Article

Efficacy of a 5-day extended therapy program during lactation with cephapirin sodium in dairy cows chronically infected with *Staphylococcus aureus*

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Abstract — This study determined the efficacy of a 5-day extended therapy with cephapirin sodium in dairy cows chronically infected with *Staphylococcus aureus.* Chronically infected cows selected from 14 dairy herds in the St-Hyacinthe region, Québec were randomly allocated to a group of 31 cows treated for 5 consecutive days with 200 mg of cephapirin per quarter BID or a group of 30 untreated control cows. Bacteriological cure was determined by 3 negative bacterial cultures at 10, 24, and 31 days after treatment. The cow cure rates were 25.8% (8/31) in the treated cows and 3.3% (1/30) in the control group ($P = 0.013$). The quarter cure rates at first sampling post-treatment were 77.6% (38/49) and 18% (9/50) in the treated and the control groups, respectively ($P < 0.0001$). A 5-day extended therapy with cephapirin is effective in treating cows chronically infected with *S. aureus.*

Résumé — Efficacité d'un programme de thérapie prolongée de 5 jours à la céphapirine sodique durant la lactation chez les vaches laitières chroniquement infectées par *Staphylococcus aureus*. L'étude a déterminé l'efficacité d'une thérapie prolongée de 5 jours à la céphapirine sodique chez des vaches laitières chroniquement infectées par *Staphylococcus aureus.* Les vaches chroniquement infectées choisies de 14 troupeaux laitiers dans la région de Saint-Hyachinthe, au Québec, ont été assignées au hasard à un groupe de 31 vaches traitées pendant 5 jours consécutifs avec 200 mg de céphapirine par quartier bid ou à un groupe témoin de 30 vaches non traitées. La guérison bactériologique a été déterminée par 3 cultures bactériennes négatives 10, 24 et 31 jours après le traitement. Les taux de guérison des vaches étaient de 25,8 % (8/31) chez les vaches traitées et de 3,3 % (1/30) dans le groupe témoin ($P = 0{,}013$). Les taux de guérison des quartiers au premier échantillon après le traitement étaient de 77,6 % (38/49) et de 18 % (9/50) dans le groupe traité et le groupe témoin, respectivement ($P < 0{,}0001$). Une thérapie prolongée de 5 jours à la céphapirine est efficace pour traiter les vaches chroniquement infectées par *S. aureus.*

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Introduction

Staphylococcus aureus is the most prevalent contagious pathogen in many countries, including Canada and the United States (1,2). It causes both clinical and subclinical intramammary infections (IMI), with subclinical chronic infection being more common, resulting in increased somatic cell count (SCC) and decreased milk production. Response to intramammary antibiotic treatments administered according to label during lactation or at dry off is poor (3). However, cure rates at dry-off are expected to be higher than during lactation because the antibiotic concentration of commercial dry-off formulations is usually higher than the equivalent lactational formulation. Also, the usually different excipient in the dry-off formulation increases the persistence of the antibiotic in the mammary gland. A quarter cure rate of 25% to 75% is reported for treatment at dry-off while a range of 3% to 63% is reported for short-term therapy during lactation (3,4). As different infection and cure definitions were used in the studies reporting these quarter cure rates, it is difficult to accurately compare them. The economic importance of *S. aureus* is very high for the industry because of the decreased quality and quantity of milk produced by infected cows, and due to increased treatment and culling costs (5).

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New treatment regimes involving extended therapy during lactation or at dry-off have been developed to try to improve cure rates for *S. aureus* IMI (3,6–8). Gillepsie et al (6) compared 2-day, 5-day, and 8-day intramammary treatments with pirlimycin hydrochloride to treat *S. aureus* infected cows during lactation. The quarter cure rates were 13.3% (2/15), 31.3% (5/16), and 83.3% (5/6) for the 2-day, 5-day, and 8-day groups, respectively. There were significant differences between the 8-day group and the other groups, but there was no difference between the 5-day or the 2-day group and the untreated control group. In another study, Oliver et al (7) evaluated the efficacy of extended ceftiofur intramammary therapy for subclinical mastitis in lactating dairy cows. The quarter cure rates for *S. aureus* infected quarters were 7% (1/15), 17% (2/12), and 36% (4/11) for 2-day, 5-day, and 8-day groups, respectively. There were no statistically significant differences between the 2-day or the 5-day group and the untreated control group. No published study evaluated cephapirin sodium in an extended therapy protocol.

