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Cefquinome sulfate behavior after intramammary administration in healthy and infected cows

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ABSTRACT

Maintenance of adequate drug concentration at the site of infection is an important problem in mastitis antibiotic therapy, and the efficacy of intramammary β-lactams can be optimized by maintaining the drug concentration at the site of infection above the minimum inhibitory concentration (MIC) as long as possible. The most important pharmacokinetic and pharmacodynamic parameter for efficacy evaluation is time during which drug concentrations exceed the MIC ($t >$ MIC). In this study, we assessed the pharmacokinetic profile of cefquinome (CFQ) after repeated intramammary administration in healthy cows and cows subclinically infected with *Staphylococcus aureus* as well as the MIC of *Staph. aureus* field strains. In addition, the degree of drug passage was investigated from udder to bloodstream by measuring systemic drug absorption in healthy and infected animals. Cefquinome concentrations were quantified by HPLC (UV-visible detection) in milk samples collected from quarters and from blood serum samples. The systemic drug absorption was negligible in healthy and subclinically infected animals (maximum concentration 0.09 ± 0.02 and 0.1 ± 0.01 μg/mL in healthy and subclinically infected animals, respectively). The MIC_{90} value for CFQ in *Staph. aureus* field strains (n = 20) was 0.24 μg/mL. The pharmacokinetic and pharmacodynamic evaluation, determined by $t >$ MIC, showed an equal persistence of CFQ in all quarters, indicating an equivalent activity of the drug regardless of the pathological status of the udder. Moreover, with literature data regarding CFQ MIC, the $t >$ MIC has been calculated for other bacterial species.

Key words: cefquinome sulfate, lactating intramammary treatment, minimum inhibitory concentration

INTRODUCTION

The success of antimicrobial therapies depends on various factors, such as the location and susceptibility of microorganisms, the animal's health, and the characteristics of the administered drug (Barragry, 1994).

This study was part of a more extensive research aiming to assess the influence of the mammary health status on the pharmacokinetic (**PK**) parameters of intramammary (**IMM**)-administered cephalosporins. In the paper by Cagnardi et al. (2010), concerning a third-generation cephalosporin (cefoperazone), we described the drug kinetic behavior in healthy and in subclinically infected lactating cows after a single IMM administration. In the present paper, we continue the investigation about cefquinome (**CFQ**), a fourth-generation cephalosporin. Cefquinome is approved only for veterinary use and it is highly stable to β-lactamases. Because of its broad spectrum, it has highly, moderate, and good activity against *Enterobacteriaceae*, *Pseudomonas aeruginosa*, and staphylococci species, respectively. In lactating cows it is used IMM for the treatment of clinical coliform and other bacterial mastitis (*Streptococcus uberis*, *Streptococcus dysgalactiae*, *Staphylococcus aureus*; Shpigel and Schmid, 1997; CVMP, 2003; Prescott, 2006) as indicated in the label of the commercial product (Cobactan L, Intervet, Milan, Italy; Summary of Product Characteristics). Cefquinome, like other fourth-generation cephalosporins, possesses improved antibacterial activity compared with second- and third-generation cephalosporins and is highly effective against gram-negative bacteria. Thus, CFQ is effectively used for the therapy of acute mastitis sustained by *Escherichia coli* (Shpigel et al., 1997); nevertheless, clinical and in vitro evidence suggests that CFQ may also be of value in the treatment of *Staph. aureus* mastitis (Limbert et al., 1991; Böttner et al., 1995; Guerin-Faublée et al., 2003; Shpigel et al., 2006).

Because cephalosporins act as time-dependent antimicrobials, the most appropriate pharmacokinetic/pharmacodynamic (**PK/PD**) parameter to describe drug efficacy is the time during which the drug's concentration exceeds the MIC (***t* > MIC**; McKellar et

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al., 2004; Wagner and Erskine, 2006). During the present study, to optimize the CFQ dosage regimen, we assessed the MIC on *Staph. aureus* and calculated the $t >$ MIC. To complete the investigation, literature data about CFQ MIC were correlated to the observed drug concentrations and $t >$ MIC for other bacterial species are proposed.

