

## Determination of Minimum Bactericidal Concentrations of Antimicrobials Used for Canine Pyoderma Against *Staphylococcus pseudintermedius*

Kazuki Harada, Aiko Iguchi and Yoshiaki Hikasa  
Department of Veterinary Internal Medicine, Tottori University,  
Minami 4-101, Koyama-Cho, Tottori-Shi, 680-8553 Tottori, Japan

**Abstract:** Minimum Bactericidal Concentration (MBC) is an important *in vitro* parameter of antimicrobial potency but has not yet been measured for *Staphylococcus pseudintermedius*, the main causative organism of canine pyoderma. This study was carried out to determine the MBC of representative antimicrobials against *mecA*-negative and -positive *S. pseudintermedius*. Fifty nine *mecA*-negative and 33 *mecA*-positive isolates were tested for Minimum Inhibitory Concentration (MIC) of eight antimicrobials by Broth Microdilution Method. Subsequently, MBC was determined as concentration at which  $\geq 99.9\%$  decrease in bacterial counts was achieved by sub-cultivating suspensions at concentrations greater than MIC. As a result, cephalexin (LEX), Amoxicillin-Clavulanic acid (AMC), Orbifloxacin (ORB) and Fosfomycin (FOF) had MBC:MIC ratio of  $\leq 4$  whereas trimethoprim-Sulfamethoxazole (SXT) had MBC:MIC ratio of  $\leq 16$ . In contrast, Minocycline (MIN), Erythromycin (ERY) and Clindamycin (CLI) had MBC:MIC ratio of  $\geq 4$ . In *mecA*-negative isolates, among the tested antimicrobials, FOF had the lowest MBC-resistance rate (8.5%) followed by AMC (13.6%) LEX (16.9%) ORB (33.9%) SXT (33.9%) whereas MIN, ERY and CLI had high MBC-resistance rates (71.2-96.6%). On the other hand, in *mecA*-positive isolates, all tested antimicrobials had  $>50\%$  of MBC-resistance rates (69.7-100%). Notably, the resistant-level MIC value of MIN was not detected in either *mecA*-negative or -positive isolates. The present data indicate that FOF is a superior bactericidal drug against *mecA*-negative isolates whereas MINO can be effective bacteriostatic drugs for *mecA*-positive and -negative isolates of *S. pseudintermedius*.

**Key words:** *Staphylococcus pseudintermedius*, minimum bactericidal concentration, minimum inhibitory concentration, canine pyoderma, organism

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### INTRODUCTION

Canine pyoderma represents one of skin diseases characterized by bacterial infection. Systemic antimicrobial treatment is generally considered necessary for the resolution of canine pyoderma. The main causative bacterium of canine pyoderma is *Staphylococcus pseudintermedius* (Bryan *et al.*, 2012). In this bacterium, methicillin resistance which is encoded by *mecA* gene has become a major therapeutic challenge for small animal practice (Bryan *et al.*, 2012; Kadlec and Schwarz, 2012).

Traditionally antimicrobial dosing regimens have been deduced from the relationship between the pharmacokinetics of the drug and some *in vitro* measures of the pharmacodynamics such as the Minimum Inhibitory Concentration (MIC) or Minimum Bactericidal Concentration (MBC) of an antimicrobial agent for important pathogens. The MIC is the concentration of drug that inhibits the growth of bacteria whereas MBC is a measure of the concentration at which bacteria are killed by the antibacterial agents

(Finberg *et al.*, 2004; Levison, 2004). Canine pyoderma is occasionally associated with the systemically and locally immunosuppressive condition such as Cushing's syndrome and hypothyroidism, corticosteroid usage and concurrent infection (Frank, 2006; Bryan *et al.*, 2012). In such immunocompromised patients, MBC rather than MIC might be available as an important pharmacodynamic parameter because these patients require bactericidal rather than bacteriostatic therapy (Ansorg *et al.*, 1990). The major antimicrobial groups commonly utilized for canine pyoderma are the macrolides, lincosamides, tetracyclines, cephalosporins, fluoroquinolones,  $\beta$ -lactamase-resistant penicillins, potentiated sulphonamides and the other miscellaneous drugs (White, 1996) but MBC values of these antimicrobials against *S. pseudintermedius* remains to be clarified.

In this study, researchers firstly determined MBC values as well as MIC values of antimicrobials used for canine pyoderma in *mecA*-negative and -positive isolates of *S. pseudintermedius* to evaluate the bactericidal capacity of these antimicrobials.

**MATERIALS AND METHODS**

**Bacterial isolates:** Researchers used 59 *mecA*-negative and 33 *mecA*-positive strains of *S. pseudintermedius* which were isolated from subjects with canine pyoderma. In addition, *S. aureus* ATCC 29213 was used as quality control strain for susceptibility testing.

