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Gastric stability and oral bioavailability of colistin sulfate in pigs challenged or not with *Escherichia coli* O149: F4 (K88)

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ABSTRACT

The aim of the present study was to investigate the *in vitro* gastric stability of colistin sulfate (CS) and its antimicrobial activity against *Escherichia coli* and to study the impact of ETEC O149: F4 (K88) infection in pigs on CS intestinal absorption. The stability profile of CS was evaluated in a simulated gastric fluid (SGF). Antimicrobial activity of CS and its degradation products were examined in a 96-well polystyrene microplate model. The effect of experimental infection with ETEC O149: F4 on CS intestinal absorption was determined by quantification of CS systemic concentration using a validated LC–MS/MS method. A rapid degradation of CS accompanied by an increase in CS antimicrobial activity by comparison with non-degraded CS ($P < 0.0001$) was observed in SGF. Additionally, CS levels were not quantifiable in systemic circulation using a highly sensitive method and concurrent oral challenge did not affect CS absorption in an induction model of subclinical post-weaning diarrhea (PWD).

1. Introduction

Colistin, also known as polymyxin E, is a polypeptide antibiotic with significant *in vitro* activity against several multi-resistant Gram-negative (MRGN) pathogens, in particular *Pseudomonas aeruginosa* (Tunyapanit et al., 2013; Walkty et al., 2009), *Acinetobacter baumannii* (Liu et al., 2014) and *Klebsiella pneumoniae* (Ku et al., 2013). For these bacterial species, polymyxins are sometimes the only available active antibiotics in human medicine (Bergen et al., 2012). Given the importance of colistin for treatment of serious bacterial infections in humans and the limited availability of alternative antimicrobials for effective treatment of MRGN pathogens, Health Canada has classified this antibiotic in the category of very high importance in human medicine (Category I) (Government of Canada, 2014).

The chemical structure of colistin consists of a hydrophilic cycloheptapeptide ring with three positively charged amine groups, a tail tripeptide moiety with two positively charged amine groups, and a hydrophobic acyl chain tail (Azzopardi et al., 2013; Biswas et al., 2012) (Fig. 1). The amino groups mediate both the bactericidal effect and toxicity to human cells (Clausell et al., 2007; Mares et al., 2009). The target of antimicrobial activity of colistin is the bacterial cell membrane. This antibiotic has a strong positive charge and a hydrophobic acyl chain allowing a high binding affinity for lipopolysaccharide (LPS) molecules (Azzopardi et al., 2013). Colistin interacts electrostatically with LPS and competitively displaces divalent cations, causing disruption of the outer cell membrane that results in an increase in the permeability of the cell envelope, leakage of intracellular contents and, subsequently, bacterial death (Clausell et al., 2007). Antimicrobial susceptibility testing for colistin can be performed using disc diffusion, E-test, agar dilution, and broth dilution (Balaji et al., 2011). Different susceptibility breakpoints for colistin have been used by different organizations (Bergen et al., 2012). The *Société Française de Microbiologie* has selected ≤2 mg/L as the susceptibility breakpoint and >2 mg/L as the resistance breakpoint, whereas the British Society for Antimicrobial Chemotherapy selected ≤4 mg/L and ≥8 mg/L as the susceptibility and resistance breakpoints, respectively (Li et al., 2005).

Colistin sulfate (CS) has been used in the livestock industry in many countries and is the recommended treatment in swine medicine for gastrointestinal tract infections, particularly for those caused by *Escherichia coli* (Belloc et al., 2008; Callens et al., 2012; Casal et al., 2007). Post-weaning diarrhea (PWD) is an economically important disease in pigs

Abbreviations: AUC, area under the curve; CS, colistin sulfate; ETEC, enterotoxigenic *E. coli*; IU, international unit; MIC, minimum inhibitory concentration; MRGN, multi-resistant gram-negative; PWD, post-weaning diarrhea.

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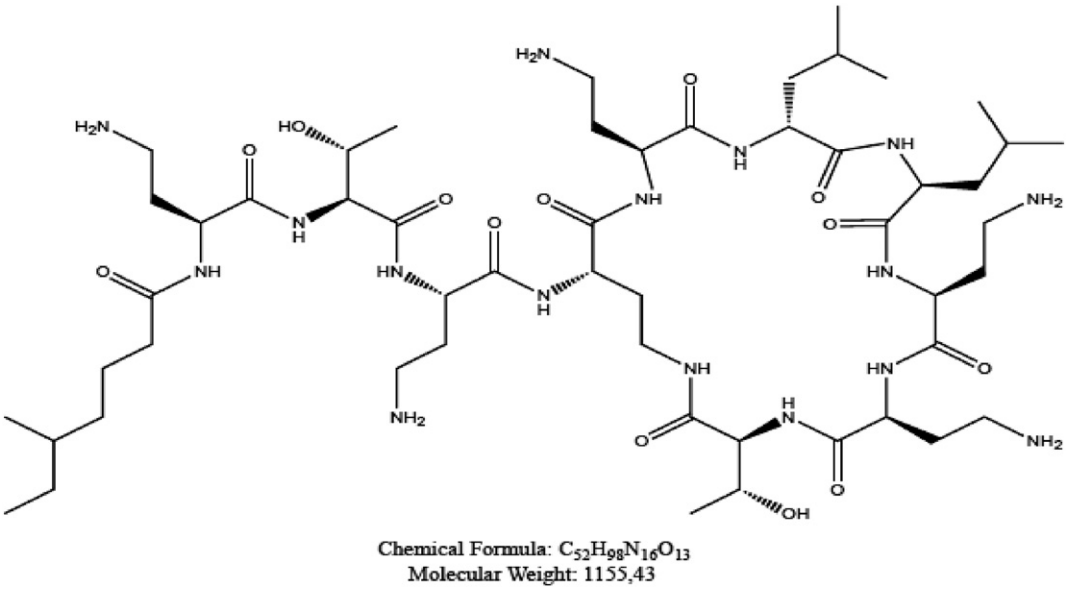