The main objective of the present study was to determine the cow cure rate of a 5-day extended therapy with cephapirin sodium in dairy cows chronically infected with *S. aureus.* A second objective was to evaluate the individual quarter cure rate with the same protocol in dairy cows with chronic infection with *S. aureus.*

Materials and methods

This study was approved by the ethical use of animals committee of the Université de Montréal (07-rech-1389).

Herds and cow selection

Preselection. A total of 103 dairy cows from 14 herds from the St-Hyacinthe area, Québec were preselected at the beginning of the study based on a previous positive milk culture for *S. aureus* and availability of individual SCC and milk production data (VALACTA; provincial DHI databank). The previous positive milk cultures had occurred on average 12.8 mo (1 to 41 mo) before the start of the project. The prior treatment history was not considered since it was not available for each cow. The participating herds were selected based on: the willingness of the farm manager to participate in the study; the presence of more than 6 lactating cows currently in the herd that were identified in the past with an IMI caused by *S. aureus;* and the geographic proximity of the farms to the researchers' laboratory. The cows preselected for the study had to be in lactation until the end of the study.

Selection. The infection status was determined by bacteriological culture of individual quarter milk samples taken at 4 and 2 wk before the beginning of the treatment period. A quarter was considered infected if 1 of the 2 milk samples was positive for *S. aureus.* A total of 28 cows were rejected because they were not infected by *S. aureus* in any quarter at either of the pre-treatment sampling times.

Treatment groups

The 75 selected cows were blocked by parity (1, 2, and 3 or more), and by days in milk (DIM) ($<$ 101, 101 to 200, $>$ 200), and were randomly allocated to treatment groups considering an a priori decision to have 1.5 treated cows for every control cow. One group consisted of 45 cows that were treated twice a day for 5 consecutive days in all 4 quarters with 1 tube of Cefa-Lak (Wyeth Animal Health, Guelph, Ontario) per quarter (200 mg of cephapirin sodium). All treatments were done by the producer immediately after each milking using an aseptic technique and a partial insertion technique. The second group consisted of 30 untreated control cows.

The cows were divided into 2 cohort groups beginning 2 wk apart. The initial objective was to select, for each herd, 1 treated cow and 2 control cows for the first cohort, and 2 treated cows and 1 control cow for the second cohort. This was done to minimize the impact on the herd's milk production losses and to test antibiotic residues on the first cohort of treated cows in order to suggest a milk withdrawal time for the second cohort of treated cows.

Sample collection

Milk samples were collected aseptically by a trained animal health technician from each quarter of all treated and control cows. Milk samples were cultured for bacterial growth 28 and 14 d prior to the beginning of the treatment, and 10, 24, and 31 d after the end of the treatment period. Milk samples were placed on ice at the farm and sent the same day to the Québec provincial bacteriology laboratory (Ministère de l'Agriculture et des Pêcheries du Québec) in St-Hyacinthe. If 1 milk sample was positive for *S. aureus* post-treatment, no further milk samples were collected from that cow, since those cows were not at risk of having their *S. aureus* status removed. Consequently, those cows were excluded from the rest of the study for calculation of quarter bacteriological cure. All clinical cases of mastitis during the trial were sampled and sent to a bacteriology laboratory for bacterial culture.

In addition, quarter SCCs were estimated on milk samples taken 2 wk pre-treatment and 10 and 31 d after the end of the treatment period. At sampling, Bronopol (2-bromo-2-nitropropane-1,3-diol) was added to milk samples as a preservative agent for SCC analysis. Milk samples were submitted within 2 days to VALACTA for an evaluation of the SCC by the Fossomatic 5000.

Definitions

A quarter was considered to be cured at a given sampling time when the post-treatment culture taken at that time and at the previous sampling time(s) were negative for *S. aureus.* A cow was considered to be cured when all infected and noninfected quarters at pre-treatment samplings were negative for *S. aureus* at the 3 post-treatment cultures. A new intramammary infection was identified when a quarter that was uninfected prior to treatment later became positive for *S. aureus* at any of the 3 post-treatment cultures.