Generally, weak acids (such as β-lactam antibiotics) penetrate the blood–mammary barrier poorly, due to the degree of ionization at physiological pH. In an in vitro study, Ehinger et al. (2006) demonstrated that CFQ did not diffuse into simulated systemic circulation after IMM administration. As in our previous study on cefoperazone (Cagnardi et al., 2010), systemic absorption of CFQ after IMM administration was investigated here, as were possible differences between healthy and subclinically infected udders due to changes in vascular permeability caused by IMI (Gehring and Smith, 2006).

Therefore, the aims of this study were to (1) assess the PK parameters after repeated IMM administration of CFQ in healthy and *Staph. aureus*-infected cows; (2) calculate the PK/PD parameters on different bacterial species; and (3) investigate the passage of CFQ through the blood–mammary barrier.

MATERIALS AND METHODS

Animal Selection

One hundred forty milking cows from a farm in northern Italy were tested for *Staph. aureus*, and 40% were infected. Twelve lactating cows (Italian Frisona), 600 to 750 kg of BW (670 ± 53 kg), in first or second lactation were selected. All cows were provided with water ad libitum and a drug-free TMR, according to their nutrient requirements (NRC, 2001). At the onset of their use, the animals were between 50 and 150 DIM (105 ± 39 d). The cows were milked twice daily in an 8 + 8 milking parlor. Milk production was 44.1 ± 7.6 L/d (range from 30 to 55 L/d).

Preliminary Bacteriology

The bacteriological sampling and testing were carried out as reported in Cagnardi et al. (2010). Infection status was defined according to the procedures recommended by National Mastitis Council (NMC, 1999), and SCC was determined for each sample by using an automated fluorescent microscopic somatic cell counter (Bentley Somacount, Bentley Instruments, Chaska, MN). Based on the presence or absence of *Staph. aureus* infection in quarters, cows were divided into healthy (**H**) and subclinically infected (**SI**) groups. Positivity for *Staph. aureus* was monitored for 3 wk during the following period by subsequent samplings in selected cows belonging to the groups.

MIC Determination

Twenty isolates from infected quarters (n = 13) and from other animals belonging to the same farm but not included in the study (n = 7) were tested for antimicrobial susceptibility to CFQ by the determination of MIC according to the microdilution broth method as recommended by NCCLS (2002). Cefquinome sulfate with purity grade of 80.1% (Intervet, Milan, Italy) was dissolved and diluted in sterile distilled water. Isolates were prepared as described by Cagnardi et al. (2010).

Treatment and Sampling

After accurate milking and teat disinfection, each quarter of each cow was administered every 12 h with 75 mg IMM of CFQ sulfate (Cobactan L, Intervet, Milan, Italy) for 3 consecutive treatments. All quarters were administered CFQ, regardless of infection status, to reach the maximum dose (300 mg of CFQ per animal each 12 h).

Milk samples were collected by hand separately from individual teats before (**t0**) and after last drug administration (2, 8, and 12 h). Then, further samples were collected every 12 h until the 10th milking. Cistern milk samples were collected after discarding the first streams of milk. Differences in milk pH due to drug administration or infection status of the udder were recorded at t0 and 0.5 h milk samples at room temperature.

Blood samples were collected from the jugular vein at t0 (before drug administration), after each drug administration at 2, 4, 8, and 12 h, and after the last treatment at 24, 36, and 48 h. Subsequently, samples were centrifuged (1,500 × *g*, 10 min at room temperature) to obtain serum and stored at −20°C pending assay.

Sample Extraction and HPLC Analyses

Cefquinome was extracted from milk and blood samples and the residues were analyzed by HPLC as reported by Cagnardi et al. (2010). To the authors' knowledge, no published paper has reported the existence of active CFQ metabolites in milk, and thus their presence was not evaluated.

Intralaboratory Validation of Analytical Method

The calibration curves were made in blank bovine milk and serum, diluting the original CFQ stock solution (1 mg/mL) to obtain final concentrations of CFQ ranging from 0.01 to 40 μg/mL. The HPLC method

was validated for both matrices and was specific, linear (range 0.01 to 40 μg/mL for both matrices), precise (CV from 2.2 to 7.6% in milk and from 1.8 to 5.7% in serum), and accurate (between +0.4% and –9.95% in milk and between +9.5% and –0.4% in serum). The lower and upper limits of quantification were 0.01 and 40 μg/mL, respectively, for milk and serum. Samples with concentrations higher than the upper limit of quantification were quantified after dilution. The limit of detection was 0.001 μg/mL for both matrices.