Fig. 1. The colistin structure is composed of a hydrophilic cycloheptapeptide ring with three positively charged amine groups, a tail tripeptide moiety with two positively charged amine groups, and a hydrophobic acyl chain tail.

due to financial losses as a result of mortalities, morbidity, reduced growth performance of surviving pigs, and cost of medication (Fairbrother et al., 2005). The predominant cause of PWD in pigs worldwide and in Canada is Enterotoxigenic *E. coli* (ETEC) of O group 149 (Fairbrother et al., 2005; Jamalludeen et al., 2007). ETEC O149 is characterized by the production of fimbriae F4 (K88) that mediates bacterial adherence to the intestinal mucosa and mediates heat stable and heat labile enterotoxins. Both families of enterotoxins enhance the secretion of sodium, chloride, and water into the intestinal lumen causing secretory diarrhea (Fairbrother and Gyles, 2012; Fairbrother et al., 2005). In pigs, CS is mainly used *per os* at a dosage of 50,000 IU/kg every 12 h for a period of 5 consecutive days for the treatment of intestinal infections caused by *E. coli*. This drug regimen has shown significant efficacy in the treatment of *E. coli* diarrhea (Belloc et al., 2008; Guyonnet et al., 2010). Colistin sulfate is used "off-label" in Canada for the treatment of PWD by transposition of data (dose, route of administration, dosing frequency) from countries where CS is approved.

In healthy pigs receiving therapeutic doses *per os*, it has been shown that CS is poorly absorbed. CS concentrations in the plasma were below the lower limit of quantitation (0.250 μg/mL) as determined by HPLC-UV (Guyonnet et al., 2010). Thus, the pig's intestinal microflora is exposed to the full dose of CS administered orally. On the other hand, there is little published data on the effect of bacterial gut infection in pigs on CS intestinal absorption. Such infections may affect bioavailability of oral antibiotics as a result of changes in intestinal hyperemia, tissue permeability, or intestinal peristalsis. Furthermore, there is no available information in the literature concerning the possible degradation of CS throughout a pig's digestive tract. This degradation may partly explain the low levels of CS systemically. In addition, there are differences in the withdrawal time between countries where this drug is approved for the treatment of colibacillosis in pigs (Committee for Medicinal Products for Veterinary Use (CVMP), 2010) due to the lack of data on CS intestinal absorption in pigs. Thus, understanding the stability of CS in the pig gastrointestinal tract is very important for interpreting results from pharmacokinetic and pharmacodynamic studies.

The first objective of this study was to investigate the *in vitro* gastric stability of CS and its antimicrobial activity with respect to two *E. coli* strains: the non-virulent strain ATCC 25922 and the virulent strain ETEC O149: F4 (K88). The second objective was to study the impact of experimental infection of piglets with ETEC O149: F4 (K88) on CS intestinal absorption levels using a highly sensitive analytical method (HPLC–MS/MS). Finally, the effect of a single oral dose of colistin (50,000 IU/kg) on the level of fecal shedding of ETEC O149: F4 (K88) and the total *E. coli* population were determined.

2. Material and methods

2.1. Stability of CS in simulated gastric fluid and antimicrobial activity of degradation products

The stability and degradation profiles of CS in simulated gastric fluid (SGF), prepared according to the United States Pharmacopoeia (United States Pharmacopeial Convention, 2009), were evaluated. Briefly, SGF was composed of 3.2 g/L pepsin and 2 g/L NaCl at a pH of 1.2. A quantity of 50,000 UI of CS (Daniel Bond & Frédéric Beaulac Inc., QC, Canada) was added to 500 mL of SGF when this solution reached 37 °C. At each time point of 0 (before adding pepsin), 5, 10, 15, 30, 45, and 60 min, three samples were taken out. Each sample was composed of 333 μL of sample solution and 666 μL of acetonitrile. Samples were centrifuged at 12,000 *g* for 5 min. The supernatant was transferred into an injection vial. Colistin sulfate concentrations were determined at each time point using an HPLC–MS/MS method. Comparatively, a concentration of 32 μg of CS was used as a stock solution to evaluate antimicrobial activity of CS after acetonitrile neutralization by evaporation. Antimicrobial assays were conducted in a sterile 96-well polystyrene microplate and 100 μL of fresh Mueller Hinton broth was added to each well. Then, 100 μL of each time point sample (0, 5, 10, 15, 30, 45, and 60 min) in duplicate was removed from the first well and double diluted from 8 μg/mL to 15 ng/mL. Two rows without CS in each plate were used as controls. One row was used as a positive control and contained *E. coli* ATCC 25922 or ECL8559 and the other row, without bacterial inoculum but containing 200 μL of Mueller Hinton broth, was the negative control. Finally, 100 μL of a bacterial count of 5.10^5 CFU/mL of *E. coli* ATCC 25922 or ECL8559 suspensions was inoculated in each well. Bacterial inocula were prepared from overnight cultures of *E. coli* ATCC 25922 and ECL8559 and were diluted in sterile saline solution (0.9%) standardized to a 0.5 McFarland standard. The bacterial cultures were then diluted one hundred-fold in Mueller Hinton broth and 100 μL of the final solution was added to each well of the 96-well plate within 10 min of inoculum preparation. In order to demonstrate the reproducibility of results,