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# Successful treatment of recurrent subclinical mastitis in cows caused by enrofloxacin resistant bacteria by means of the sequential intramammary infusion of enrofloxacin HCl-2H<sub>2</sub>O and ceftiofur HCl: a clinical trial

Edgar Alfonseca-Silva <sup>1</sup>, Juan Carlos Cruz-Villa <sup>1</sup>, Lilia Gutiérrez <sup>2</sup>, Hector Sumano <sup>2,\*</sup>

<sup>1</sup>Department of Microbiology and Immunology, School of Veterinary Medicine, National Autonomous University of Mexico, Mexico City 04510, Mexico

<sup>2</sup>Department of Physiology and Pharmacology, School of Veterinary Medicine, National Autonomous University of Mexico, Mexico City 04510, Mexico

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\*Corresponding author:

Hector Sumano

Department of Physiology and Pharmacology,  
School of Veterinary Medicine, National  
Autonomous University of Mexico, Avenida  
Insurgentes Sur 3000, Mexico City 04510,  
Mexico.

E-mail: sumano@unam.mx


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cited.

ORCID iDs

Edgar Alfonseca-Silva 

<https://orcid.org/0000-0003-3274-9596>

Juan Carlos Cruz-Villa 

<https://orcid.org/0000-0001-6644-8180>

Lilia Gutiérrez 

<https://orcid.org/0000-0002-4823-0388>

Hector Sumano 

<https://orcid.org/0000-0002-8802-5274>

## ABSTRACT

**Background:** Recurrent subclinical mastitis (RScM) due to resistant bacteria has low clinical and bacteriological cure rates, often requiring the culling of cows. The sequential intramammary administration of enrofloxacin hydrochloride-dihydrate (enro-C) followed by ceftiofur HCl may be useful for treating these cases.

**Objectives:** This study assessed the bacteriological and clinical cure-efficacies of the sequentially intramammary administration of enro-C, followed by ceftiofur HCl to treat RScM in Holstein/Friesian cows.

**Methods:** This trial was conducted in a herd with a high prevalence of RScM, and 20 Holstein/Friesian cows were included: 45% suffering subclinical mastitis and 38.9% of the mammary quarters affected. Twenty-nine bacterial isolates *in vitro* resistant to enro-C were obtained (coagulase-negative *Staphylococcus* spp, 55.2%; *Staphylococcus aureus*, 27.6%; *Escherichia coli*, 6.9%; *Streptococcus uberis*, 6.9%; *Corynebacterium bovis*, 3.4%). Polymerase chain reaction-isolated the following genes linked to enro-C resistance: chromosomal (*gyrA*) and plasmid (*aac(6')-Ib-cr*). The treatments were as follows: twice-daily intramammary infusions of enro-C (300 mg/10 mL) for 5 days. Cows clinically considered treatment failures were also treated with intramammary ceftiofur (125 mg/10 mL, twice daily for 5 days). The clinical and bacteriological cure rates were carried out when completing each treatment phase and at 14 and 21 days, aided by a California mastitis test, somatic cell count, and failure to identify the initially causative bacteria.

**Results:** Enro-C achieved 65% clinical and bacteriological cure rates, and 100% cure rates were obtained after the rescue treatment with ceftiofur HCl.

**Conclusions:** Outstanding clinical and bacteriological cure rates in cows affected by RScM were achieved with the consecutive intramammary infusions of enro-C, followed by ceftiofur HCl.

**Keywords:** Fluoroquinolone; mastitis; bovine; bacterial-resistant

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### Conflict of Interest

The authors declare no conflicts of interest. The National Autonomous University of Mexico (UNAM), owner of the patent for enro-C, is open to licensing it.

### Author Contributions

Conceptualization: Sumano H, Alfonseca-Silva E, Gutiérrez L; Data curation: Alfonseca-Silva E, Gutiérrez L; Investigation: Sumano H, Alfonseca-Silva E, Cruz-Villa JC, Gutiérrez L; Methodology: Alfonseca-Silva E, Cruz-Villa JC; Project administration: Sumano H; Validation: Cruz-Villa JC; Writing - original draft: Sumano H, Alfonseca-Silva E; Writing - review & editing: Sumano H.