**MIC and MBC determination:** Each organism was determined for MIC values by the Broth Microdilution Method using a custom-designed, commercially prepared microtiter panel (Eiken Chemical, Japan) followed by MBC determination. The tested antimicrobials and ranges were cephalexin (LEX; 0.06-128 µg mL<sup>-1</sup>) Amoxicillin-Clavulanic acid (AMC; 0.06/0.03-64/32 µg mL<sup>-1</sup>) Orbifloxacin (ORB; 0.03-64 µg mL<sup>-1</sup>), Minocycline (MIN; 0.12-256 µg mL<sup>-1</sup>), Erythromycin (ERY; 0.06-128 µg mL<sup>-1</sup>), Clindamycin (CLI; 0.25-512 µg mL<sup>-1</sup>), trimethoprim-Sulfamethoxazole (SXT, 0.08/0.004-152/8 µg mL<sup>-1</sup>) and Fosfomycin (FOF; 0.25-512 µg mL<sup>-1</sup>). Plates were inoculated with a standard inoculum of approximately 5×10<sup>5</sup> colony-forming units (cfu)/mL and incubated at 35°C for 18-20 h. MIC was defined as the lowest concentrations at which visible growth of isolate is inhibited.

MBC determination was performed as described elsewhere (CLSI, 1999; Jones, 2006). The bacterial suspension of clear wells at concentrations greater than the defined MIC was sub-cultivated on Muller-Hinton agar (Becton, Dickinson and Company, Japan). Petri dishes were incubated at 35°C for 24 h and colonies were counted. The limit of detection was 10 cfu mL<sup>-1</sup>. MBC was determined as the concentration at which a ≥99.9% decrease in bacterial counts (i.e., 3 log<sub>10</sub> reduction in cfu/mL) was achieved.

**Interpretative criteria:** In this study, resistance rates were calculated based on both MIC and MBC values as previously reported (Ansorg *et al.*, 1990). The veterinary breakpoints (µg/mL) for AMC, ORB, ERY, CLI and SXT were used (i.e., ≥1, ≥8, ≥8, ≥4 and ≥4/76, respectively)

according to Clinical and Laboratory Standards Institutes guideline (CLSI, 2013). On the other hand, the breakpoints for the other antimicrobials have not yet been established in veterinary field. Thus, the breakpoints for LEX and FOF (i.e., both are ≥32) were set based on each peak plasma concentration which was obtained from the previous pharmacokinetic studies (Carli *et al.*, 1999; Gutierrez *et al.*, 2008). In addition, the breakpoint for MIN (i.e., ≥32) was set according to the report by Weese *et al.* (2013).

**Statistical analysis:** Fisher's exact test was used to compare data between two groups. The threshold for significance was set at p<0.05 in all analyses.

**RESULTS**

**MBC:MIC ratio:** MBC:MIC ratios were determined in the isolates whose both values of MIC and MBC were fallen within test range of each antimicrobial (Table 1). LEX, AMC, ORB and FOF had MBC:MIC ratio of ≤4 whereas SXT had MBC:MIC of ≤16. In contrast, MIN, ERY and CLI had MBC:MIC ratio of ≥4.

**MIC and MBC of *mecA*-negative isolates:** In *mecA*-negative isolates, the percentages of resistant-level MIC for ORB, ERY, CLI, SXT, LEX, FOF and AMC were 32.2, 27.1, 23.7, 22.0, 16.9, 8.5 and 6.8%, respectively (Fig. 1a). None of the isolates had resistant-level MIC for IN.

The percentages of resistant-level MBC for ERY, CLI, MIN, ORB, SXT, LEX, AMC and FOF were 96.6, 81.4, 71.2, 33.9, 33.9, 16.9, 13.6 and 8.5%, respectively. In MIN, ERY and CLI, MBC-resistant isolates were significantly more prevalent than MIC-resistant isolates (p<0.05).

**MIC and MBC of *mecA*-positive isolates:** In *mecA*-positive isolates, the percentages of resistant-level MIC values for ERY, SXT, CLI, ORB, AMC, LEX and FOF were 97.0, 97.0, 97.0, 93.9, 81.8, 66.7 and 66.7%, respectively (Fig. 1b). None of the isolates had resistant-level MIC only for MIN.