Bacteriological analysis

Bacteriological analysis was performed by the Québec provincial bacteriology laboratory in St-Hyacinthe according to NMC guidelines (9). A volume of 10 μL of milk was plated on

trypticase soy blood agar plates containing 5% sheep blood. In order to confirm and validate the bacteriological status of those chronically infected cows, only *S. aureus* growth was looked for. Beta-hemolytic and double-hemolytic colonies were counted on trypticase soy blood agar plates, then streaked onto mannitol salt agar plates (PML microbiologicals, Mississauga, Ontario) and incubated aerobically for 24 h at 35°C. A coagulase test was performed on yellow colonies. The initial milk sample was incubated for 24 h at 35°C and was plated on blood agar plates if the primary culture was negative for *S. aureus.*

Antibiotic residue analysis

For antibiotic residue testing, milk samples from the first cohort of treated cows (13 cows) were collected by the producer immediately before the milking following the end of the treatment period at 0.5, 4, 4.5, 5, 5.5, 6, 6.5, 7, 7.5, 8, 8.5, 9, 9.5, 10, and 10.5 days. The samples were kept frozen (-20°C) until analysis. The milk samples were sent to the Faculté de médecine vétérinaire of the Université de Montréal for quantification of cephapirin by liquid chromatography tandem mass spectrometry. The milk samples of 4 randomly selected cows were assayed and a tentative withdrawal period was determined. To reduce the number of milk samples analyzed, milk samples from the other selected cows were assayed based on the withdrawal period determined by the results from these first 4 cows. The maximum limit of cephapirin residues in milk tolerated in Canada is 20 ng/mL (0.02 ppm). The limit of quantification was set at 4 ng/mL (0.004 ppm) for the purpose of the study.

Analysis of cephapirin in milk by liquid chromatography tandem mass spectrometry instrumentation

The HPLC system consisted of a Perkin Elmer Series 200 autosampler (Boston, Massachusetts, USA) and a Thermo Separation System P2000 (San José, California, USA). The LC-MS/MS system used was a *PE*Sciex API 3^+ (Applied Biosystem/MDS Sciex, Concord, Ontario). Computer data were analyzed using MassChrom 1.0 (Concord, Ontario). Calibration curves were calculated from the equation y = ax + b, as determined by weighted (1/x) linear regression of the calibration line constructed from the peak-area ratios of the drug and the internal standard.

Sample preparation. Using a protein precipitation method, cephapirin was extracted from the milk. A total of 100 μL of milk sample was mixed with 500 μL of internal standard solution (500 ng/mL of reserpine in acetonitrile) in a 1.5 mL centrifuge tube. The sample was then vortexed vigorously and allowed to sit for 10 min at room temperature prior to centrifugation. Samples were centrifuged at approximately 12 000 × *g* for 10 min and 300 μL of the supernatant was transferred to a 400 μL injection vial and transferred to an autosampler for LC-ESI/MS/MS analysis.

Chromatographic conditions. An isocratic mobile phase was used with a Waters Symmetry C18 150 × 3.9 mm column with a particle size of 5 μm. The mobile phase consisted of acetonitrile and 0.5% formic acid in water at a ratio of 80:20. The flow rate was fixed at 0.9 mL/min and cephapirin eluted at 1.3 min and the internal standard reserpine at 1.2 min. The column flow was split 1:10 prior to introduction into the electrospray source. A 25 μL volume of the extracted sample was injected and the total run time was set for 2 min.

Mass spectrometry conditions. The mass spectrometer was interfaced with the HPLC system using a pneumatic-assisted electrospray ion source. The N_2 pressure of the nebulizer gas was set at 40 psi and the ESI electrode was set to 4000 V. The nebulization was assisted with heated nitrogen gas set at a flow rate of 3 L/min and at 400°C. The declustering potential was set at 20 V and the collision energy (E_{lab}) at 20 V. The collision gas used was argon at 2.7×10^{14} molecules/cm^2. The SRM transitions were m/z 424 → 292 and 609 → 174 for cephapirin and reserpine, respectively. The dwell time was set at 150 ms and the pause time at 5 ms.

