aquaculture yields, the pollution levels with metals, such as Hg, Cd, Cu, and Zn, have reached skyrocketing values. As a result, the microbiotas, especially from the soil, have undergone bigger challenges than any precedent earthly experience (Barreto and Rodríguez, 2010; Seiler and Berendonk, 2012). These kinds of environments have produced bacteria with a behavior in accordance with CS1 and CS2 development.

Images of bacterial growth, snapped at the end of weeks two, and three, especially (Fig. 1 b and c) prove adaptation to antimicrobials, observed along an exuberant culture line. 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). These genes may be located in plasmids or the nucleoid, also known as bacterial chromosome. Most research report a predominance of the former in terms of copper resistance. The latter also have some part in it, according to a review made by Cooksey (2006).

Any of the above mentioned variants may have influenced the results (previous experiment), that involved atypical *E. coli* strains producing H2S resistant to $CuSO_4$ and Apramycin (also known as Nebramycin II, or Apralan). Tolerance to both antimicrobials was regulated by a plasmid that disappeared when the bacteria underwent successive cultures in culture media with no metal and antibiotics. The opposite occurred when either of the two was present (Barreto, 1988b).

To prevent cell damage in the presence of frequently occurring antimicrobials, bacteria have developed tolerance mechanisms that can explain their resistance to heavy metals:

1. Complex formation

When the metal is "trapped", the concentration of free ions in the cytoplasm is minimum, way below lethal levels. This choice is associated with an increase in biofilm production in the presence of these elements; this mucilage is where most fixation takes place (Harrison et al, 2007; Barreto and Rodríguez, 2010). Regarding *E. coli* (Fig. 2) a diffuse area with biofilm-characteristic mucilaginous exopolysaccharide (EPS) accumulation was observed along the growth line, in the third week of Cu2+ exposure. It is an extreme choice made to counteract the very high accumulation of such toxic metal (Harrison et al., 2007; Barreto *and Rodríguez, 2010).*

2. Reduction ion intracellular concentration

One example is the production of mercury-reductase in gram negative bacteria. It can reduce the very toxic Hg_2+ to less aggressive Hg_0, which easily disposed of by the cell due to its low evaporation point (Seiler and Berendonk, 2012).

2. Efflux mechanisms

They are very commonly observed in gram negative bacteria, helping with metal extraction from the cytoplasm, through the inner and outer membrane, to the surrounding environment (Seiler and Berendonk, 2012). This case is a very probable choice in theory, but it lacks confirmation.

What is clear is that successive contacts with the Cu2+ cation triggers responses in the strains studied that help tolerate very high concentrations of the metal. One of them is the production of biofilm, clearly observed in the A-1 strain. Generally, this result has implications far beyond resistance to this heavy metal. It is a fact that the presence of heavy metals in natural media stimulates co-selection of resistant bacteria to them, which, in turn, are tolerant to certain antibiotics, though they are not present (Seiler and Berendonk, 2012). In turn, antimicrobial resistance and virulence are mechanisms that play critical roles in bacterial infections. Both factors are highly favored when biofilm is produced (Reis et al., 2014). In particular case of *E. coli*, a correlation between the existence of virulence factors and biofilm production has been reported (Naves et al., 2008).

Although present day animal production demand alternatives like the one exposed in this paper, it is important to keep in mind that there are other less aggressive choices to the target species, consumers and the environment; such as, the use of pre-biotics, probiotics, and efficient microorganisms (Rodriguez *et al.*, 2013).

CONCLUSIONS

In vitro exposure of *E. coli* to increasing concentrations of $CuSO_4$ induces resistance to the metal, even at very high concentrations. Two of the wild strains were able to grow freely since the first contact, evidence of previous contact with the cation in nature. Biofilm production is one of the mechanisms involved in tolerance, thanks to its important role as virulence factor for colibacillosis and other bacterial diseases. The previous, is another element to consider in swine production systems that use this salt as growth promoter.

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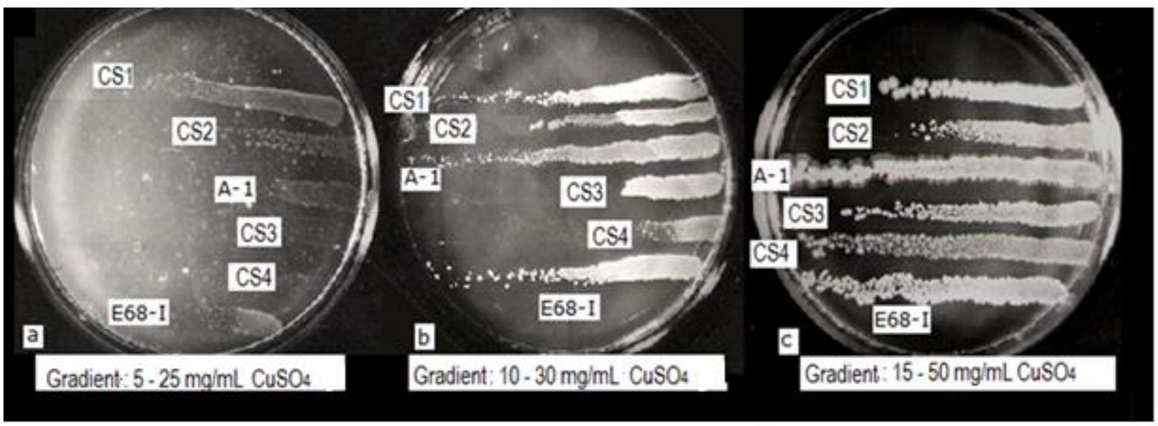

Fig. 1 Growth of six E. coli strains at increasing concentrations of $CuSO_4$ at the end of the first (a), second (b), and third (c) week of challenging. Contact prolongation leads to increased approach to areas with greater cation concentration (left end of the plate), also, to greater growth exuberance along the culture line.

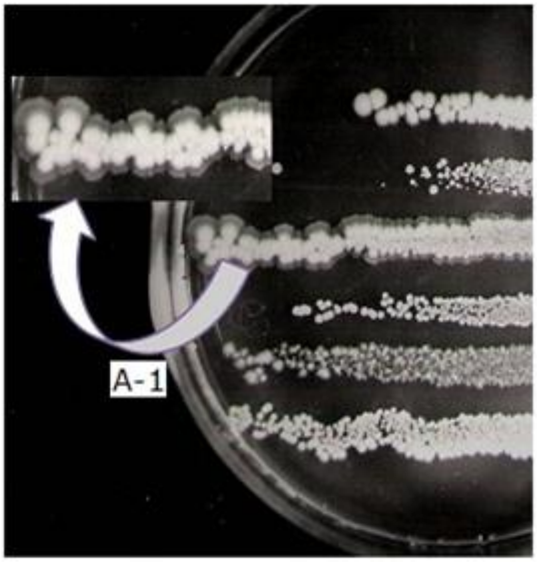

Fig.2. Formation of exopolysaccharides around A-1 growth. The mucoide area grows as copper concentration increases