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Dry Period Therapy Based on Antimicrobial Susceptibility for Cows with Subclinical Mastitis Caused by *Mycoplasma bovis*

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ABSTRACT

Cows with mastitis caused by *Mycoplasma bovis* (*M. bovis*) are generally subjected to preventive culling rather than treatment even if the infection is subclinical as they can be a source of severe infection, thereby causing significant economic loss to dairy farmers. We performed dry period therapy based on antimicrobial susceptibility on cows with subclinical mastitis caused by *M. bovis*. Marbofloxacin and pyrulimycin were selected as susceptibility antibiotics in this study. Overall, 16 cows with subclinical *M. bovis*udder infection were categorized into treatment (n=8, administered with antibiotics for 5 days before routine dry cow therapy) and control (n = 8, administered with routine dry cow therapy only) groups. Postpartum milk was *M. bovis*-positive in 0% (0/8) and 25% (2/8) cows in the treatment and control groups, respectively. The findings of this study suggest that the correct choice of antibiotics and treatments can completely cure *M. bovis* subclinical udder infections, thereby preventing unnecessary culling.

Keywords: Antibiotics, Dairy cow, *Mycoplasma bovis*, Subclinical mastitis.

INTRODUCTION

Mycoplasma bovis (*M. bovis*) is a well-known causative agent of pneumonia, otitis media, arthritis, and mastitis in cows (Maunsell et al., 2011). Clinical mastitis is challenging to cure, and cows with this disease can spread the infection to other cows, thereby warranting culling rather than treatment (Gonzalez and Wilson, 2003; Fox et al., 2005). Furthermore, cows with subclinical mastitis may also spread a large amount of *Mycoplasma* (Gonzalez and Wilson, 2003), and hence, many farmers choose culling to eliminate the infection source altogether. Therefore, both symptomatic and asymptomatic udder infection caused by *M. bovis* results in great loss to dairy farmers. However, appropriate antibiotic selection and treatment of cows based on mastitis severity may improve the therapeutic outcomes of *M. bovis*-related udder infections, especially in cows with subclinical mastitis.

We encountered a farm where in *M. bovis* was detected in 60 of 200 milking cows, of

which 40 were asymptomatic. Non-pregnant cows were culled, whereas symptomatic pregnant cows were stopped milking early and subsequently culled following parturition. The pregnant cows with subclinical mastitis were quarantined and monitored for disease progression. We treated the cows exhibiting subclinical infection based on antimicrobial susceptibility, and their milk was examined for the presence of *M. bovis* following parturition.

MATERIAL AND METHODS

Antimicrobial susceptibility tests:

Antimicrobial susceptibility tests were performed using three *M. bovis* strains isolated from farm cows with clinical udder infection. Using the conventional method (Hannan, 2000), the minimum inhibitory concentrations (MICs) of oxytetracycline, marbofloxacin, thiamphenicol, florfenicol, tylosin, tilmicosin, chlortetracycline, and lincomycin were investigated. Additionally, specific mutations in the 16S rRNA (*rrs*), 23S rRNA (*rrl*), DNA gyrase (*gyrA*), and topoisomerase IV (*parC*) genes responsible for decreased susceptibility to the antimicrobials were analyzed as described previously (Hata et al., 2019), and antimicrobial resistance to tetracyclines, spectinomycin, macrolides, lincosamides, and fluoroquinolones were confirmed.

Methods of dry period therapy:

The antibiotics were selected based on the results of previously described antimicrobial susceptibility test. A sensitive antibiotic was administered for 5 consecutive days, i.e., 5 days before the last milking of the lactation period. Each antibiotic was administered based on the standard administration method and dose. Then, as routine dry period therapy, the intramammary suspension of dry cows (Ceplavin Dry Cow, MSD Animal Health Japan, Tokyo) was infused after the last milking of the lactation period.

Animals:

This study included 16 cows with subclinical udder infection that reached the dry period within 1–3 months. Of the 16 cows, 8 were prescribed the abovementioned dose before the routine dry cow therapy (treatment group) and the remaining eight were administered only the routine dry cow therapy (control group). We isolated *M. bovis* and mastitis-causing bacteria and enumerated the somatic cells three to seven times per cow using milk (including transitional milk) collected within 1 month following parturition. The sampling frequency of milk was higher in cows with suspected clinical mastitis. Furthermore, the somatic cells of the last milk before the dry period were counted. The cows were considered *M. bovis*-positive if the bacteria were detected even once following parturition.

Polymerase chain reaction:

Polymerase chain reaction was used to detect *M. bovis* in milk, as described previously (Itoh et al., 2019).