Statistical analysis

Computer software (SAS version 8.02; SAS, Cary, North Carolina, USA) was used for all analyses. A chi-squared test was used for cure rate analysis. To measure the effect of treatment on SCC, the SCC was converted to a logarithmic scale (L2 score) at the end of the sampling period and tested in a mixed model (SAS mixed proc) where the quarter was treated as a repeated measure in the cow, and the initial L2 score was used as an explanatory variable. The significance level of all analyses was set at $P < 0.05$.

Results

Seventy-five cows were enrolled in the study and 61 cows completed it. Cows were lost to the study because of producer withdrawal from the study (n = 10), culling (n = 1), early dry-off (n = 2), and death of the cow (n = 1). The 10 cows withdrawn by the owner were withdrawn before treatment at the time when the provincial federation of dairy producers announced a milk quota increase of 2%. The owners preferred not to treat these cows because of sudden additional revenues. As milk loss was not a problem for control cows, none of the cows in the control group were withdrawn. All of these reasons were unrelated to the study except for the producer withdrawals; these were related to the milk losses associated with the milk withdrawal time. A total of 31 dairy cows were treated and 30 cows were untreated negative controls.

The 2 groups were similar with respect to lactation number, DIM, the number of quarters infected, and the SCC prior to treatment (Table 1). One cow in the treated group and 2 cows in the control group each had one blind quarter.

The cow cure rates were 25.8% (8/31 cows) and 3.3% (1/30 cows) in the treated and control groups, respectively. The difference between the 2 groups was statistically significant (P = 0.013). A tendency to a higher cow cure rate was also observed in the treated group when cows had only 1 infected quarter (36.8%, 7/19 cows), as compared with cows that had > 1 infected quarter (8.3%, 1/12 cows) (P = 0.1). The quarter cure rate for the treated cows that had 1 infected quarter (36.8%, 7/19 cows) was statistically higher than the quarter cure rate for the control cows that had 1 infected quarter (5.6%, 1/18) (P = 0.02).

Table 1. Descriptive data for the treatment and control groups of cows

	Control group (n = 30)	Treatment group (n = 31)	Cured cows from the treatment group (n = 8)
Mean lactation number	2.1 (1–4)[a]	2.8 (1–9)	2.1 (1–3)
Mean DIM[b]	166 (21–311)	180 (96–335)	171 (98–250)
Mean number of infected quarters	1.7 (1–4)	1.6 (1–4)	1.1 (1–2)
Mean SCC[c] 2 wk prior to treatment ($\times 10^3$ cells/mL)	1519 (11–15 950)	2167 (1–26 634)	1200 (1–10 259)
Mean SCC 10 d after treatment ($\times 10^3$ cells/mL)	2863 (10–19 432)	1814 (27–25 172)	1436 (115–25 172)
Mean SCC 31 d after treatment ($\times 10^3$ cells/mL)	4216 (18–29 234)	2646 (31–29 633)	689 (45–2806)

[a] Range is given in parentheses.
[b] DIM = days in milk.
[c] SCC = somatic cell count.

The quarter cure rates at the first sampling post-treatment were 77.5% (38/49 quarters) and 18.0% (9/50 quarters) in the treated and control groups, respectively (Table 2). The difference between the 2 groups was statistically significant ($P < 0.0001$). There was a follow-up loss of 16 and 7 pre-treatment infected quarters in the treated and the control group, respectively. In the treated group, 8 quarters were lost after being negative at the 1st sampling and 8 quarters were lost after being negative at the 1st and the 2nd samplings. In the control group, all 7 quarters were lost after being negative at the 1st sampling. These follow-up losses were caused by cessation of sampling when another quarter on the same cow was found infected in a previous sampling post-treatment. About 69% (11/16) of the potentially cured quarters in the treated group that were lost to follow-up were lost because of non cured infections in other quarter(s) of the same cow. The other 31% (5/16) were lost because of new intramammary infection (NIMI) in other quarter(s) of the same cow. In the control group, 71% (5/7) of the cured quarters that were lost to follow-up were lost because of non-cured infections in other quarter(s) of the same cow. The other 29% (2/7) were lost because of NIMI in other quarter(s) of the same cow.

