Inhibition of *E. coli* by brief antimicrobial exposure and canine polymorphonuclear leukocytes

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KEY WORDS: Antimicrobial pre-exposure, dogs, *Escherichia coli*, polymorphonuclear leukocytes

ABSTRACT

This study was conducted to assess the impact of canine polymorphonuclear leukocytes (PMNs) on the growth of *Escherichia coli* that were pre-treated with antimicrobials, including amoxicillin, cephalexin, or orbifloxacin. The growth of *E. coli* ATCC25922 was evaluated during 2 h after a 10 min exposure to each drug (at maximum blood concentration in dogs) followed by the addition of canine PMNs. In the absence of antimicrobials, bacterial growth was significantly inhibited by 2 h co-culture with canine PMNs ($p < 0.01$ or 0.05). The exposure with amoxicillin or cephalexin had no significant impact on bacterial growth either in the presence or the absence of PMNs ($p > 0.05$). On the other hand, the exposure with orbifloxacin significantly inhibited bacterial growth either in the presence or the absence of PMNs ($p < 0.01$ or 0.05). Our results revealed that the canine PMNs were the primary contributors to bacterial growth inhibition. However, brief exposure to orbifloxacin (but not amoxicillin or cephalexin) resulted in significant PMN-independent bactericidal activity. We believe that the relationship with PMN may vary by antimicrobial drug, and should be taken into account, together with its antibacterial activity and risk of antimicrobial resistance, in antimicrobial treatment for bacterial infections in dogs.

INTRODUCTION

Antimicrobial drugs are essential for the treatment of bacterial infections that emerge frequently in companion animals, as well as humans. Growth of bacteria can be inhibited by antimicrobial drugs in vivo via complex interactions with the host immune system.[1-4] Notably, specific antimicrobials can induce postantibiotic leukocyte enhancement (PALE), which is the inhibition of bacterial growth via their capacity to enhance of leukocyte phagocytosis following a brief exposure.[5] Interactions between antimicrobials and leukocytes have been reported for several agents used to treat human infections.[6-9] However, this has not yet been explored for any of the most common antimicrobials used in veterinary practice.

Table 1. *The minimum inhibitory concentration (MIC) and Cmax values determined for each antimicrobial for E. coli ATCC25922*

Antimicrobials	MIC (μg/ml)	C_{max} (μg/ml)	References
Amoxicillin	2	18.6	Küng and Wanner (1994)
Cephalexin	4	31.5	Papich et al. (2010)
Orbifloxacin	0.063	3.3	Matsumoto et al. (1997)

Escherichia coli is a frequent source of infection among companion animals.[10] Among the best known drug classes, β-lactams and fluoroquinolones have been approved for the treatment of *E. coli* infection in many countries including Japan;. These drugs can be administered orally and have a strong safety profile and high activity against this pathogen.[11] The purpose of our study was to evaluate the impact of canine polymorphonuclear cells (PMNs) on *E. coli* growth after brief exposure to amoxicillin, cephalexin, or orbifloxacin.

MATERIALS AND METHODS

In this study, minimal inhibitory concentrations (MICs) for amoxicillin (Sigma-Aldrich Co. LLC, MO, USA), cephalexin (Wako Pure Chemical Industries, Ltd., Osaka, Japan), and orbifloxacin (Tokyo Chemical Industry Co., Ltd., Tokyo, Japan) were determined for the *E. coli* ATCC25922 strain using the agar dilution method according to the guidelines of the Clinical and Laboratory Standards Institute.[12]

