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Abstract

We determined the maximum inhibitory dilutions (MID) of bamboo pyroligneous acid (BPA) against 104 *Escherichia Coli*, 112 *Staphylococcus Pseudintermedius* and 58 *Pseudomonas Aeruginosa* strains isolated from dogs and cats. The MID was determined by inoculating each strain on agar containing serial dilutions of BPA. The MIDs for BPA against *S. pseudintermedius*, *E. coli* and *P. aeruginosa* were 103.4 ± 7.41 (100–150), 109.4 ± 3.64 (100–120) and 147.2 ± 8.87 (140–160) -fold, respectively. Our results indicated that BPA have significant effects on inhibiting the growth of representative bacterial pathogens from companion animals, although the inhibition differed among species.

Keywords: Bamboo pyroligenous acid, cats, dogs, *Escherichia Coli*, maximum inhibitory dilution, *Pseudomonas Aeruginosa*, *Staphylococcus Pseudintermedius*.

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Introduction

Antimicrobials are used routinely to treat bacterial infections in companion animals. However, the use of antimicrobial drugs has promoted the emergence of antimicrobial resistant bacteria, which may not only reduce the efficacy of veterinary antimicrobial treatments but may also have implications for human health because of the close contact between companion animals and humans (Guardbassi *et al.*, 2004). Therefore, it is desirable to identify alternative antimicrobial materials to replace traditional antimicrobial drugs.

Bamboo pyroligneous acid (BPA) is a brown-red transparent liquid, which is produced as a by-product during the pyrolysis of bamboo charcoal. BPA contains more than 200 different organic compounds, such as phenolic, alkane, alcohol and aldehyde compounds as well as acetic acid (Mun and Ku, 2010). Several studies have previously demonstrated that BPA has antiviral (Marumoto *et al.*, 2012) and antifungal effects (Komagata and Motoyama, 2004). However, little is known about the antibacterial effects of BPA.

In addition to other researchers, we previously reported the prevalence of antimicrobial resistance in several representative pathogens isolated from companion animals, including *Staphylococcus Pseudintermedius*, *Escherichia Coli* and *Pseudomonas Aeruginosa*, (Harada *et al.*, 2012a and 2012c; Kawakami *et al.*, 2010). In the present study, we investigated the maximum inhibitory dilution (MID) of BPA using these veterinary pathogens to assess the suitability of BPA as an alternative to antimicrobial drugs.

Materials and Methods

In this study, BPA distilled from moso bamboo (*Phyllostachys pubescens*), which was harvested in Ishikawa Prefecture, Japan, was provided by Nakata Engine Ltd. (Ishikawa, Japan). BPA was produced in accordance with the guideline of the Japan Mokusaku-eki Association. In brief, bamboo charcoal was produced by pyrolyzing bamboo at 100°C–400°C in oxygen-limiting conditions, and condensation of the smoke from pyrolysis yielded the crude BPA. The

solution was distilled to increase the purity for 1 h at 105°C, filtered to remove any impurities and used in this study. The pH of the distilled BPA was 2.3, and it contained approximately 8.73% acetic acid, 0.14% phenol compounds and 0.014% formaldehyde compounds.

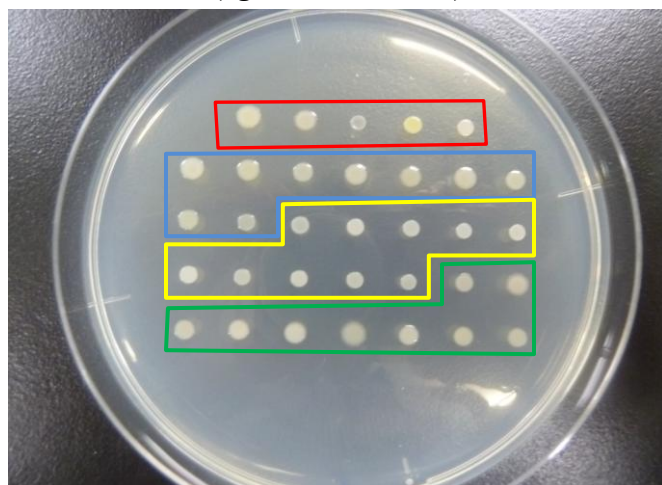
We used 112 *S. pseudintermedius* strains, including 60 methicillin-resistant strains; 104 *E. coli* strains, including 11 that produced ESBLs and 58 *P. aeruginosa* strains. *E. coli* strains were collected from dogs and cats with urogenital infections, whereas *P. aeruginosa* strains were collected from animals with skin, ear and urinary tract infections in our previous studies (Harada *et al.*, 2012a and 2012c). *S. pseudintermedius* strains were isolated from subjects with canine pyoderma (Kawakami *et al.*, 2010) and were kindly provided by Prof. Fukata, Gifu University. The MIDs were determined by the previous protocol (Watanabe *et al.*, 2008) with several modifications. Serial dilutions of BPA (i.e. 1/2, 1/3, 1/4, 1/5, 1/6, 1/7, 1/8, 1/9, 1/10, 1/11, 1/12, 1/13, 1/14, 1/15, 1/16, 1/17, 1/18, 1/19, and 1/20) were prepared in test tubes with sterile distilled water. Two millilitres of each dilution were mixed with 18.0 mL of Muller–Hinton Agar medium (Difco, USA) in Petri dishes. Microbial inocula with turbidities equivalent to a #0.5 McFarland standard (approximately $1-2 \times 10^8$ CFU/mL) were prepared in test tubes with saline, using *E. coli*, *P. aeruginosa* and *S. pseudintermedius* strains. Using inoculum replicators (1-mm pin), approximately 0.1 µL microorganisms were deposited onto the surface of agar, which contained different dilutions of BPA. The dishes were subsequently incubated for 18 h at 35°C, and the MID was determined as the highest dilution of BPA that was capable of inhibiting the growth of test strains. *E. coli* ATCC25922, *P. aeruginosa* ATCC27853, *Enterococcus faecalis* ATCC29212, *Staphylococcus aureus* ATCC29213 and *S. pseudintermedius* LMG 22219 were used as quality controls. Figure 1 shows one example of determination of MIDs. One-way analysis of variance (ANOVA) was used to compare the MIDs among the three bacterial species. The Tukey–Kramer test was used to evaluate differences among the geometric means of the MIDs. The