New IMI was diagnosed at the first sampling post-treatment in 1.4% (1/74 quarters) and 10.3% (7/68 quarters) of the quarters in the treated and control groups, respectively (Table 2). This difference was significant ($P = 0.021$). After the first sampling, 20 and 62 noninfected quarters were lost in the treated and control groups, respectively. After the second sampling, 23 and 1 noninfected quarters were lost in the treated and control groups, respectively. Eighty-seven percent (n = 55/63) of the noninfected quarters lost to follow-up in the control group were lost because of uncured infections in other quarter(s) of the same cow and 13% (8/63) were lost because of NIMI. In the treated group, 78% (33/43) of the noninfected quarters were lost because of uncured infections in other quarter(s) of the same cow. The other 23% (10/43) in the treated group were lost because of NIMI.

The effect of treatment on SCC on day 31 post-treatment was evaluated. The groups were not statistically different ($P = 0.12$) when the SCC was converted to a logarithmic scale (L2 score).

Clinical mastitis was reported in 4 treated cows (12.9%) between 5 and 8 d after the end of the treatment. Three of the cows had mild clinical mastitis and recovered with no treatment, but the 4th cow had an acute mastitis. The cow recovered but she had a lower milk production from the affected quarter. Yeast was the cause of the mastitis in each case. No follow-up culture was done on those 4 cases. No clinical mastitis case was reported in the control group during the treatment or the post-treatment follow-up period.

Antibiotic residues in milk were tested on the first group of 13 treated cows. Only 1 cow had cephapirin residues above the MRL limit of 4 d (96 h) after the end of the treatment period with a level of cephapirin of 59.4 ng/mL (0.0594 ppm). This cow was under the accepted limit at 4.5 d (108 h) after the end of the treatment (14.5 ng/mL or 0.0145 ppm). Four days (96 h) after the end of the treatment period, the other 12 cows had cephapirin residues levels lower than 14.2 ng/mL (0.0142 ppm). Based on these results, the milk withdrawal period was set at 5 d for the 2nd cohort of treated cows.

Discussion

The overall cow cure rates and the quarter cure rates at the first sampling post-treatment in the treated group were significantly different from those in the controls. In other studies using pirlimycin or ceftiofur for a 5-day extended therapy, there was no statistical difference between the treated quarter cure rate and the untreated quarter cure rate and the overall cow cure rates were not reported (6,7). Although comparative studies reported their results as quarter cure rate and not cow cure rate, the authors believe that overall cow cure rate is more meaningful to veterinarians and producers than quarter cure rate. It would be very interesting to evaluate cephapirin in different extended therapy protocols, such as an 8-day extended therapy, to see the impact on cure rates and to compare with results reported for other lactating formulations.

As positive cows were removed from the trial because of the occurrence of new intramammary infection or the persistence of intramammary infection in one or more quarters, several potentially cured quarters were lost for follow-up in both groups after the first milk sample. This prevented us from accurately

Table 2. Cow cure rate, quarter bacteriological cure rate, and new intramammary infection (NIMI) rate (%)

Groups		First sampling			Second sampling	Third sampling	
		Number/ Total number	Quarter cure rate	NIMI rate	Number/ Total number	Number/ Total number	Cow cure rate
Controls	Cows cured	2/30			1/30	1/30	3.3[a]
	Quarters cured	9/50	18.0[a]		2/2	2/2	
	Number of cured quarters lost after sampling	7			0		
	NIMI	7/68		10.3[a]	1/6	1/5	
Treated	Cows cured	21/31			9/31	8/31	25.8[b]
	Quarters cured	38/49	77.5[b]		22/30	13/14	
	Number of cured quarters lost after sampling	8			8		
	NIMI	1/74		1.4[b]	9/54	0/31	

[a,b] Means with different superscripts within a column differ statistically ($P < 0.05$).

calculating and reporting overall quarter cure rates. Since about 2 times more cured quarters were lost to follow-up in the treated versus the control group and 50% of the cured quarters lost in the treated group were lost after 2 negative cultures while none was lost after the 2nd sampling in the control group, it can be speculated that the calculation of the overall quarter cure rate could have underestimated the overall quarter cure rate for the treated group.