## INTRODUCTION

Bovine mastitis is caused mainly by pathogenic bacteria and occasionally by injury, allergic reactions, fungal infections, and other causes. The disease is the most prevalent and costly of the dairy industry because of the direct costs and for the reduction in milk production because a single episode of clinical mastitis can reduce milk production by 30% or more [1-3], with a negative impact on milk quality, i.e., high somatic cell count (SCC). Furthermore, mastitis increases the overall costs of herd health care and causes milk withholding and the premature culling of animals [4-6]. In Mexico, the loss of milk production in a herd can be as high as 30%, and an annual cost of USD 61.00 per cow per episode of mastitis has been estimated [1].

Clinical mastitis is characterized by a sudden onset, changes in the composition and appearance of the milk, decreased milk production, and signs of udder inflammation in the infected quarters. In contrast, recurrent subclinical mastitis (RScM) due to resistant bacteria does not present conspicuous signs, either in the udder or in the milk. On the other hand, milk production is reduced, and SCC increased [4,7,8]. This latter form of mastitis has been regarded as having a greater economic impact than the more recognizable clinical mastitis; it also has a higher prevalence and, in the long term, has a more deleterious effect on the economic viability and health of the herd [8]. The number of bacteria regarded as pathogens of RScM is increasing, and their former classification as contagious, environmental, or opportunistic pathogens is now imprecise [4,5].

Traditionally, antibacterial drugs are considered the treatment of choice for intramammary infections. On the other hand, their continuous use, and misuse, contributed to a noticeable decline in their efficacy [4,9]. As an evolutionary and adaptive process of bacterial pathogens, the appearance of bacterial resistance is constantly challenging the clinical introduction of new or pharmacological modified preparations of antimicrobial agents [10]. In this context, a new recrystallized enrofloxacin polymorphic form, enrofloxacin hydrochloride-dihydrate (enro-C) [11], has shown higher water solubility than the parent compound and superior bioavailability than the reference enrofloxacin in broiler chicken, dogs, and cows [12-14]. As a 1.5% suspension, enro-C exhibits a pH of 6.5, and it does not irritate the mammary gland when 300 mg of this drug are infused intramammary [15]. In addition, enro-C, pharmaceutically formulated for intramammary infusion, has high bacteriological and clinical efficacy in non-severe clinical mastitis [15]. This drug has not been tested as the sole intramammary treatment in cases of RScM in dairy cows, particularly against bacteria with resistance genes to fluoroquinolones. Considering the high concentration of enrofloxacin from enro-C in the 300 mg/10 mL infusions, which were proposed as the first treatment phase in this study, the concentrations achieved by this drug at the infection site could surpass the bacterial resistance found. In those cases, the treatment resulted in an ineffective rescue. Hence, a sequential treatment with ceftiofur HCl for another 5 days is proposed. Ceftiofur may contribute to the efficacy of the RScM treatment because of its direct effect [16] and its favorable association with fluoroquinolone derivatives [17].

## MATERIALS AND METHODS

### Animals

Study design and animal handling complied with the Mexican prescripts (NOM-062-ZOO-2001). This trial was implemented at a Holstein/Friesian dairy farm in the Agricultural

and Industrial Complex of Tizayuca SA, State of Hidalgo, Mexico, from January 2020 to July 2020. The chosen farm is within an industrial complex of several dairies with high bovine population densities and deficient hygiene and management approaches. Under these conditions, it was predictable that an acceptable number of RScM cases could be gathered and provide this trial with a more demanding challenge in evaluating the efficacy of enro-C alone or with the subsequent infusion of ceftiofur because chosen cows were cases of RScM, caused by fluoroquinolone-resistant bacteria. Overall, this trial included twenty Holstein/Friesian cows with a mean production of 30 L/day and 3–4 lactational periods. The absence of previous treatments with antibacterial drugs or any other drug for the last 21 days was ensured.

### Subclinical mastitis diagnosis

To detect subclinical cases of mastitis, the California mastitis test (CMT) was carried out before milking, following the protocol suggested by Schalm and Noorlander [18] and The National Mastitis Council-USA [19]. In addition, SCCs were obtained using the De Laval DCC device (De Laval DCC, Sweden), following the instructions provided by the manufacturer, and moments before milking after discarding 3 milk ejections.