Pharmacokinetic Analysis

As reported by Stockler et al. (2009a), no specific PK model is available to describe IMM kinetics. Thus, a noncompartmental analysis was carried out on milk and serum drug concentrations using the WinNonLin 5.2.1 software (model 201; Pharsight Corp., Mountain View, CA) to obtain comparable parameters after kinetic analyses of the concentration–time profile in both matrices. Mean residence time (MRT) was determined from the following equation (Gibaldi and Perrier, 1982):

$$\text{MRT} = \text{AUMC}/\text{AUC},$$

where AUMC is area under the moment curve and AUC is area under serum or milk concentration–time curve.

Statistical Analysis

Differences were investigated between healthy and infected animals. Unpaired *t*-test with Welch correction (variances unequal) was performed on elimination half-life, maximum concentration, AUC_{last} and MRT_{last}, where last = calculated from 0 to last time point, and on pH values before and after treatment (InStat 3.0 GraphPad, La Jolla, CA). The ANOVA test was performed to evaluate differences among groups of quarters. In both tests, differences with $P < 0.05$ were considered significant.

RESULTS

Drug quantification was carried out in samples from single quarters, thus results from group H were identified as healthy quarters (**HQ**, n = 24), whereas results from group SI were divided into infected quarters (**IQ**, n = 13) and suspected quarters (**SQ**; i.e., healthy quarters of infected animals, n = 11).

Cefquinome was detected in milk until 108 h (ninth milking) in IQ and HQ and until 96 h (eighth milking) in SQ (Figure 1). Serum concentrations were variably quantifiable with low values in all animals (Figure 2). In serum of healthy subjects, CFQ was detectable in all animals until 32 h and in only 1 subject until 60 h. In infected cows, all animals showed quantifiable concentrations until 48 h and in 4 subjects until 72 h.

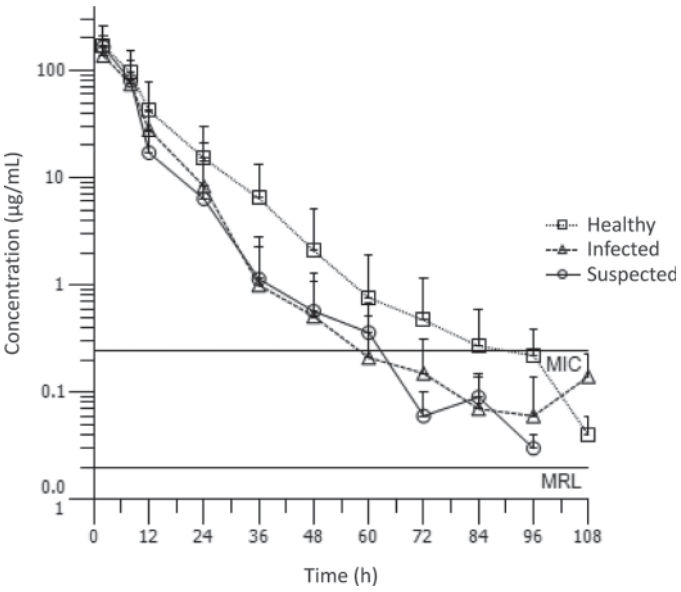

Figure 1. Milk concentration in healthy (HQ), infected (IQ), and suspected quarters (SQ) after the last administration of cefquinome at the dose of 75 mg/quarter plotted with MIC (0.24 μg/mL) and maximum residual limit (MRL; 0.02 μg/mL).

Mean milk and serum PK parameters are shown in Table 1. The kinetic analysis in serum was carried out on data after the last treatment. Mean milk pH values measured at t0 and 0.5 h after first treatment are shown in Table 2. The drug inhibitory concentrations

