Effects of Fragrance Ingredients on the Uptake of Legionella pneumophila into Acanthamoeba castellanii

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Received 21 April, 2018/Accepted 20 June, 2018

Acanthamoeba castellanii, a ubiquitous organism in water environments, is pathogenic toward humans and also is a host for bacteria of the genus Legionella, a causative agent of legionellosis. Fragrance ingredients were investigated for their antibacterial activity against planktonic Legionella pneumophila, amoebicidal activity against A. castellanii, and inhibitory effect against L. pneumophila uptake into A. castellanii. Helional® exhibited relatively high antibacterial activity (minimum inhibitory concentration (MIC), 32.0 µg/mL). Anis aldehyde, canthoxal, helional® and vanillin exhibited amoebicidal activity (IC₅₀ values, 58.4±2.0, 71.2±14.7, 66.8±8.3 and 49.1±2.5 µg/mL, respectively). L. pneumophila pretreatment with sub-MICs (0.25×MIC) of anis aldehyde, canthoxal, cortex aldehyde® 50 percent or vanillin evidently reduced L. pneumophila uptake into A. castellanii (p < 0.01). Thus, fragrance ingredients were good candidates for disinfectant against L. pneumophila and A. castellanii.

Key words : Fragrance ingredients / Antibacterial activity / Amoebicidal activity / Legionella pneumophila / Acanthamoeba castellanii.

Legionella pneumophila is a gram-negative bacterium, which is widely distributed in natural aquatic environments, such as hot springs, as well as in artificial water systems, such as air-conditioning equipment, fountains and public baths. The contamination of artificial water systems by this bacterium may lead to legionellosis outbreaks; thus, controlling the number of L. pneumophila in natural and artificial water systems is crucial to prevent such outbreaks: Various disinfectants have been used to control L. pneumophila: however, control is difficult because L. pneumophila forms a biofilm or grows inside biofilm and amoeba cells, making it resistant to various disinfectants (Barker et al., 1992; Cooper and Hanlon, 2010; Kim et al., 2002; Wright et al., 1991).

Acanthamoeba is a free-living amoeba that is commonly found in various environmental water sources worldwide. Acanthamoeba serves as a host for of L. pneumophila, and those are often isolated from the same location (Rowbotham, 1980; Sasaki et al., 2003; Thomas et al., 2006). Intracellular L. pneumophila is tolerant to adverse conditions, such as treatment of disinfectants, (Thomas et al., 2004) and shows decreased sensitivity to disinfectants because of its intracellular location and phenotypic modifications, such as inducing regulator against stress and exert cyst-like form in which decreased metabolic activity, (Bandyopadhyay et al., 2004; Garduño et al., 2002).

Since L. pneumophila multiplies in Acanthamoeba cells, controlling Acanthamoeba and inhibiting bacteria uptake into Acanthamoeba cells are important.

Fragrance ingredients are known antibacterial agents against gram-positive and gram-negative bacteria. In our study, five fragrance ingredients of aldehyde derivatives are examined and showed high antibacterial activity against planktonic L. pneumophila JCM7571 (Shimizu et al. 2009), for their amoebicidal activity against A. castellanii trophozites, inhibitory effects against L. pneumophila uptake into A. castellanii with an
aim of developing a novel disinfectant that may prevent legionellosis by controlling the numbers of both *L. pneumophila* and *A. castellanii*.

All fragrance ingredients (anis aldehyde, canthoxal, cortex aldehyde\(^{50}\) percent, helional\(^{8}\) and vanillin) were provided by Ogawa & Co., Ltd. (Chiba, Japan) and stored at 4°C until use. These fragrance ingredients were dissolved in dimethylsulfoxide (DMSO), and the final concentration of DMSO showed no effect on the number and form of the amoebae cells used in this study (data not shown). The names and structures of these ingredients are presented in Fig.1. Ampicillin (ABPC, Wako Pure Chemical Co., Ltd., Osaka, Japan) was used as a reference compound.

*L. pneumophila* ATCC BAA-74 and *A. castellanii* ATCC 30234 were obtained from the American Type Culture Collection (Manassas, VA, USA). *L. pneumophila* was grown on buffered charcoal yeast extract agar supplemented with \(\alpha\)-ketoglutarate and L-cysteine (BCYE-\(\alpha\), Nomura et al., 2013) at 37°C for 3 days. *A. castellanii* was cultured as trophozoites cells in peptone-yeast extract glucose (PYG) medium (ATCC medium 712) using a cell culture flask at 25°C for 4 days. Amoeba cells were harvested by centrifugation at 250 X g for 5 min, washed with Acanthamoeba (Ac) buffer (PYG medium without peptone and yeast extract) and suspended with Ac buffer at a required concentration depending on the experiment.