Many new IMI, which negatively affected the cow cure rates, were observed in both groups. The cow cure rate in the treated group was more affected as 7 cows in the treated group were removed because of new IMI in the other quarters. One of these cows was removed at the first sampling, and 6 were removed at the second sampling, compared with 2 cows having been removed in the control group: 1 cow at each of the 2 first samplings. Those new infections could be true new IMI or pre-existing infections not diagnosed by the 2 previous milk cultures. Because NIMI were detected in noninfected quarters, it is also possible that some quarters were classified as uncured because of NIMI in that quarter and not because of a persistent infection as no identification of the infecting *S. aureus* strain was done. No particular management practice or milking procedure, including milking order, was suggested to the producers during the study. We can speculate that if management, handling, and milking procedures would have been implemented, some new *S. aureus* infections could have been prevented from developing during or following treatment.

Clinical mastitis caused by yeast was diagnosed in 4 cows (12.5%) following cephapirin extended therapy. However, only 1 cow was severely affected and no gram-negative infection following cephapirin extended therapy was diagnosed. In a study using pirlimycin for extended therapy, 9.5% to 15.5% of the cows treated for 5 d and 8 d, respectively, developed a new IMI mainly caused by *Escherichia coli* or *Klebsiella pneumoniae* (6). Among those cows, 1 was dried off early and 3 died (1 in the 5-day treatment and 2 in the 8-day treatment). Another study reported a 26% incidence rate of clinical mastitis following a 5-day treatment with Pirlimycin (10). Pirlimycin has a spectrum of activity limited to gram-positive bacteria, whereas cephapirin has a broader spectrum. This could be an advantage in favor of cephapirin when choosing an antibiotic for extended therapy in a specific herd. However, excellent aseptic infusion technique must be used to minimize the risk of development of clinical mastitis during extended therapy, regardless of which antibiotic is used.

The authors elected to treat all 4 quarters of a cow despite the fact that for some quarters, both pre-treatment cultures were negative for *S. aureus*. This approach was chosen because: 1) to the author's knowledge, it is the most common recommendation made in the field by bovine practitioners; 2) it is the method used in many, if not all, comparable studies on extended therapy; 3) a cow is more at risk to develop NIMI caused by *S. aureus* in another quarter when she already has 1 infected quarter; and 4) some negative quarters might be positive as sensitivity of milk bacterial culture is less than 100% while often, the decision to treat a cow in the field is based on only 1 milk culture.

Based on the results of antibiotic residue testing, the suggested milk withdrawal time for this study was set at 5 d (120 h) after the end of the treatment period. This is 24 h longer than the approved withdrawal time in Canada. With this 5-day milk withdrawal, no violative residues in milk bulk tank occurred in any of the trial farms during the study.

If we consider the cost of the antibiotics (40 tubes at $4.00 each) and the cost of milk withdrawal for 10 d (5-day treatment + 5-day withdrawal time) with a cow producing 30 kg of milk/d ($0.75/L), the total cost for a 5-day extended therapy using cephapirin is approximately $385.00.

A 5-day extended therapy with cephapirin sodium is effective for treatment of chronically infected *S. aureus* cows. However, the overall cost of extended therapy protocols, including drug and milk withdrawal for milk residues, is high. Therefore, the selection of cows for extended therapy should be based on the evaluation of the presence of risk factors influencing cure rate. One should remember that cows with chronically infected quarters due to *S. aureus* have lower cure potential than cows with NIMI (11,12). Moreover, cows infected with *S. aureus* in many quarters are at lower risk for a cure (3). Young cows with only

1 quarter recently infected by *S. aureus* and a low SCC should be the best candidates for extended therapy (13). These particular factors were not evaluated in this study as the number of cows studied was too low to allow proper analysis of the risk factors except for the number of infected quarters. This study demonstrated that cows infected in only 1 quarter had a significantly better chance for cure with the treatment (36.8%) compared with controls (5.6%), and a tendency to a higher cow cure rate as compared with treated cows with more than 1 infected quarter (8.3%). On those selected cows, an excellent aseptic infusion technique must also be carried out to minimize the risk of development of clinical mastitis following extended therapy protocols. Also, management, handling, and milking procedures should be implemented to help prevent new *S. aureus* infections from developing during or following treatment.

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