Cephalosporin susceptibility of Staphylococcus aureus *strains isolated from commercial rabbit and goat farms in Spain*

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Summary
Antimicrobial drug resistance is an important problem that challenges veterinary clinicians to provide effective treatments without further spreading this resistance to other animals and people. The most commonly used pharmacodynamic parameter to define potency of antimicrobial drugs is minimum inhibitory concentration (MIC). The aim of this study was to evaluate the antibiotic susceptibility of thirty-six strains of *Staphylococcus aureus* isolated from dairy goats with mastitis and rabbits with chronic staphylococcosis. Four cephalosporins were tested: cephalexin, cephalotin, cefonicid and ceftiofur. MIC tests were performed according to the microdilution broth method. The calculated values of sensitivity in goats and rabbits were 66.67% and 72.22% for cephalexin, 72.22 % and 94.44% for cefonicid, 77.78% and 94.44% for cephalotin and 77.78% and 100% for ceftiofur, respectively. For all antibiotics, MIC_{90} of *S. aureus* from rabbits were lower than MIC_{90} from goats. These data suggest that more antibiotics are used in goat milk production than in rabbit farming. According to MIC values obtained in this study, ceftiofur and cephalotin may be the best option for treating *S. aureus* infections in lactating goats. For rabbits, ceftiofur showed lowest MIC values, but cephalosporins can produce fatal diarrhoea in this species, therefore additional studies are needed to evaluate the effects of repeated ceftiofur administration on microflora of rabbits before recommending the use of this antibiotic in this species.

Staphylococcus aureus is one of the most important pathogenic *Staphylococcus* species in veterinary medicine. In rabbits, it mainly causes skin infections, pododermatitis and mastitis with subsequent economic losses in industrial rabbitries (Hermans *et al.* 1999). In goats, *Staphylococcus aureus* is probably the most important infectious agent (Mørk *et al.* 2005), since it causes a chronic and deep infection in the mammary glands which implies an evident economic loss in relation to the decrease in milk production and health status of animals (Seegers *et al.* 2003).

Cephalosporins are a broad group of antimicrobial agents with a bactericidal mechanism of action similar to all β-lactam antibiotics inhibiting cell-wall synthesis. In accordance with their antimicrobial spectrum, they are divided into first (cephalexin, cephalothin) second (cefonicid), third (ceftiofur) and fourth generation compounds displaying a variable activity *in vitro*, structural similarities and the time of their introduction into the market (Caprile 1988). From first to fourth generation, the spectrum of activity against gram negative organisms and the stability against β-lactamase increase commonly together with the same or reduced spectrum of activity against gram positive organisms, except for fourth generation agents, which have enhanced activity against gram positive organisms (Papich and Lindeman 2018). Cephalotin (CL) and cefonicid (CD) are developed for use in human medicine, cephalexin (CX) for use in human and veterinary medicine and ceftiofur (CR) for exclusive use in animals and approved for use in food animals in Europe.

According to the European Medicines Agency (EMA), antibiotics are classified based on the potential consequences for public health of increased antimicrobial resistance due to their use in animals and depending on the necessity to use them in veterinary medicine. In the EMA categorization, there are four categories, A (Avoid), B (Restrict), C (Caution), and D (Prudence), with the latter as the lowest risk category (EMA 2019). Antibiotics from the A category are reserved for human treatment only, and their use is not allowed in food-producing animals. B category antibiotics (as third and fourth-generation cephalosporins) are critically important antimicrobials, and should be used only as a last option after susceptibility testing has been conducted when no other antibiotic would be clinically effective. C category antibiotics (as first and second-generation cephalosporins) should be considered only when there are no antibiotics in D Category that could be clinically effective. Because of this, the antibiotic consumption in animal production should be progressively reduced in order to decrease the antimicrobial resistance against the antibiotics to which the bacteria have been exposed. Thus, the purpose of this study was designed to evaluate the degree of *in vitro* activity of CD, CX, CL and CR against 36 *S. aureus* strains, isolated from rabbits with chronic staphylococcosis and goats with clinical mastitis in Spain.

A number of 18 *S. aureus* strains were isolated from rabbit farms with mastitis, subcutaneous abscesses and pododermatitis in the southeast region of Spain. Similarly, 18 *S. aureus* strains were isolated from milk of goats with clinical mastitis. The plates were incubated aerobically at 37 °C to be examined at 24, 48, and 72 h. Bacteria with target hemolysis were identified (presumptive) by catalase test and Gram staining. Commercial latex agglutination kit (Staphytec Plus, Oxoid) was used by specific identification of *S. aureus*. The strains were stored at - 80 °C in a nutrient broth enriched with 15% glycerol.