The effects of antimicrobial exposure and/or PMN-mediated growth inhibition were evaluated according to the following protocols6,8 with several modifications. Briefly, the *E. coli* strain in logarithmic growth phase (approximately 10^8 cells/mL) was exposed to each drug at maximum blood concentration (C_{max}; Table 1) in Muller-Hinton II broth (Becton, Dickinson and Company, MD, USA) with continuous shaking. After a 10-minute exposure, the bacterium was pelleted by centrifugation at 2000×g for 10 min and re-suspended in sterile saline. This was repeated for a total of three washes. PMNs were prepared from heparinized blood from four healthy beagles that were purchased from Kitayama Labes Co., Ltd. (Nagano, Japan). Pooled blood samples were layered on Polymorphprep™ (Axis-Shield Diagnostics Ltd., Dundee, Scotland) and centrifuged at 500×g for 30 min. The PMN layer was isolated and washed with sterile saline according to the manufacturer's protocol. Erythrocytes were eliminated by hypotonic lysis in sterile water for 30 sec. PMNs were then resuspended in 5.0 mL of Hanks' balanced salt solution (Sigma-Aldrich Co., St. Louis, MO, USA) supplemented with 0.1% gelatin and 20% pooled canine serum to a final concentration of approximately 106 PMNs/mL; 0.1 mL of the 5 mL PMN suspension was removed and replaced with the same volume of bacterial cells in suspension to achieve a ratio of 2 bacterial cells:1 PMN. Bacterial counts (colony forming units (CFU)/mL) of cultures containing bacteria that were exposed to antibiotic and unexposed controls, and those treated with PMNs and those that were not were determined during incubation at 0 and 2 hours by plating serial dilutions on Mueller-Hinton agar.

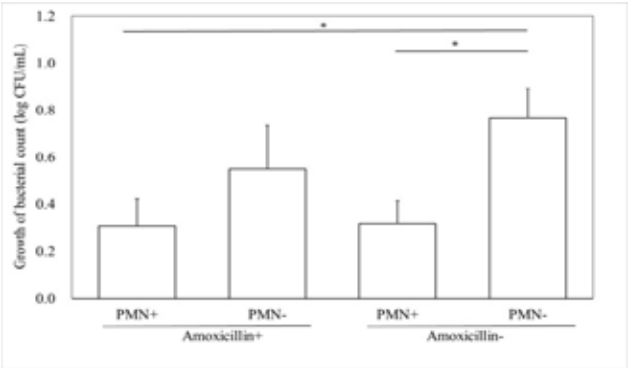
The animal experiments included in this study were conducted under an ethics committee-approved protocol in accordance with the Tottori University Animal Use Committee (approval number: 17-T-30) and care was taken to minimize the number of animals used. Results are presented as log10 (CFU/mL) at t = 2 hours (mean ± standard error) after subtraction of the counts determined at t = 0 hours; data presented are from three or four independent experiments. Repeated analysis of variance with Bonferroni's correction was used to compare bacterial growth among the four different conditions described; $p < 0.05$ was considered as statistically significant throughout.

RESULTS AND DISCUSSION

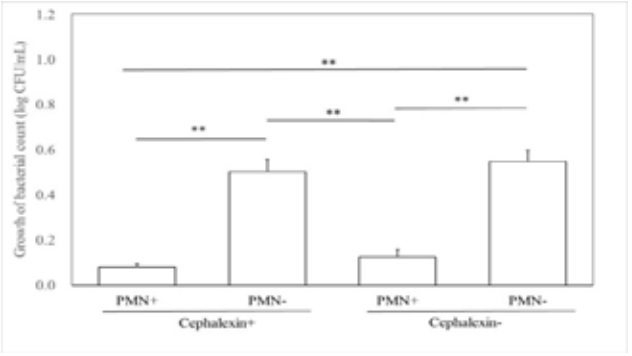
The MICs of each of the three antimicrobials targeting *E. coli* ATCC25922 are shown in Table 1, together with the Cmax of each drug as determined from in vivo evaluations carried out in previous studies.[13-15] The results in Fig. 1 reveal the growth of bacterial colony forming units (log CFU/mL) in cultures grown for 2 h either with or without canine PMNs and with or without pre-treatment with each antimicrobial drug. No significant differences were observed among the three drugs when comparing growth of bacteria that were not pre-treated with antimicrobial agent and were not grown in the presence of canine PMNs (Fig. 1a–1c). In the absence of amoxicillin and cephalexin, bacterial growth was significantly inhibited by 2 h co-culture with canine PMNs (**$p < 0.01$; *$p < 0.05$). As such, growth inhibition by canine PMNs, potentially via phagocytosis is likely to be a major feature of the endogenous antibacterial response in the absence of antibiotic treatment.