### Bacteriological analyses

At this time, bacteriological analyses were obtained by milk sampling from each mammary gland according to the guidelines of the National Mastitis Council [19], and collecting it in sterile capped-Falcon tubes. The samples were kept at 4°C during transport to the laboratory and processed within 4 h. For analysis, the milk samples were incubated at 37°C for 30 min to detach bacteria from fat. Each sample was vortexed for 5 min to achieve a homogeneous mixture. The samples were seeded using the striatum technique on blood agar and MacConkey agar plates and incubated under aerobic conditions at 37°C for 24–48 h. Bacteria colonies were identified initially based on the results of gram staining and their morphological and physiological characteristics. The bacteria were then fully identified using the method described by Carter and Cole [20].

### Bacterial susceptibility to enro-C

The cows to be treated were selected based on the existence of bacterial resistance to enrofloxacin from enro-C. To that end, susceptibility tests to enro-C were performed in triplicate in all isolates, using a modification to the Kirby-Bauer method. Briefly, wells, 5 mm in diameter, were drilled into the Mueller-Hinton agar. Subsequently, 50 µL of enro-C aliquots were added at 2 concentrations, 0.2 µg/mL and 1.0 µg/mL. The control bacteria were *Escherichia coli*, ATCC-10536 (minimum inhibitory concentration [MIC] ≤ 0.2 µg/mL), and *Staphylococcus aureus* ATCC-25923 (MIC ≤ 1.0 µg/mL), which have a susceptible phenotype to fluoroquinolones. The interpretation of the inhibition halo for bacterial growth was carried out based on standardized patterns, which are equivalent to the MIC as laid out by the National Committee for Clinical Laboratory Standards.

### Molecular resistance to quinolones

The detection of genes for resistance to quinolones, whether from a plasmid (*aac(6)-Ib-cr*) or chromosomal (*gyrA*) origin, was carried out by extracting the total DNA from the bacterial cultures of each resistant bacteria, using cetyltrimethylammonium bromide-chloroform-isoamyl alcohol. The concentration and integrity of the DNA were measured by spectrophotometry (NanoDrop) over a reading range of 260–280 nm and 1% agarose gel electrophoresis. The DNA samples were standardized at 40 ng/µL. The quinolone resistance genes were detected by polymerase chain reaction (PCR) using a MyTaq DNA Polymerase kit

(Bioline; Meridian Life Science, USA). The utilized primers were F- TAC ACC GGT CAA CAT TGA GG and R- TTA ATG ATT GCC GCC GTC GG (648 bp) [21] for the *gyrA* gene, and F- TTG CGA TGC TCT ATG AGT GGC TA and R- CTC GAA TGC CTG GCG TGT TT (482 bp) [22] for the *aac(6')-Ib-cr* gene. The *groEL* gene was used as an internal control.

The PCR conditions were as follows: activation 95°C for 2 min, followed by 35 cycles of denaturation, 95°C for 45 sec; alignment, 62°C for 30 sec; amplification, 72°C for 45 sec; final extension, 72°C for 2 min. The size of the amplicons was checked by 1% agarose gel electrophoresis, stained with GelRed.

### Intramammary treatments and clinical evaluation

The cows identified as having fluoroquinolone-resistant bacteria were treated in each milking (twice per day) for 5 days as follows. Twenty IU/cows injecting IM with oxytocin (Oxitocina 20 U.I.; Virbac Mexico S.A. de C.V., Mexico) [23]. The cows were milked, and the milk discarded. When the mammary quarters were emptied, 300 mg of enro-C in 10 mL (Enromastic; Laboratorios Aranda S.A. de C.V., Mexico) were administered through the intramammary route (in all, ten infusions). On day 6, the health status of the mammary gland was evaluated clinically by a visual inspection, gland palpation to detect changes in the consistency of the mammary tissue, and by the strip-cup test, CMT, and SCC. If a cow presented with clumps in the milk or any other deviation from a normal appearance or was positive to CMT or had SCC above 200,000/mL, the particular case was quantified as treatment failure. The cow was then incorporated into the treatment-rescue scheme based on the intramammary infusion of ceftiofur HCl (125 mg/10 mL, twice daily for 5 days) (Spectramast; Zoetis, Mexico). The same clinical evaluation was performed at the end of treatment and at 14 and 21 days later.