The antibiotics selected for the study were provided by manufacturers in this way: CR and CL by Sigma-Aldrich (ST. Louis, USA), CX by Tokyo Chemical Industry (Tokyo, Japan) and CD by Santa Cruz Biotechnology (Heidelberg, Germany). According to the Clinical and Laboratory Standards Institute's recommendations, antibiotic stock solutions were prepared at concentrations of 1,280 mg/L by dissolution in suitable solvents and then diluted in sterile distilled water (CLSI 2015). Minimum inhibitory concentration (MIC) tests were performed according to the microdilution broth method recommended by the CLSI (CLSI 2015) using U-bottomed, 96-well microtiter plates. Serial two-fold dilutions of the antimicrobial agents were prepared starting from the stock solution of each drug (concentrations ranging from 0.03 to 128 mg/l).

Inocula were prepared by diluting an overnight Mueller-Hinton Broth culture in buffered saline solution to a density of 0.5 McFarland turbidity scale and finally diluting again 40-fold before testing.

The MIC was defined as the lowest concentration at which bacterial growth was completely inhibited, that is the lowest concentration of an antimicrobial agent that prevents visible growth of a microorganism in a broth dilution susceptibility test (CLSI 2015). The MIC values of these drugs that inhibited 50% and 90% of the isolates were expressed as MIC_{50} and MIC_{90}, respectively. In relation to breakpoints of susceptibility and resistance, a strain was considered resistant when the MIC was ≥ 8 mg/L for CD, CX, CL and CR (CLSI 2018).

Reference strains of *S. aureus* (ATCC 29213) and *E. coli* (ATCC 25922) were used as controls for each plate. In this case, all results for *S. aureus* ATCC 29213 and *E. coli* ATCC 25922 were within the accepted range (CLSI 2018).

The specific MIC values for each selected antimicrobial agent are showed in Table I, where also MIC_{50} and MIC_{90}, and the minimum and maximum MIC values (range) are included. In goats, it was

Table I. *Frequency of* Staphylococcus aureus *strains in relation to their minimal inhibitory concentrations and MIC_{50}, MIC_{90}, and MIC range values of cefonicid, ceftiofur, cephalotin and cephalexin for rabbits (n = 18) and goats (n = 18).*

Antibiotics	Animals	Number of strains with MIC (mg/L)													MIC_{50} (mg/L)	MIC_{90} (mg/L)	MIC range (mg/L)
		0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	128			
Cefonicid	Goats	-	-	-	-	1	3	3	6	1	-	-	-	4	4	128	0.5-128
	Rabbits	-	-	-	1	-	3	5	8	-	-	-	-	1	2	4	0.25-128
Ceftiofur	Goats	-	-	-	-	3	8	3	-	-	2	1	1	-	1	32	0.5-64
	Rabbits	-	-	-	1	3	11	2	1	-	-	-	-	-	1	2	0.25-4
Cephalotin	Goats	-	-	3	7	4	-	-	-	-	1	2	-	1	0.25	32	0.12-128
	Rabbits	-	-	2	10	4	-	-	1	-	1	-	-	-	0.25	4	0.12-16
Cephalexin	Goats	-	-	-	-	-	1	3	8	2	-	-	2	2	4	128	1-128
	Rabbits	-	-	1	-	-	-	2	10	4	-	-	1	-	4	8	0.12-64

observed four resistant strains for CR and CL each, five for CD and six for CX. In contrast, the number of resistant strains in rabbits was considerably lower, one resistant strain for CD and CL each, zero for CR and five resistant strains for CX were found. The calculated values of sensitivity in goats and rabbits were 66.6% and 72.2% for CX, 72.2 % and 94.4% for CD, 77.7% and 94.4% for CL and 77.7% and 100% for CR, respectively. The lowest MIC_{90} were obtained in goats for CR and CL (MIC_{90} = 32 mg/L). Instead in rabbits, CR showed a lower value (MIC_{90} = 2 mg/L) than CD (MIC_{90} = 4 mg/L), CL (MIC_{90} = 4 mg/L) and CX (MIC_{90} = 8 mg/L). Indeed, for all antibiotics, MIC_{90} of *S. aureus* from rabbits were lower than MIC_{90} from goats. These evidences suggest that more antibiotics are used in goat milk production than in rabbit farming.

