Short Communication

Benzalkonium Chloride Resistance in *Listeria monocytogenes* Isolated in Japan

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Summary

Benzalkonium chloride (BC) is widely used in medical settings, food processing facilities, livestock farming, and industry. In this study, *Listeria monocytogenes* (Lm) resistance to BC was investigated by a screening test using stored laboratory strains. Results indicated that 3 of 219 tested strains demonstrated growth on plates containing 10 µg/ml of BC. Therefore, BC-resistant Lm strains were confirmed to exist in Japan.

Keywords : benzalkonium chloride, Japanese strain, *Listeria monocytogenes*, resistance

Ingestion of food contaminated with *Listeria monocytogenes* (Lm) is considered the main route of listeriosis, although identification of the route of infection is difficult. Lm is an environmental bacterium widely distributed in nature and can grow at temperatures lower than 4°C and salt concentration of 11.5%. This bacterium is resistant to disinfectants due to biofilm formation⁹⁾ and can survive in areas with poor cleaning and disinfection. In the latter setting, Lm can be a continuous source of contamination. Furthermore, at 4°C, a small number of bacteria can reach a sufficiently large number to cause infection within 2 weeks. Thus, listeriosis becomes a problem with so-called ready-to-eat food, such as dairy, processed meat, and cooked products, which are stored at low temperatures. The disinfectant benzalkonium chloride (BC) is used as a countermeasure against infectious diseases in food processing facilities, medical sites, livestock farming, and industry. Because BC is highly versatile and widely used as a countermeasure against infectious diseases, the emergence of drug-resistant bacteria has become a problem, and the emergence of bacteria resistant to BC has recently been reported^{1-4, 6-8, 12-15)}. Mínavová et al.⁶⁾, Rodríguez-Melcón et al.¹²⁾, and Aarestrup et al.¹⁾ reported that BC minimum inhibitory concentrations (MICs) of Lm ranged 1.26–12.5 µg/ml, 3–13 µg/ml, and exhibited bimo-

dality at 4 µg/ml and 16–32 µg/ml, respectively. However, to our knowledge, there is no report about BC-resistant Lm in Japan. Since 1989, we have continuously investigated Lm contamination in domestic marketed meat and preserved the isolates. BC resistance in preserved Lm strains was, therefore, examined in this study.

We collected 547 Japanese Lm isolates derived from 820 meat samples (pork, beef, chicken, minced beef, and pork) purchased at 105 stores from June 1996 to June 2007 and December 2017 to August 2018, in metropolitan Tokyo and Kanagawa, Chiba, Saitama, Gunma, and Shizuoka prefectures. These 547 strains had been stored at –80°C until analysis. We then randomly selected 219 of the 547 strains for analysis in this study; these 219 strains comprised the following serotypes (numbers of strains): 1/2a (53), 1/2b (81), 1/2c (50), 4b (33), and 4d/e (2). The screening test used for BC resistance was performed according to the method of Mullapudi et al.⁸⁾. Mueller Hinton medium (Difco/BBL) containing sterilized BC (Nacalai Tesque Co., Ltd.) with a final concentration of 10 µg/ml and 2% sheep blood (Japan Bio Research Institute Co., Ltd.) was used. Bacterial growth was confirmed by three culture methods: spotting 5 µl of the bacterial suspension on the medium, puncture, and streaking. Disk and broth micro dilution methods for determining MIC were carried out according to the method for conducting drug resistance test reference (National Veterinary Assay Laboratory, Japanese Ministry of Agriculture, Forestry, and Fisheries)^{10, 11)}. A disk (Advantec, Co., Ltd.; diameter = 8 mm) was soaked in sterilized BC of

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(a)

(b)

Fig. 1 Examinations for BC-resistant *L. monocytogenes*;
 (a) shows the screening test by spotting, streak and puncture methods using a Mueller Hinton medium containing 2% sheep blood and sterilized BC with a final concentration of 10 $\mu\text{g}/\text{ml}$.
 (b) shows the broth micro dilution method. Upper numbers, concentrations (mg/ μl) of benzalkonium chloride; left side, Lm strains.