The bacteriological cure was determined by the absence of clinical signs, negative SCC, and CMT, as well as the absence of the corresponding bacteria in the milk samples. This was done when finalizing either of the antibacterial treatment phases, and both 14 and 21 days after the last treatment of either enro-C and ceftiofur.

### Statistical analysis

Statistical analyses were conducted using the general linear model procedure of SAS version 9.3 (SAS Inst, USA) for an analysis of the difference, analysis of the covariance, and survival analysis (Cox regression model) as well as using 2-sample *t*-tests and Mann-Whitney-Wilcoxon *U* tests. All SCC data was processed based on the geometric means.

## RESULTS

Of the 20 cows included in this study, 9 (45%) were suffering from subclinical mastitis in a clinical phase. The number of mammary-quarters presenting clinical signs was 14 (38.9%), with CMT grade 1 (19%), grade 2 (29%), and grade 3 (50%). The average SCC was  $2.7 \times 10^6$  cells/mL of milk (range,  $5.8 \times 10^5$ – $4.8 \times 10^6$ ). Overall, 29 bacterial isolates from the 14 affected quarters were obtained. Most of the affected quarters showed a mixed infection, and *Staphylococcus* coagulase-negative was the commonest identified microorganism. **Table 1** lists the data. The *in vitro* susceptibility test showed that 100% of the isolated bacteria presented phenotypic resistance at 2 concentrations of enro-C from enro-C (0.2 µg/mL and 1.0 µg/mL). Furthermore, most strains showed a combination of resistance to other antibacterial drugs, such as benzylpenicillin G, sulfathiazole, neomycin, tetracycline, and ceftiofur (data

**Table 1.** Frequency and percentage bacteria present in the cows' milk with recurrent subclinical mastitis and fluoroquinolone resistance genes, as identified by polymerase chain reaction

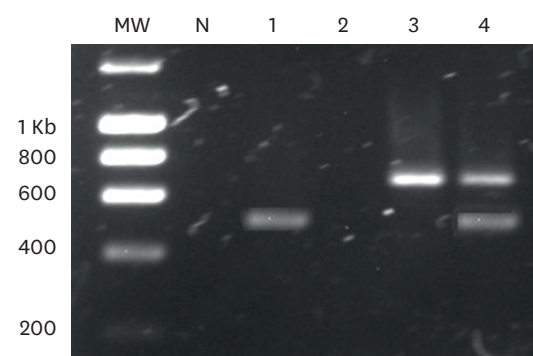
Bacteria	Microbiological isolates	Presence of fluoroquinolone resistance genes
<i>Staphylococcus</i> spp. coagulase (-)	16 (55.2)	2 genes (37.5)
		1 gene (37.5)
		Negative (25.0)
<i>Staphylococcus aureus</i>	8 (27.6)	2 genes (12.5)
		1 gene (50.0)
		Negative (37.5)
<i>Escherichia coli</i>	2 (6.9)	2 genes (50.0)
		1 gene (50.0)
<i>Streptococcus uberis</i>	2 (6.9)	2 genes (50.0)
		1 gene (50.0)
<i>Corynebacterium bovis</i>	1 (3.4)	1 gene (100.0)
Total	29 (100)	

Values are presented as number (%).

not shown). The control strains of *E. coli* ATCC-10536 and *S. aureus* ATCC-25923 exhibited the expected susceptibility to enro-C.

The presence of 2 enrofloxacin resistance genes was identified by PCR. Twenty out of 29 isolated bacteria showed the presence of the gene *gyrA* (69%), and 11 out of 29 presented the gene aminoglycoside N-acetyltransferase (*aac(6')-lb-cr*) (38%). These results are gathered in **Fig. 1**. **Tables 2** and **3** list the resistance genes by genus and species.

At the end of the treatment with enro-C, 9 of 14 quarters were graded as clinically cured (65%), and 5 out of 14 quarters (35%) were regarded as clinically positive to intramammary infection. On the other hand, there were no clinical signs of mastitis upon a visual inspection or palpation. Of the latter cases, one mammary quarter exhibited a mixed infection by *S. aureus*, *Streptococcus uberis*, and *E. coli*; 2 other quarters presented a simple infection by coagulase-negative *Staphylococcus*; another presented infection by *S. aureus* and another by *S. uberis*. In all cases, the bacteria presented the 2 resistance genes already referred to, and SCC was above  $4.8 \times 10^6$  cells/mL. After the rescue treatment with ceftiofur, all mammary quarters were clinically and bacteriologically cured and remained on days 14 and 21 post-treatment.