There are reduced reported data on cephalosporin susceptibility of *S. aureus* isolated from milk of goats with subclinical or clinical mastitis. For CL, our data were higher than those obtained in a previous study (MIC_{50} = 0.25 mg/l, MIC_{90} = 0.25 mg/l, range 0.06-128 mg/l, Virdis *et al.* 2010). There are no reported data with CD, CR and CX in *S. aureus* isolated from goats. However, published research papers on cephalosporin susceptibility of *S. aureus* isolated from bovine milk are numerous. In these studies, reported MIC values were lower than those obtained in this research for CR (MIC_{50} = 1 mg/L, MIC_{90} = 1 mg/L, range 0.06-64 mg/L, de Oliveira *et al.* 2000; MIC_{50}= 1 mg/L, MIC_{90} = 1 mg/L range 0.25-8 mg/L, De Jong *et al.* 2015), CX (MIC_{50} =2 mg/L, MIC_{90} = 4 mg/L, range 0.25-64 mg/L, De Jong *et al.* 2015) and CL (MIC_{50} = 0.25 mg/L, MIC_{90} = 0.5 mg/L, Lindeman *et al.* 2013). Cefonicid has not been studied in veterinary medicine, but susceptibility tests for *S. aureus* from human isolates showed values of MIC_{50} = 4 mg/l and MIC_{90} = 8 mg/l (Barry *et al.* 1986). There are no reported MICs for four cephalosporins tested in this study against isolated bacteria from rabbits. However, the obtained values of MIC_{90} were high compared to the cefiofur and cephalexin breakpoints [≤ 2 µg/mL (susceptible), 4 µg/mL (intermediate), and ≥ 8 µg/mL (resistant)] reported for other bacterial species isolated from other animals (De Jong *et al.* 2014, Papich and Lindeman 2018).

S. aureus affects rabbits producing important dermal lesions and even invading subcutaneous tissues, resulting in suppurative dermatitis, mastitis, multisystemic abscessation and pododermatitis (Segura *et al.* 2007). In addition, *S. aureus* is probably the most significant infectious agent that can cause mastitis in cows, goats, and sheep, because it produces a chronic and deep infection in the mammary glands that is extremely difficult to treat (Mørk *et al.* 2005, Aires de Sousa *et al.* 2007). Due to antibiotic resistance, treatment of infections caused by Gram-positive bacteria is becoming a difficult problem. The situation is particularly critical in infections caused by *S. aureus* which is also frequently resistant to multiple groups of antibiotics (Livermore 2000). One of the strategies proposed for the control of *S. aureus* infections is the re-evaluation of older antimicrobial agents and develop them for using against this type of microorganisms with high degrees of resistance (Chopra *et al.* 1997). Therefore, first and second generation of cephalosporins and ceftiofur have become a good option for the treatment of infectious diseases in veterinary medicine. The ratio T > MIC is the best parameter for predicting the antimicrobial effect of time-dependent antimicrobial agents as β-lactam antibiotics. It has been previously determined that the maximal bactericidal action for cephalosporins is reached when plasma concentrations are greater than the MIC of the pathogen for 60-70% of the dosing interval and a bacteriostatic effect is observed when T > MIC is 30-40% of the dosing interval (Drusano 2004).

Distribution of cephalosporins to the mammary gland and the consequent access to milk is limited. Pharmacokinetic parameters of these four cephalosphorins in lactating goats (Lacchini *et al.* 2007, Doré *et al.* 2011, Fernández-Varón *et al.* 2016, Badillo *et al.* 2020) after different routes of administration showed a low elimination through the milk. Therefore, the treatment of mastitis in goats with these cephalosporins should be performed by intramammary administrations. According to MIC values obtained in the present study, CR and CL may be the best options for treating *S. aureus* infections in these species due to their lowest MIC values.

Considering MIC values obtained in this study and pharmacokinetic parameters in rabbits (Dzierzanowska *et al.* 1982, Meneses *et al.* 2013, Gardhouse *et al.* 2017) after different routes of administration, CR could be an option for treating *S. aureus* infections on account to the lowest MIC value, high plasma levels and long half-life showed in this species. However, CR (B category) should be used only as a last option after susceptibility testing has been conducted when no other antibiotic would be clinically effective. Moreover, cephalosporins can disrupt the caecal microflora in rabbits (because of biliary elimination of some members of this group) resulting sometimes in fatal diarrhoea. Although single-dose parenteral administration of CR resulted in minimal adverse effects (Gardhouse *et al.* 2017), additional studies are needed to evaluate the effects of repeated CR administration in rabbits and to develop and implement appropriate specific dosing regimens that can maximize its clinical efficacy for use in production animals and reduce the risk of selection of resistant pathogens.

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