The minimum inhibitory concentrations (MICs) of fragrance ingredients and ABPC against planktonic *L. pneumophila* were evaluated according to the modified broth microdilution method (Shimizu et al., 2009) on the basis of the standard method of the Clinical and Laboratory Standards Institute (CLSI, 2000). Fragrance ingredients were dissolved at a concentration of 12.8 mg/mL (ABPC was dissolved in phosphate buffer of pH 8.0) and then diluted to a final concentration of 512 \(\mu\)g/mL in BYE-\(\alpha\) broth (BCYE-\(\alpha\) without charcoal and agar). Samples were serially diluted with BYE-\(\alpha\) broth as described previously. Each diluted sample (50 \(\mu\)L) was mixed with 50 \(\mu\)L of *L. pneumophila* suspension adjusted to \(6.0 \times 10^5\) colony forming units (CFU)/mL with BYE-\(\alpha\) broth and incubated at 37°C for 48 h. Antibacterial activities of the tested compounds are presented in Table 1. All compounds exhibited antibacterial activities against planktonic *L. pneumophila*, with the MICs ranging from 32.0 to 256.0 \(\mu\)g/mL. Helional\(^{50}\) showed markedly high antibacterial activity, with MIC values of 32.0 \(\mu\)g/mL. Reportedly, cinnamic aldehyde acts upon and permeabilizes bacterial cell membranes (Kwon et al., 2003). These aldehyde compounds that showed antibacterial activity in this study belong to the same aldehyde family to which cinnamic aldehyde belongs; thus, we hypothesize that these compounds acts upon bacterial cell membranes in a similar manner.

The amoebicidal activity of fragrance ingredients was evaluated by the alamarBlue\(^{8}\) assay method (Nomura et al., 2015). Fragrance ingredients were dissolved at a concentration of 100 mg/mL (ABPC was dissolved in phosphate buffer of pH 8.0). These samples were diluted with Ac buffer to 200, 20, 2 and 0.2 \(\mu\)g/mL. At total 50 \(\mu\)L of *A. castellanii* suspension (\(4.0 \times 10^5\) cells/mL) was added seeded in duplicate on 96 well microtiter plate (Greiner Bio-One Co. Ltd., Frickenhausen, Germany) and the amoebae cells were allowed to adhere to the wells at 37°C for 3 h. Then, 50 \(\mu\)L of the test sample solutions in Ac buffer were added to each well and incubation was done at 37°C for 24 h. At 6 h prior to the end of incubation, 10 \(\mu\)L of alamarBlue\(^{8}\) reagent (Invitrogen, Carlsbad, CA, USA) was added to each well and the plates were further incubated at 37°C for 6 h in the dark. The intensity of the fluorescence of the reduced alamarBlue\(^{8}\) dye was measured using a SpectraMax M5 (Molecular Devices Japan, Tokyo, Japan) at an excitation wavelength of 560 nm and emission wavelength of 590 nm. The data are reported as means ± standard deviation of three separate observations. Amoebicidal activities of the tested compounds are presented in Table 1. Anis aldehyde, canthoxal, helional\(^{8}\), and vanillin exhibited amoebicidal activity against *A. castellanii*, with the IC\(_{50}\) values ranging.
from 49.1±2.5 to 71.2±14.7 µg/mL. Cortex aldehyde\(^6\) 50 percent did not exhibit any amoebicidal activity even at the concentration of 100 µg/mL. Vanillin and anis aldehyde showed high amoebicidal activity. It was suggested that the side chain length containing aldehyde group was more active when shorter than longer.