**Fig. 1.** Detection of bacterial genes associated with resistance to fluoroquinolones (including enrofloxacin from enro-C) (*gyrA* or *aac(6')-lb-cr*) in bacteria isolated from cows' milk affected of recurrent subclinical mastitis, as shown by polymerase chain reaction on 1% agarose gel. MW, 1 kb molecular weight marker; N, negative control; 1, *aac(6')-lb-cr* gene 482 bp; 2, negative sample; 3, *gyrA* gene 648 bp; 4, *gyrA* and *aac(6')-lb-cr* positive control.

**Table 2.** Fluoroquinolone resistance genes in bacteria isolated from subclinical cases of mastitis after total DNA extraction

Cow's ID	Isolated bacteria	<i>gyrA</i>	<i>aac(6')-lb-cr</i>
1, 13, 15, 25, 26, 28	<i>Staphylococcus coagulase</i> (-)	✓	✓
14, 16, 17, 29	<i>Staphylococcus coagulase</i> (-)	✓	✗
2	<i>Staphylococcus coagulase</i> (-)	✗	✓
3	<i>Staphylococcus coagulase</i> (-)	✗	✓
5, 6, 7, 8	<i>Staphylococcus coagulase</i> (-)	✗	✗
18	<i>Staphylococcus aureus</i>	✓	✓
11, 12, 23, 27	<i>Staphylococcus aureus</i>	✓	✗
4, 9, 10	<i>Staphylococcus aureus</i>	✗	✗
20	<i>Streptococcus uberis</i>	✓	✓
19	<i>Streptococcus uberis</i>	✓	✗
21	<i>Escherichia coli</i>	✓	✓
24	<i>Escherichia coli</i>	✓	✗
22	<i>Corynebacterium</i> spp.	✓	✗

Genes were identified by polymerase chain reaction: chromosomal *gyrA* (648 bp) and plasmid *aac(6')-lb-cr* (482 bp).

**Table 3.** Presence of bacterial genes associated with resistance to fluoroquinolones (*gyrA* or *aac(6')-lb-cr*) in milk from cows affected by recurrent subclinical mastitis

Resistance gene	Frequency (n = 29)
<i>gyrA/aac(6')-lb-cr</i>	9 (31)
<i>gyrA</i>	11 (37)
<i>aac(6')-lb-cr</i>	2 (7)
Negative	7 (24)

Values are presented as number (%).

## DISCUSSION

Despite the increase in bacterial resistance to antimicrobial drugs in dairy farms, their use continues to be the first-line defense in cases of intramammary infections. The incidence of subclinical mastitis has a wide range, with reports of up to 50% in commercial dairies [1,8]. This study was carried out in a production complex with poor hygiene and biosafety conditions, and 47.4% of the cows were diagnosed with subclinical mastitis in the evaluated sample as detected by the SCC, with values that could go as high as  $4.8 \times 10^6$  cells/mL. These counts are considered a reliable indicator of an intramammary infection [8,24,25] and coincide with the fact that the cows did not show any signs of clinical mastitis.

The microbiological examination shows that the isolation of coagulase-negative *Staphylococcus* spp was the most frequent pathogen, which concurs with the current trend reported by other authors [26-28]. Predictably, the evaluation of *in vitro* susceptibility to antibiotics demonstrated multiple resistance, including to enrofloxacin and ceftiofur, as was the case for the isolated bacteria in this trial presenting phenotypic resistance to enro-C. A literature review confirmed that the resistance to quinolones encoded in the chromosome is higher than that presented in plasmids, i.e., 76% vs. 37%. A high frequency of the *aac(6')-lb-cr* gene was observed. Other authors have reported up to 32% of isolates positive for the same quinolone resistance gene in Enterobacteriaceae [29]. Approximately 24% of the bacteria isolated in this test did not show the resistance genes *gyrA* or *aac(6')-lb-cr*. On the other hand, these bacteria presented *in vitro* phenotypic resistance towards enro-C. Therefore, it is reasonable to speculate that these bacteria possess other resistance mechanisms not detected in this trial [30-32].