Table 1: Distribution of MBC:MIC ratio of eight antimicrobials in *S. pseudintermedius* from canine pyoderma

Antimicrobials*	No. of isolates according to MBC:MIC ratio <sup>†</sup>									
	1	2	4	8	16	32	64	128	UD+S	UD+R
LEX	47 (7)	24 (7)	4 (2)	-	-	-	-	-	-	17 (17)
AMC	56 (21)	18 (5)	7 (6)	-	-	-	-	-	11 (1)	-
ORB	44 (12)	26 (3)	2 (1)	-	-	-	-	-	-	20 (17)
MIN	-	-	1 (0)	1 (0)	5 (0)	6 (3)	12 (5)	17 (10)	41 (12)	9 (3)
ERY	-	-	1 (0)	-	4 (0)	11 (1)	21 (0)	7 (0)	-	48 (32)
CLI	-	-	3 (0)	-	1 (0)	-	2 (0)	-	45 (1)	41 (32)
FOF	26 (17)	11 (8)	1 (0)	-	-	-	-	-	53 (7)	1 (1)
SXT	15 (2)	22 (2)	7 (0)	3 (0)	1 (0)	-	-	-	-	44 (29)

\*LEX: cephalexin; AMC: Amoxicillin-Clavulanic acid; ORB: Orbifloxacin; MIN: Minocycline; ERY: Erythromycin; CLI: Clindamycin; FOF: Fosfomycin; SXT: trimethoprim-sulfamethoxazole; <sup>†</sup>Numbers in the parenthesis mean the number of *mecA*-positive *S. pseudintermedius*; <sup>‡</sup>UD+R, MBC:MIC ratio was undermined because MIC and/or MBC values were beyond the upper limit of each test range; UD+S, MBC:MIC ratio was undermined because MIC and/or MBC values were under the lower limit of each test range

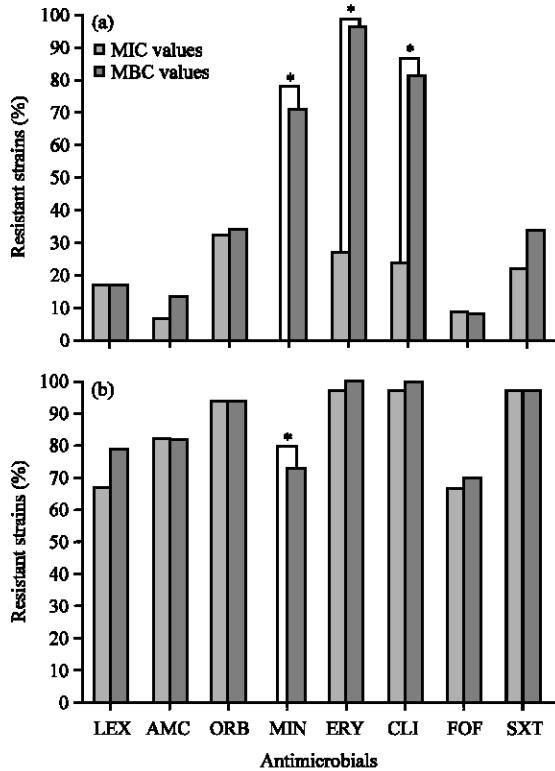


Fig. 1: a) Resistance rates of 59 *mecA*-negative and b) 33 *mecA*-positive isolates of *S. pseudintermedius* to eight antimicrobials. LEX: Cephalexin; AMC: Amoxicillin-Clavulanic acid; ORB: Orbifloxacin; MIN: Minocycline; ERY: Erythromycin; CLI: Clindamycin; FOF: Fosfomycin; SXT: Trimethoprim-sulfamethoxazole. \*Significance difference in prevalence between resistance rates according to MIC and MBC values ( $p < 0.05$ )

The percentages of resistant-level MBC values for ERY, CLI, SXT, ORB, AMC, LEX, MIN and FOF were 100, 100, 97.0, 93.9, 81.8, 78.8, 72.7 and 69.7%, respectively. Only in MIN, MBC-resistant isolates were significantly more prevalent than MIC-resistant isolates ( $p < 0.05$ ).

### DISCUSSION

For bactericidal drugs, the MBC values are usually  $\leq 4$  times the MIC values. In contrast, the MBC values of bacteriostatic drugs are  $> 4$  times higher than the MIC values (Levison, 2004). These properties of antimicrobial drugs can be influenced by the infecting bacterium because a given antimicrobial agent may be bactericidal to one organism but bacteriostatic to another (Finberg *et al.*, 2004; Pankey and Sabath, 2004). The bactericidal or

bacteriostatic activity of antimicrobials used for the treatment of canine pyoderma has not been demonstrated in *S. pseudintermedius*.

LEX, AMC and ORB belong to the cephalosporin,  $\beta$ -lactamase-resistant penicillins and fluoroquinolone classes, respectively. The antimicrobials belonging to these classes have a bactericidal effect on a wide range of pathogens (Lees *et al.*, 2008). The data presented here demonstrated that these antimicrobials have substantially low MBC:MIC ratios (i.e.,  $< 4$ ). Thus, these drugs are likely to have a strong bactericidal effect on *S. pseudintermedius* when it is exposed to drug concentrations above the MIC.