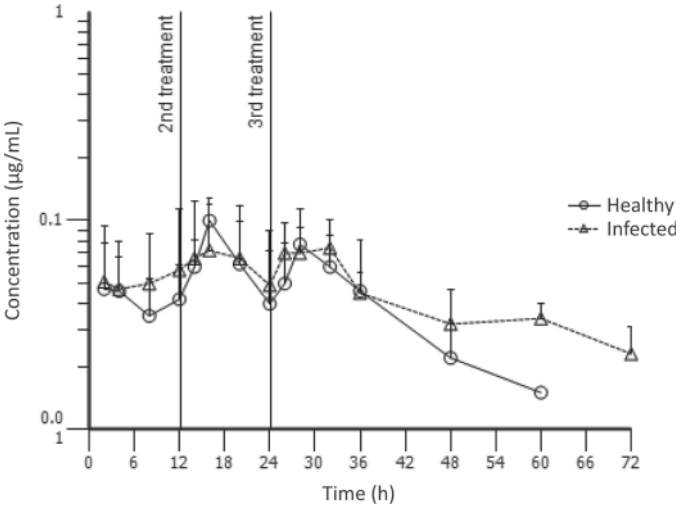

Figure 2. Mean serum concentration (±SD) in healthy and infected animals during intramammary administration of cefquinome at a dose of 75 mg/quarter each 12 h. Vertical lines indicate second and third treatment (first treatment corresponds to time 0).

Table 1. Mean (±SD) milk and serum pharmacokinetic parameters after intramammary administration of cefquinome in healthy (HQ), infected (IQ), and suspected quarters (SQ) in healthy and infected animals

Parameter[1]	Healthy quarter (n = 24)	Infected quarter (n = 13)	Suspected quarter (n = 11)
Milk			
$t_{1/2\lambda z}$ (h)	6.21 ± 3.95	4.20 ± 2.48	4.43 ± 2.1
T_{max} (h)	2*(SQ) ± 0	2*(SQ) ± 0	3.2*(HQ; IQ) ± 2.68
C_{max} (μg/mL)	171.89 ± 41.01	137.77 ± 48.34	168.34 ± 89.55
AUC_{last} (h·μg/mL)	1,592.99 ± 673.4	1,201.79 ± 425.53	1,242.62 ± 618.81
AUC_{inf} (h·μg/mL)	1,780.74 ± 832.57	1,337.78 ± 669.64	1,320.75 ± 704.78
$AUMC_{last}$ (h·h·μg/mL)	11,436.07 ± 7,628.29	16,413.12*(SQ) ± 8,006.82	7,114.22*(IQ) ± 4,330.01
MRT_{last} (h)	6.58 ± 2.05	6.39 ± 1.47	5.79 ± 2.12
$t > MIC$ (h)	54 ± 34	42 ± 18	43 ± 16

Serum	Healthy cow (n = 6)	Infected cow (n = 6)
$t_{1/2\lambda z}$ (h)	11.43 ± 7.09	17.3 ± 7.7
T_{max} (h)	29.50 ± 3.00	29.5 ± 3.00
C_{max} (μg/mL)	0.09 ± 0.02	0.1 ± 0.01
AUC_{last} (h·μg/mL)	1.07 ± 0.50	2.39 ± 1.07
$AUMC_{last}$ (h·h·μg/mL)	9.54§ ± 6.76	58.46§ ± 33.75
MRT_{last} (h)	8.31 ± 3.10	14.72 ± 8.93

[1]$t_{1/2\lambda z}$ = elimination half-time; T_{max} = observed time for C_{max}; C_{max} = maximum drug concentration; AUC_{last} = area under milk/serum concentration-time curve; AUC_{inf} = area under the concentration-time curve from 0 to infinity; $AUMC_{last}$ = area under the moment curve; MRT_{last} = mean residence time; $t > MIC$ = time during which drug concentrations exceeded the MIC.

*Statistically significant ($P < 0.05$) difference between quarters (ANOVA); letters in parentheses show groups.

§Statistically significant ($P < 0.05$) difference between healthy and infected animals (*t*-test Welch correction).

toward *Staph. aureus* field strains (n = 20) ranged from 0.12 to 0.48 μg/mL; the calculated MIC_{90} was 0.24 μg/mL. The $t > MIC$ values (Table 1) in milk were 54 ± 34 h, 42 ± 18 h, and 43 ± 16 h in HQ, IQ, and SQ, respectively ($P > 0.05$).

The MIC_{90} collected from literature data on various bacterial strains and the calculated $t > MIC$ are reported in Table 3. Considering MIC_{90} and $t > MIC$, the activity recorded against *Staphylococcus* spp. and *Staph. aureus* was relatively poor.