Fig. 1*. Growth of bacterial count (log CFU/mL ± SE) of E. coli ATCC25922 during 2 h after a 10-minute exposure or non-exposure to (a) amoxicillin, (b) cephalexin, or (c) orbifloxacin at maximum blood concentration. PMN+, presence of PMN; PMN-, absence of PMN; 'drug name'+, exposure to each drug; 'drug n*

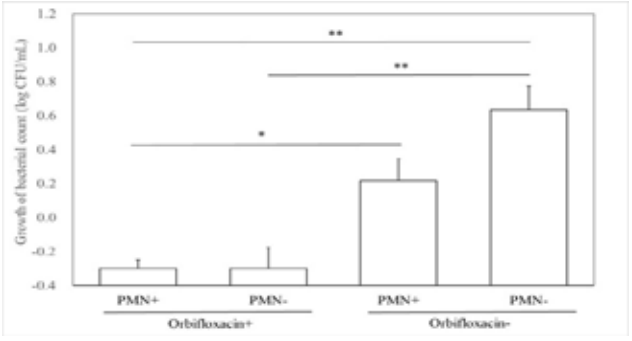
(a)



(b)



(c)



By contrast, brief exposure to orbifloxacin was profoundly bactericidal. Growth of bacteria exposed to this antimicrobial agent was significantly inhibited regardless of the presence or absence of canine PMNs ($p < 0.05$, $p < 0.01$). Interestingly, we previously found that this drug can exert a post-antibiotic effect (PAE; i.e., growth inhibition

We next examined the impact of antimicrobial pre-exposure and its role in promoting PMN-mediated growth inhibition. Interestingly, the 10-minute pulse exposure with amoxicillin or cephalexin had no significant impact on bacterial growth either in the presence or the absence of PMNs (Fig. 1a and 1b). These findings indicate that brief pulses with these antibiotics have no impact on bacterial growth nor can they contribute to growth inhibition mediated by canine PMNs.

of canine *E. coli* isolates following brief exposure).[16] This effect is not observed in response to beta-lactam antibiotics, including amoxicillin and cephalexin.[17]

Our findings suggest that transient exposure to orbifloxacin can greatly reduce bacterial viability even in the absence of PMNs. Notably, there was no significant difference in growth of orbifloxacin-treated bacteria between in the presence and in the absence of PMNs, and thus we could not clarify the PMN activity in the orbifloxacin-treated bacteria. One possible reason for this finding is that even the bacteria without PMNs was strongly inhibited by exposure to orbifloxacin, unlike amoxicillin and cephalexin. Therefore, the importance of PMN activity in antibiotic-exposed bacteria may vary greatly by drug.

The PALE is considered to occur through antibiotic-induced metabolic changes to the bacterial surface structures, which enhances susceptibility to phagocytosis or intracellular killing (Herrera-Insúa et al., 1997; Sasahara et al., 2003), although the detailed mechanisms have not yet been clarified. The previous studies revealed that brief exposure to antimicrobial agents used in humans, including lomefloxacin and meropenem, can enhance PALE for *E. coli* (Pruul and McDonald, 1990; Novelli et al., 2000).

In contrast, we observed no synergy between the actions of any of these antimicrobials and the isolated canine PMNs, and thereby could not demonstrate PALEs. This discrepancy may be related to the nature of the specific drugs and/or to as yet unexamined species-specific properties of PMNs. In any cases, our and previous findings may imply that the degree of PALE is different between humans and dogs, and thus data on PALE of human drugs should not be extrapolated to veterinary drugs. In addition, Novelli et al. (2000) found that the PALE activity can be related not only to antimicrobial agents but also to bacterial species. Further studies using diverse pathogens including Staphylococcus spp. and Streptococcus spp. are needed in order to comprehend the relationship between veterinary antimicrobials and canine PMNs.

In conclusion, our studies were designed to evaluate the relationship between brief antimicrobial exposure and PMN-mediated growth inhibition using agents commonly employed as treatments for *E. coli* infections in dogs. Our results demonstrated that isolated canine PMNs contribute to bacterial growth inhibition in vitro. However, brief exposure to beta-lactam antibiotics, including amoxicillin and cephalexin, had no impact on bacterial growth when used alone or prior to addition of canine PMNs. By contrast, brief exposure to orbifloxacin is profoundly bactericidal via a mechanism that does not rely on PMNs. We strongly believe that the relationship with immune cells may vary by antimicrobial drug, and should be considered, together with its antibacterial activity and risk of antimicrobial resistance, for the antimicrobial treatment of bacterial infections in dogs.

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