FOF and SXT which belong to the antimicrobial classes of phosphonic drugs and potentiated sulfonamides, respectively have a bacteriostatic effect on *S. aureus* and *S. epidermidis* (Molina-Manso *et al.*, 2012). In this study, FOF had an MBC:MIC ratio of  $< 4$  and thus is likely to have a bactericidal effect on *S. pseudintermedius*. In contrast, the MBC:MIC ratio of SXT ranged between 1 and 4 and additionally, 8-16, suggesting that this drug is bactericidal for some *S. pseudintermedius* isolates and bacteriostatic for others. Taken together, these results suggest that FOF and SXT exert excellent and partial bactericidal effects, respectively on *S. pseudintermedius* unlike those on other species of staphylococci.

In *mecA*-negative isolates, the prevalence of resistant-level MBC values against the tested bactericidal antimicrobials was relatively low (i.e., 8.5-33.9%) indicating that these drugs maintain strong bactericidal efficacy during the treatment of canine pyoderma caused by *mecA*-negative *S. pseudintermedius* isolates. In contrast, a high proportion (i.e., 69.7-97.0%) of *mecA*-positive isolates had resistant-level MBC and MIC values against these bactericidal drugs. This result is demonstrated by the high prevalence of multidrug resistance in *mecA*-positive isolates (Papich, 2012). In a comparison of bactericidal drugs, resistant-level MBC values against FOF were relatively less common in both *mecA*-negative and -positive isolates. These observations indicate that FOF may have the most potential as a bactericidal drug for canine pyoderma, although further clinical trials are needed to demonstrate its efficacy.

In general, the prevalence of the *mecA* gene in staphylococci is closely related to  $\beta$ -lactam drug resistance, including cephalosporins. In this study, however, researchers found some discrepancies with regard to the relationship between isolates possessing the *mecA* gene and demonstrating LEX resistance. Eleven of

33 *mecA*-positive isolates had susceptible-level MIC values for LEX. Furthermore, a previous study identified *mecA*-positive *S. pseudintermedius* isolates that were susceptible to cephalosporin (Ishihara *et al.*, 2010). In contrast, researchers demonstrate here that 10 of 59 *mecA*-negative isolates had a resistant-level MIC value for LEX. Previous studies have reported on *mecA*-independent  $\beta$ -lactam resistance mechanisms such as the hyper-production of penicillinase and inducible oxacillin resistance (Liu *et al.*, 1990; Ghoshal *et al.*, 2004). These resistance mechanisms have been identified in *S. aureus* and coagulase-negative staphylococci but not in *S. pseudintermedius*. Thus, further investigation would help to elucidate the *mecA*-independent resistance mechanisms present in *S. pseudintermedius*.

ERY and CLI are members of the macrolides and lincosamides antibiotic classes, respectively whereas MIN belongs to the tetracycline family. These antimicrobials generally have a bacteriostatic effect on several pathogens (Lees *et al.*, 2008). In this study they each had extremely high MBC:MIC ratios (>4) demonstrating that they also have a bacteriostatic effect on *S. pseudintermedius*. The data showed that most of the isolates tested had resistant-level MBC values for these antimicrobials which occurred in the presence and absence of the *mecA* gene. In these bacteriostatic drugs, a significant difference was observed in the prevalence of resistant-level MBC and MIC values in *mecA*-negative isolates because most of the MIC-susceptible isolates had resistant-level MBC value. These findings may have a significant implication on canine pyoderma treatment with these antimicrobials, particularly in immune-compromised hosts, regardless of appropriate dosing or the MIC of the infecting bacterium.

It is noteworthy that in this study, the resistant-level MIC value of MIN was not detected in either *mecA*-negative or -positive isolates. This high susceptibility to MIN by methicillin-resistant *S. pseudintermedius* isolates was also reported by Weese *et al.* (2013). Researchers results combined with those of the current study imply that MIN can be used as an effective bacteriostatic drug for the treatment of canine pyoderma caused by *mecA*-positive *S. pseudintermedius* isolates, although this drug has no or weak bactericidal effects.

### CONCLUSION

Researchers first determined the MBC values of multiple antimicrobials used for the treatment of canine pyoderma caused by *S. pseudintermedius*. Results analysed on the basis of CLSI breakpoints or peak serum

concentrations indicated that more than half of the *mecA*-negative isolates were killed by LEX, AMC, ORB, FOF and SXT. In particular, FOF may have the highest potential as a bactericidal drug against *mecA*-negative isolates because of the low prevalence of resistant-level MBC values. On the other hand, more than half of the *mecA*-positive isolates had resistant-level MBC values for all drugs whereas none of those isolates had resistant-level MIC values for MIN. This result predicts that MIN may hold promise as a superior drug to inhibit the growth of *mecA*-positive isolates but not kill them. Results of the present study may be helpful in choosing the optimum antimicrobials for the treatment of canine pyoderma.

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