Somatic cells count:

Enumeration of the somatic cells was performed using fully automatic fluoro-optoelectronic somatic cell counter (FOSSOMATICTM 7, Foss Electric, Denmark). For detection of cows with subclinical mastitis.

Blood agar culture:

For the general mastitis bacteria, milk samples were cultured in blood agar (Trypticase Soy Agar with 5% Sheep Blood, Nippon Becton Dickinson, Tokyo), and the isolates were identified using Gram stain reaction and biochemical tests (Oliver et al., 2004).

Statistical analysis:

Statistical analysis was conducted using Fisher's exact test and Mann–Whitney U test for comparing the *M. bovis*-positive rate and prepartum and postpartum somatic cell counts (SCC) between the treatment and control groups.

RESULTS

Antimicrobial susceptibility results:

Following antimicrobial susceptibility tests, three strains exhibiting similar antimicrobial susceptibility and high sensitivity to marbofloxacin (MIC, 0.5–1 mg/ml) and lincomycin (MIC, 1–2 mg/ml) were identified (Table 1). No mutation decreased fluoroquinolone and lincosamide susceptibilities (i.e., the coexistence of missense mutations in both *gyrA* and *parC* and adenine mutations at positions 2058 and 2059 in both *rrl*) (Table 2). Moreover, the mutation involved in decreased spectinomycin susceptibility was not confirmed (i.e., cytosine mutations at positions 1192 in both *rrs*). Conversely, mutations decreasing tetracycline and 16-membered macrolide susceptibilities were confirmed (i.e., adenines at positions 965 and 967 in both *rrs* mutated to thymines [A965T and A967T, respectively] and guanines at position 748 in both *rrl* mutated to adenines [G748A]). These results also support the findings of the conventional method (oxytetracycline MIC, 32 mg/ml; chlortetracycline MIC, 32–64 mg/ml, tylosin MIC, 16–64 mg/ml; and tilmicosin MIC, >128 mg/ml) (Table 1, Table 2) (Lysnyanskyet al., 2009; Amram et al., 2015; Sulyok et al. 2017; Hata et al., 2019).

This study selected fluoroquinolone-based marbofloxacin and lincomycin-based pirlimycin as susceptibility antibiotics, and consequently, marbofloxacin (2 g) (Marbocyl 10%, Meiji Seika Pharma Co., Tokyo) was intramuscularly administered and pirlimycin hydrochloride (50 mg) (Pirsue, Zoetis, Parsippany, NJ) was infused via the intramammary route for 5 consecutive days.

Effect of dry period therapy:

M. bovis was detected in the milk in none of the cows (0/8, 0%) in the treatment group and in two cows (2/8, 25%) in the control group within 1 month following parturition (Table 3). No significant difference was observed in the *M. bovis*-positive rate between the treatment and control groups.

The median (minimum–maximum) SCC linear score immediately before dry-off was 2.5 (1–6) and 2.0 (1–4) in the treatment and control groups, respectively. Similarly, within 1 month following parturition, the highest SCC linear score was 6.0 (2–9) and 2.5 (0–7) in the treatment and control groups, respectively (Table 3). The postpartum SCC linear score was higher in the treatment group than in the control group, although the difference was not statistically significant ($p = 0.06$). *Prototheca**zopfii*, yeast-like fungi, and *Streptococcus* sp. were isolated from the postpartum milk of two, one, and one cow, respectively, in the treatment group. These cows also exhibited high SCC, and hence, were diagnosed with clinical mastitis. *M. bovis* was isolated from two cows in the control group, one with *Streptococcus* sp. demonstrating high SCC and diagnosed with clinical mastitis (Table 3) and another with subclinical mastitis demonstrating low SCC.

Table (1): Minimum inhibitory concentration of *Mycoplasma bovis* isolated from milk:

Strain	MBFX	OTC	TP	FFC	TS	TMS	CTC	LCM
M. bovis 1	1	32	16	8	64	>128	64	2
*M. bovis*2	0.5	32	16	8	16	>128	32	1
*M. bovis*3	0.5	32	16	8	64	>128	64	2

M. bovis: Mycoplasma bovis.

MBFX, marbofloxacin; OTC, oxytetracycline; TP, thiamphenicol; FFC, florfenicol; TS, tylosin; TMS, tilmicosin; CTC, chlortetracycline; LCM, lincomycin.