Welch test was used to make pairwise comparisons of the MIDs. The threshold for significance was set

at $P < 0.05$ in all analyses.

Fig. 1: One example of determination of minimum inhibitory dilution (MID) of bamboo pyroligneous acid (BPA).

A: Control (agar without BPA).



Red box: Type strain. Starting from the left, *E. coli* ATCC25922, *P. aeruginosa* ATCC27853, *E. faecalis* ATCC29212, *S. aureus* ATCC29213, and *S. pseudintermedius* LMG22219.

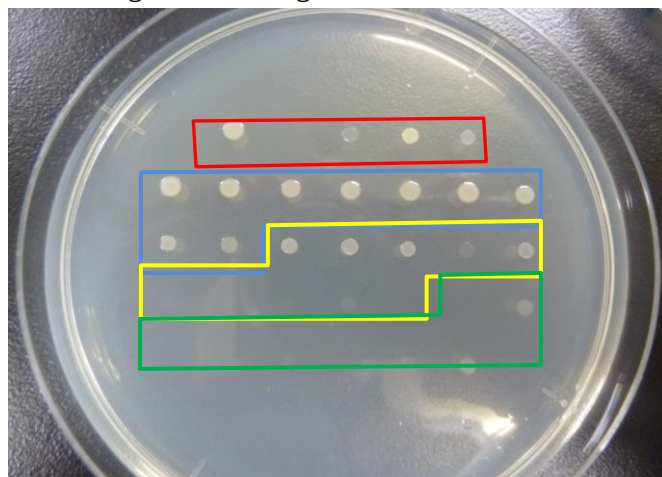
Blue box: Field strains of *E. coli* (n=9).

Yellow box: Field strains of *S. pseudintermedius* (n=10).

Green box: Field strains of *P. aeruginosa* (n=9).

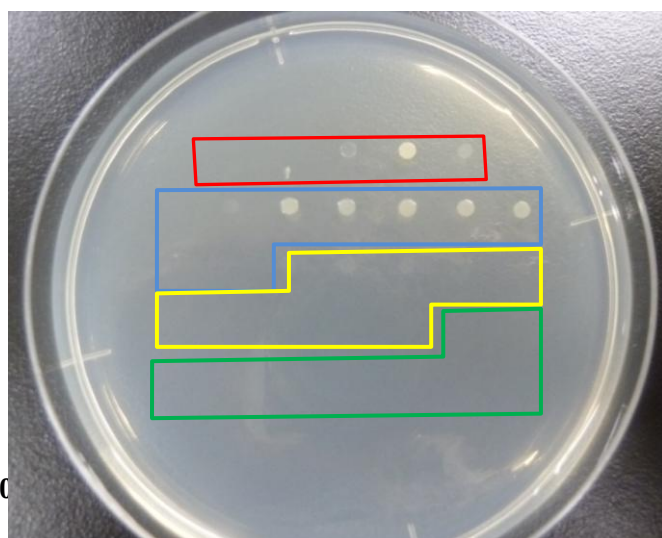
Remark: All isolates grew.

B: Agar containing 150-fold diluted BPA.



Remark: In type strains, the growth of *P. aeruginosa* ATCC27853 was first completely inhibited. In field strains, 4 of 10 *S. pseudintermedius* isolates and 7 of 9 *P. aeruginosa* isolates were first completely inhibited. These strains were judged to exhibit MID of 150-fold with BPA.

C: Agar containing 120-fold diluted BPA.