Further, the effects of these fragrance ingredients on \textit{L. pneumophila} uptake into \textit{A. castellanii} were evaluated. \textit{L. pneumophila} uptake experiments were performed using a partially modified method of Nomura et al. (2015). \textit{A. castellanii} suspension (1×10\(^6\) cells/mL) were inoculated into a 96-well microtiter plate (Sumitomo Bakelite Co. Ltd., Tokyo, Japan). \textit{L. pneumophila} (3.0×10\(^8\) CFU/mL) was incubated in BYE-a broth containing 0.25×MIC (sub-MIC) of the test sample at 37°C, 150 rpm for 24 h. The added and incubated with 0.25×MIC (sub-MIC) of the test sample was no effect on the growth of \textit{L. pneumophila} (data not shown). The bacterial cells were harvested by centrifugation at 3,220 rpm for 5 min to remove of fragrance ingredients, and were suspended in sterile saline at a final concentration of 2.0×10\(^7\) CFU/mL. The bacterial suspension was added to each well containing \textit{A. castellanii} [a multiplicity of infection (MOI) of 20]. The plates were centrifuged at 250×g for 20 min and incubated at 37°C for 1 h. After infection, the extracellular bacteria were killed by gentamicin (100 µg/mL). The amoeba cells were washed and lysed by adding 0.04% Triton X-100. Aliquots of the amoebal cell lysates were immediately diluted with buffered saline supplemented with 0.01% gelatin, plated on BCYE-α plates and incubated at 37°C for 72 h for colony enumeration. All assays were performed in triplicate, and the numbers (CFU/mL) of viable intracellular bacteria were analyzed using the Student’s t-test. Data are reported as means ± standard deviation of three separate experiments. Following \textit{L. pneumophila} pretreatment with sub-MIC concentrations of anis aldehyde, canthoxal, cortex aldehyde\(^6\) 50 percent, and vanillin, \textit{L. pneumophila} uptake into \textit{A. castellanii} was significantly inhibited (p < 0.01) (Fig.2). The mean value of \textit{A. castellanii} uptake observed for the untreated control (1.4±1.0×10\(^3\) CFU/mL) was reduced to 3.7±5.3×10\(^2\) CFU/mL (anis aldehyde), <1.0×10\(^2\) CFU/mL (canthoxal and cortex aldehyde\(^6\) 50 percent), and 7.0±7.0×10\(^1\) CFU/mL (vanillin). These activities were equivalent to that of the reference ABPC (4.1±1.2×10\(^2\) CFU/mL), which is known to reduce \textit{L. pneumophila} uptake into \textit{A. castellanii} (Lück et al., 1998). Helional\(^6\) also showed inhibitory activity against \textit{L. pneumophila} uptake (2.0±2.2×10\(^2\) CFU/mL); however, its activity was lower than that of other compounds. Lück et al. (1998) have reported that \textit{L. pneumophila} pretreatment with sub-MICs of antibiotics, such as ABPC and imipenem, reduced bacterial uptake into \textit{A. castellanii}. \(\beta\)-Lactams inhibit D-alanine transpeptidase, resulting in the disruption of murein synthesis and the subsequent structural disturbance of the outer bacterial membrane. They have speculated that the outer membrane and/or lipopolysaccharide structures of bacteria that may play a role in the adhesion and/or uptake of bacteria into host cells could be affected by sub-MICs of \(\beta\)-lactams. \textit{L. pneumophila} pretreatment with canthoxal, or cortex aldehyde\(^6\) 50 percent, anis aldehyde, helional\(^6\), and vanillin, significantly inhibited bacteria uptake into \textit{A. castellanii}. Since these compounds are not antibiotics, their inhibitory mechanism may differ from those of \(\beta\)-lactams. The uptake inhibitory effect of these five compounds was stronger than that of ABPC. To date, cinnamic aldehyde has been reported to degenerate the bacterial cell membrane by reducing its fluidity through changes in its fatty acid composition (Di et al., 2007). Thus, these five compounds may act in the same manner as the cinnamic aldehyde. Furthermore, comparison of the uptake inhibiting effect and the structure of these compounds allowed to suggest that the presence of phenoxy affects the inhibitory effect on \textit{L. pneumophila} uptake into \textit{A. castellanii}. There was no correlation between antibacterial activity, amoebicidal activity, and inhibitory effect on \textit{L. pneumophila} uptake into \textit{A. castellanii} in these compounds. Thus, these activities may be due to the different mechanisms of action, but the detailed mechanisms are unknown.
Taken together, the compounds examined in the present study exhibited antibacterial and amoebicidal activities as well as inhibitory effect against *L. pneumophila* uptake into *A. castellanii*, indicating that a combination of these fragrance ingredients act as a novel type of disinfectant to inhibit the growth of *L. pneumophila* and *A. castellanii*.

**ACKNOWLEDGMENTS**

This study was supported by the supply of fragrance ingredients from Ogawa & Co. Ltd.

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