Table (2): Existence of nonsynonymous mutations or single nucleotide polymorphisms in target genes confirmed in *Mycoplasma bovis* isolated from milk:

strain	16S rRNA gene(*rrs*) mutations*		23S rRNA gene(*rrl*) mutations**	DNA gyrase gene(*gyrA*) mutations†	Topoisomerase IV gene(*parC*) mutations†
M. bovis 1	A965T	A967T	G748A	None	None
*M. bovis*2	A965T	A967T	G748A	None	None
*M. bovis*3	A965T	A967T	G748A	None	None

M. bovis: Mycoplasma bovis
* *rrs* A965T and A967T mutations: low susceptibility to tetracyclines.
***rrl*G748A mutation: low susceptibility to 16-membered macrolides.
† Amino acid substitution in both *gyrA* and *parC*: low susceptibility to fluoroquinolones.

Table (3): Number of cows detected with *Mycoplasma bovis* in milk following parturition and somatic cell enumeration before dry-off and after parturition:

	Number of cows	Number of *M.bovis* positive cows	*M. bovis* positive rate (%)	SCC linear score (median [min–max])		Clinical mastitis in postpartum
				Before dry-off	After* parturition	
Treatment group	8	0	0	2.5 [1–6]	6.0 [2–9][a]	2: *Protothecazoffi* 1: Yeast-Like fungi 1: *Streptococcus* sp.
Control group	8	2	25	2.0 [1–4]	2.5 [0–7][b]	1: *Streptococcus* sp. ＋*M. bovis* (mixed infection)

M. bovis: *Mycoplasma bovis.*
SCC: somatic cell count.
* The highest values within 1 month after parturition.
ab: p=0.06

DISCUSSION

Bulk milk is regularly screened for *Mycoplasma* udder infection in some areas of Japan. If *Mycoplasma* is detected in the bulk milk, all milking cows on the farm are tested. Such tests enable the early detection of *Mycoplasma* mastitis. Furthermore, these tests detect subclinical mastitis in cows, which are usually culled to prevent the spread of infection to other cows even if they are asymptomatic. However, such culling is undesirable to dairy farmers.

In this farm, *M. bovis* udder infection was detected in 60 cows, of which 40 had subclinical infection. The latter were only quarantined and not culled. Some *M. bovis*-positive cows became negative in the subsequent milk tests; therefore, the farmers wanted to transfer those cows back to their original herd. However, it has been reported that previously *M. bovis*-positive cows intermittently shed *M. bovis* (Gonzalez and Wilson, 2003). Therefore, these cows were treated during the dry period to eradicate *M. bovis* udder infection, and the presence of *M. bovis* was confirmed in the postpartum milk. This attempt was based on a previous report of treating cows with *M. bovis* udder infection using a 5-day intramammary infusion of oxytetracycline and systemic

administration of fluoroquinolone (Ishiyama et al., 2014). However, the resistance of *M. bovis* to these antibiotics has been recently reported (Gerchman et al., 2009; Kawai et al., 2014; Lerner et al., 2014); hence, antimicrobial susceptibility tests were conducted in advance to enable the use of effective antibiotics. Ourantimicrobial susceptibility test only included three *M. bovis* strains detected from the milk of this farm before starting the survey. As the farm had not introduced pregnant heifers or multiparous cows from other farms, we assumed that only one type of *M. bovis* strain existed in the milk. Consequently, pulsed-field gel electrophoresis (Itoh et al., 2019) and multilocus sequence typing (Register et al., 2015) analyses performed for all *M. bovis* isolates (strains used in antimicrobial susceptibility test and strains detected in cows with following parturition) confirmed the same genotype for all the isolates. Our most notable finding was the absence of *M. bovis* in the postpartum milk of the cows in the treatment group. Hence, the dry period therapy using antibiotics appeared to have been effective for cows with subclinical *M. bovis* mastitis. We continuously followed up on the recurrence and reinfection of these cows, and to date (24 months), no cases of recurrence or reinfection have been observed. Meanwhile, although no significant difference was observed in the postpartum SCC between the two groups, the value was higher in the treatment group, and clinical mastitis caused by microorganisms other than *M. bovis* was observed in four cows in the treatment group. Whether intractable mastitis other than that caused by *Mycoplasma* had coincidentally occurred on this farm or was triggered by the dry period therapy should be investigated. The time and effort required to administer antibiotics for 5 days were also a limitation of this study. In future, we would like to consider shortening the treatment period.

CONCLUSION

Cows with subclinical udder infection by *M. bovis* and treated with marbofloxacin and/orpyrulimycin for 5 consecutive days before dry-off. *M. bovis* was not detected in the milk of all cows after parturition. The findings of this study suggest that the correct choice of antibiotics and treatments can completely cure *M. bovis* subclinical udder infections, thereby preventing unnecessary culling.

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