DISCUSSION

Fourth-generation cephalosporins are valuable extended-spectrum drugs for treatment of serious human infections and not first-choice antimicrobial agents in animals. To limit the selection of resistant bacteria, fourth-generation cephalosporins should be reserved for use where susceptibility testing indicates that alternatives are not available (Prescott, 2006; FDA, 2008). Cefquinome is developed solely for veterinary use and is licensed in Europe but not in the United States.

Pharmacokinetic studies are usually carried out in healthy animals. However, drug behavior in unhealthy animals could be modified by various factors, as might occur, for example, during subclinical IMI in cows. Therefore, in this study, we investigated IMM administration in healthy cows and in cows infected with *Staph. aureus*. The subclinical stage was preferred to an acute IMI phase, because serious gland modifications could affect results in an acute infection.

When planning the study, we supposed that SQ could be considered as control quarters for infected animals and thus were considered as a separate group; conversely to what we supposed, similar profiles in the different pathological conditions (HQ, IQ, and SQ) and a negligible influence of the udder environment were observed in kinetics of CFQ after IMM treatments.

After administrations, CFQ was generally quantifiable in milk for 108 h in HQ and IQ and for 96 h in SQ. Some evidence suggests that cows with low milk production eliminate IMM drugs more slowly than cows with higher production (Gehring and Smith, 2006). In this study, as observed in a similar study with cefoperazone (Cagnardi et al., 2010), no correlation was

Table 2. Mean (±SD) milk pH in healthy (HQ), infected (IQ), and suspected quarters (SQ) before (t0) and after the first treatment (0.5 h)

Time	HQ (n = 24)	IQ (n = 13)	SQ (n = 11)
t0	6.98*(IQ) ± 0.09	6.91§*(HQ) ± 0.07	6.92§ ± 0.05
0.5 h	6.96 ± 0.18	7.02§ ± 0.15	7.01§ ± 0.12

*Statistically significant ($P < 0.05$) difference between quarters (ANOVA); letters in parentheses show groups.

§Statistically significant ($P < 0.05$) difference between healthy and infected animals (*t*-test Welch correction).

Table 3. Minimum inhibitory concentrations (MIC_{90}) of cefquinome against common mastitis-causing bacteria from Europe and North America between 1990 and 2002 (Shpigel and Schmid, 1997; Schmid and Thomas, 2002; Ehinger et al., 2006) and the calculated $t >$ MIC (time during which drug concentrations exceeded the MIC)

Species (n)	MIC_{90} (μg/mL)	$t >$ MIC (h)
Streptococcus spp. (759)	0.25 to 0.5	46–54
Streptococcus agalactiae (281)	<0.03 to 0.13	94–64
Streptococcus dysgalactiae (404)	<0.008 to 0.25	94–54
Streptococcus uberis (529)	<0.03 to 0.25	94–64
Enterococcus spp. (127)	1.0 to >4.0	44–28
Staphylococcus spp. (580)	0.5 to 2.0	46–32
Staphylococcus aureus (229)	0.5 to 1.0	46–44
Actinomyces pyogenes (43)	1.0	44
Enterobacteriaceae (323)	0.13	64
Escherichia coli (637)	0.06 to 0.13	72–64
Klebsiella spp. (31)	0.13	64
Pseudomonas spp. (55)	>4.0	28

observed between milk production and drug elimination after IMM administration.

Residue depletion studies have demonstrated that CFQ can be detected in kidney at 24 h after IMM administration (CVMP, 1995), thus confirming the systemic absorption of the drug. Drug amounts in serum samples were low in healthy and infected animals with a maximum concentration (Cmax) of approximately 0.1 μg/mL in both groups (Table 1). In healthy animals, serum concentrations were detected for a short time (i.e., in only 1 subject until 60 h). In infected cows, serum concentrations were more persistent and in 4 subjects the drug was still quantifiable 72 h after administration (Figure 2). However, considering the kinetic results and, in particular, the parameter of the total exposure of the body to the drug (as AUC), no differences were observed and thus a negligible influence of udder status in the absorption of CFQ could be demonstrated in our animals.