Remark: In type strains, the growth of *E. coli* ATCC25922 was first completely inhibited. In field strains, 3 of 9 *E. coli* isolates were first completely inhibited. These strains were judged to exhibit MID of 120-fold with BPA.

Results and Discussion

The results are summarized in Table 1.

Table 1: Summary of the maximum inhibitory dilutions of bamboo pyroligneous acid against type strains and pathogens isolated from companion animals.

		No. of isolates	Maximum inhibitory dilution (MID)
			Mean ± SD (range)
Type strains	<i>Escherichia Coli</i> ATCC25922	1	110–120
	<i>Pseudomonas Aeruginosa</i> ATCC27853	1	140–150
	<i>Enterococcus faecalis</i> ATCC29212	1	80–90
	<i>Staphylococcus aureus</i> ATCC29213	1	70
	<i>S. pseudintermedius</i> LMG 22219	1	110–120
Field strains	<i>E. coli</i>		
	All isolates	104	109.4 ± 3.64 (100–120) ^{a,b}
	Extended-spectrum β-lactamase producing isolates	11	106.4 ± 4.81 (100–110)
	<i>S. pseudintermedius</i>		
	All isolates	112	103.4 ± 7.41 (100–150) ^{a,c}
	Methicillin-resistant isolates	60	104 ± 8.21 (100–150)
	<i>P. aeruginosa</i>		
	All isolates	58	147.2 ± 8.87 (140–160) ^{b,c}

^{a,b,c} There were significant differences among the mean values of the MID's with the same superscript.

S. pseudintermedius is the main causative bacterium of canine pyoderma. In recent years, the spread of methicillin-resistant isolates, which have broad multidrug resistance, has become a complex challenge for small animal practice (Frank and Loeffler, 2012). The present study demonstrated that the growth of *S. pseudintermedius* strains, including methicillin-resistant strains, was inhibited by a 100–150 (mean, 103.4 $\pm$ 7.41) -fold dilution of BPA. There was no significant difference in the MID's of methicillin-resistant and -susceptible isolates ($P > 0.05$). This result is important for the efficacy of BPA in treating infections caused by methicillin-resistant *S. pseudintermedius*.

Extraintestinal *E. coli* is frequently isolated from dogs and cats with urogenital infections, and it has high rates of resistance against veterinary antimicrobials such as β -lactams and fluoroquinolones in Japan (Harada *et al.*, 2012c). In particular, extended-spectrum β -lactamase (ESBL)-producing *E. coli* isolates exhibit multidrug

resistance, which may be a major therapeutic problem in veterinary medicine (Harada *et al.*, 2012c). The present results showed that the growth of all *E. coli* strains, including ESBL-positive strains, was inhibited by a 100–120 (mean, 109.4 $\pm$ 3.64) -fold dilution of BPA, which was significantly higher than that for *S. pseudintermedius* ($P < 0.05$). There was no significant difference between the MID's of ESBL-positive and -negative strains ($P > 0.05$). Thus, BPA is likely to have significant antibacterial effects on *E. coli* strains with and without ESBLs.

P. aeruginosa is the major opportunistic bacterium in companion animals. This bacterium exhibits inherent resistance against multiple drugs because of intrinsic resistance mechanisms (Hancock, 1998). Our study demonstrated that *P. aeruginosa* isolates exhibited MID's of 140–160 (147.2 $\pm$ 8.87) -fold with BPA, which were significantly higher than those of the other two bacterial species ($P < 0.05$). Unfortunately, we have

no explanation for these differences in the susceptibility to BPA among bacterial species. However, our study indicated that *P. aeruginosa* was more susceptible to BPA compared with *E. coli* and *S. pseudintermedius*; therefore, BPA may have greater efficacy against *P. aeruginosa* infections.

The antibacterial components of BPA include phenol and formaldehyde compounds as well as acetic acid. Acetic acid can acidify the interior of bacterial cells, which results in degeneration and loss of bacterial components (Cherrington *et al.*, 1991). Phenol is involved in the inactivation of bacterial enzymes and depletion of metabolites from bacterial cells (Rutala, 1996). Furthermore, formaldehyde has antibacterial effects, possibly by binding DNA, RNA and proteins within bacterial cells (McDonnell and Denver Russell, 1999). All three of these components have a broad antibacterial spectrum; thus, BPA is likely to have antibacterial effects against various species of pathogens isolated from companion animals. It is known that BPA contains several other chemicals in addition to those mentioned above (Akakabe *et al.*, 2006; Mun and Ku, 2010). Further studies will be required to identify these antibacterial components and to clarify any synergistic effects that occur between BPA components.

In this study, we evaluated the efficacy of BPA against representative pathogenic bacteria, including multidrug-resistant strains, which were isolated from companion animals. Our study demonstrated that BPA significantly inhibited the growth of *S. pseudintermedius*, *E. coli* and *P. aeruginosa*, although the susceptibility to BPA differed among bacterial species. Therefore, we consider that BPA may be an alternative antimicrobial for the treatment of bacterial infections in companion animals.

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