Related to the observed clinical and bacteriological efficacy, the initial treatment achieved reasonably good clinical efficacy (65%) given the known difficulties in treating RScM and

considering that phenotypic and genotypic resistance to fluoroquinolones was previously demonstrated in the bacteria involved. Those cases of RScM having *S. aureus* as the etiology show resistance to antibiotic therapy. Moreover, it is not recommended to treat them as the reported efficacy percentages are very low [9]. Similarly, the discrepancy between the *in vitro* data showing phenotypic and genotypic resistance to enro-C, and the results obtained at the clinical level with bacteriological and clinical cures were attributed to the high dose of intramammary infusions of enro-C (300 mg in 10 mL twice daily), as well as the number of days of treatment. Hence, the concentrations of enro-C at the tissue-target level surpass the bacterial resistance and become therapeutic. From a pharmacokinetics/pharmacodynamics perspective, the optimal antibacterial efficacy of enrofloxacin obeys the concentration-dependent ratios [33]. Therefore, it is consistent with using high doses for this fluoroquinolone when infused through the mammary route [15]. There is no data on the concentrations in tissue or milk of enrofloxacin or enro-C after its intramammary infusion. On the other hand, the dose of 300 mg in 10 mL was much higher than the doses of more or less equipotent preparations, such as ceftiofur HCl (125 mg/10 mL). Nevertheless, it has already been shown that these concentrations of enro-C do not harm the mammary epithelium and have very high clinical efficacy [15]. In contrast, the clinical cure of intramammary infections treated with parenteral administration of enrofloxacin (5 mg/kg) has been reported to be low (8%–21%) [34,35], even considering that useful concentrations of enrofloxacin and its metabolite ciprofloxacin in milk have been reported, after parenteral administration of enrofloxacin [36]. The bacteria that did not respond to the enro-C treatment were coagulase-negative *Staphylococcus*, *S. aureus*, and *S. uberis*. To the best of the authors' knowledge, no reported studies have assessed their rescue-treatment using intramammary ceftiofur infusions, which itself has similar overall efficacy in treating RScM as the one obtained in this trial with enro-C [9]. In this context, 100% clinical and bacteriological efficacy was obtained with the consecutive treatments of enro-C followed by ceftiofur HCl, summing up a total of 10 days of treatment with antibiotic(s). The absolute clinical and bacteriological cure of RScM has not been reported previously. This study could not explain the underlying pharmacology of the successful efficacy observed. Nevertheless, the initial aggressive treatment with enro-C weakened bacteria and bacterial biofilms, despite these being resistant bacteria. Ceftiofur completed the antibacterial effects after an extended treatment period as described elsewhere [16]. The selection of ceftiofur as a rescue antimicrobial drug was based on the proposal of not combining bacteriostatic and bactericidal active principles [37], and because the combined effects of enrofloxacin and ceftiofur have been described as an additive, after *in vitro* susceptibility testing in biofilm-producing bacteria, through fractional inhibitory concentration indices [17]. This proposal should be explored in subsequent trials, evaluating the effects of the extended treatment with enro-C on biofilm production and the treatment-rescue effects with ceftiofur on this structure and the resistant bacteria. The combined effect cannot be attributed only to ceftiofur because extended treatment with intramammary infusions of this cephalosporin in cows with mastitis caused by *S. aureus* has been much lower than those observed in this trial [16,38].

In conclusion, these results show that even in the presence of bacteria genotypically and phenotypically resistant to fluoroquinolones, the intramammary infusion of enro-C at 300 mg/mammary-quarter twice per day for 5 days can achieve a clinical and bacteriological cure in a surprisingly high percentage (65%). Moreover, after a consecutive treatment with ceftiofur HCl, all cases of REcM included in this trial presented clinical and bacteriological cures. The population sample treated in this study can be considered relatively small. On the other hand, there are no data of absolute efficacy in the formal literature for RScM.

Therefore, clinical and bacteriological tests will be needed on a larger scale with this double antibiotic treatment scheme. Furthermore, it is necessary to evaluate the cost: benefit ratio that this dual treatment might offer, as well as the environmental suitability of using this combination for cows that are normally culled. Lastly, this and other antibiotic treatments should be accompanied by stringent hygiene and animal-handling maneuvers.

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