Considering milk pH (Table 2), our results are not straightforward. Unexpectedly, a more acidic milk was observed in IQ ($P < 0.05$) at t0, and the behavior of milk pre- and posttreatment in SQ and IQ was similar, supporting the finding, documented by Oshima and Yoshida (1988) and Holdaway et al. (1996), that pH is not a good indicator of mastitis. The slight acidification we expected after IMM administration was not observed in HQ; on the contrary, a slight basification ($P < 0.05$) was reported in the other quarters (IQ and SQ). The reason for these changes is not completely clear and because the pH evaluation was conducted only after the first treatment, we are not able to estimate the influence of the 2 further treatments on milk pH, even though a possible milk acidification could be supposed. As stated above, in our SI cows, no differences in absorption were observed, and milk pH and CFQ acid dissociation constant ($pK_a = 2.51$ and 2.91) did not seem to influence drug diffusion from milk to serum (Gehring and Smith, 2006). On the contrary, in the study with cefoperazone, slightly higher absorption was observed in infected animals and attributed to the chronic alteration of mammary membrane due to subclinical infections (Cagnardi et al., 2010).

This relevant finding could be important when considering that withdrawal times are generally established in healthy animals and, in the case of CFQ, correspond to 4 and 5 d for meat and milk, respectively. Because no differences were observed in systemic CFQ absorption in healthy and infected animals and in milk kinetics among HQ, IQ, and SQ, we can confirm the validity of these withdrawal times for infected animals also.

The MIC_{90} value of CFQ for *Staph. aureus* field strains was low (0.24 μg/mL) and, as shown in the Figure 1, milk drug concentrations were maintained above the MIC for a long period. The elimination half-lives in milk from all groups of quarters were similar: 6.21 ± 3.95 h in HQ, 4.20 ± 2.48 h in IQ, and 4.43 ± 2.1 h in SQ ($P > 0.05$), and the calculated $t >$ MIC were comparable: 54 h in HQ, 42 h in IQ, and 43 h in SQ ($P > 0.05$). This means that in all quarters the microorganisms were exposed to drug activity for a long period and indicated good efficacy of CFQ against *Staph. aureus*. At the end of the study, the SI group animals were monitored for health conditions and degree of infection. The results were negative; that is, no *Staph. aureus* strains were isolated in milk samples collected from IQ, thus confirming the efficacy of CFQ treatment, even though CFQ is not the elective drug for subclinical mastitis therapy. The overall positive CFQ performance after IMM injection against *Staph. aureus* infections was also confirmed by Bradley and Green (2009). As reported by Shpigel et al. (2006), the cure rate was low after systemic administration of CFQ at drying-off in dairy cows affected by subclinical *Staph.*

aureus mastitis; thus, the IMM therapeutic scheme seems to be more appropriate.

As reported by Stockler et al. (2009b), milk fraction seems to influence the PK and residue depletion of cephapirin, and bucket milk seems to be the best sample, because it reflects the whole condition of the mammary gland. In our study, we collected cisternal milk and it would be interesting to evaluate if this choice could have influenced our PK and antimicrobial results.

The MIC_{90} collected from literature data on various bacterial strains and the $t >$ MIC calculated are reported in Table 3. These data confirm the good activity of CFQ, particularly against *Strep. agalactiae*, *Strep. dysgalactiae*, *Strep. uberis*, *E. coli*, and *Enterobacteriaceae*. It should be noted that the standard procedure for MIC determination uses Mueller-Hinton broth. Constable and Morin (2003) stated that the presence of milk during in vitro MIC determination could markedly decrease the activity of antibiotics, mainly due to drug protein or lipid binding or to the difference in pH between Mueller-Hinton broth and milk. Thus, milk, and particularly milk collected from IQ, would most likely represent the best test medium to investigate the efficacy of drugs administered IMM.

CONCLUSIONS

The CFQ sulfate product administered every 12 h IMM in every quarter at the maximum approved dose of 75 mg of CFQ per quarter did not cause any adverse effects and was well tolerated. Systemic CFQ absorption was comparable in healthy and infected animals. The MIC_{90} value of CFQ for *Staph. aureus* field strains was low (0.24 μg/mL). The 3 IMM administrations of CFQ in H and SI animals showed a similar $t >$ MIC in all quarters (54, 43, and 42 h in HQ, SQ, and IQ, respectively), indicating that *Staph. aureus* strains were exposed to antibiotic activity for a long time in all animals. At the end of our study, all the SI animals were negative for *Staph. aureus*, confirming the validity of dosage scheme adopted.

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