10, 10^2 , 10^3 , or 10^4 $\mu\text{g}/\text{ml}$, heat dried, and placed on the Mueller Hinton medium and cultured at 37°C for 24 h. The diameter of the formed inhibition circle was then measured using a caliper. For the broth micro dilution method, Mueller Hinton broth (95 μl) containing 4% horse lysate (Japan Bio Research Institute Co., Ltd.) and BC (64, 32, 16, 8, 4, 2, 1, and 0.5 mg/ μl) was first placed in each well of a 96-well microplate. Then, pre-cultured bacterial solution (5 μl ; McFarland 0.5 to McFarland 1) diluted with physiological saline was added, incubated at 37°C , and observed for bacterial growth after 24 h.

The diameter obtained using the disk method is expressed as mean $\pm$ standard error of the mean (SEM) in millimeters. Stu-

dent's t-test was used to analyze significant differences between diameters.

Although the negative control EGD strain did not grow, three strains (1.4%), 779C2, 633C3, and 868C4, formed colonies characterized by a transparent to round shape with smooth edges were observed in the area where the bacterial suspension spread in spots (Fig. 1a). Similar results were obtained by puncture or streaking. The three abovementioned strains grew on the medium, and β -hemolysis was observed in the culture by puncture. Using the disk method, 779C2, 633C3, and 868C4 displayed obvious resistance to BC at BC concentrations of 10^3 and 10^4 $\mu\text{g}/\text{ml}$. Diameters of inhibition circles at 10^3 and 10^4 $\mu\text{g}/\text{ml}$ were 10.3

± 0.3 and 13.0 ± 0.0 mm ($n = 3$), respectively, in 633C3, which were significantly smaller ($P < 0.05$) than those of negative control EGD (15.7 ± 0.7 and 18.3 ± 1.7 mm; $n = 3$, respectively). Similar results were obtained for 779C2 and 868C4. The broth micro dilution method also confirmed BC resistance in these three strains. All three strains grew at $16 \mu\text{g/ml}$, though EGD was inhibited at a BC concentration of $4 \mu\text{g/ml}$. Thus, BC MICs of the three strains were $32 \mu\text{g/ml}$ (Fig. 1b). Romanova et al.¹³⁾ and To et al.¹⁵⁾ reported that MICs of BC-sensitive Lm strains were $\leq 1.25 \mu\text{g/ml}$ ¹³⁾ and $\leq 4 \mu\text{g/ml}$ ¹⁵⁾, respectively. Therefore, the present results indicate that BC-resistant Lm already exists in Japan.

BC-resistant 633C3 and 779C2 strains were both serotype 1/2a strains isolated from chickens. The former strain was purchased in 2004 and the latter strain was purchased in 2007 from different stores of the same chain store. Processing plants were different between these two stores. Although the origin of both strains is unknown, a previous study found that 779C2 and 633C3 strains are genetically related¹⁶⁾. Therefore, this BC-resistant Lm strain is possibly a resident in the marketing channel from the farm to this chain store. The 868C4 strain was a chicken-derived serotype 1/2b strain, and the meat was purchased in 2018. The store from where this chicken meat was purchased differed from that of the other two strains. The isolation of BC-resistant Lm strains from another store may be because BC is highly versatile and widely used as a disinfectant in Japan.

In Japan, the Food Sanitation Law was amended in 2018. In 2021, all food-handling businesses will begin to fully institute hygiene management protocols established by the Hazard Analysis Critical Control Point (HACCP). BC may be further used in post-HACCP sanitation. Thus far, no outbreak of food poisoning caused by Lm has occurred in Japan. However, sporadic listeriosis has occurred. A previous study⁵⁾ reported that Lm carrying the BC-resistant gene *emrC* is associated with a worse prognosis. Although the rate of resistant strains was 1.4% in this study, which is lower than those of foreign reports (10%²⁾ and 22%⁴⁾), the potential increase in BC-resistant strains is a concern in Japan. Therefore, further surveillance of BC-resistant Lm is required.

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短 報

日本において分離された *Listeria monocytogenes* の
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要旨

塩化ベンザルコニウム (BC) は, 医療現場, 食品加工施設, 畜産および産業で広く使用されている。本研究では, 我々の研究室に保管されていたリステリアモノサイトゲネス (Lm) 株の BC に対する耐性をクリーニングテストで試験した。その結果, 219 株のうちの 3 株が 10 µg/ml の

BC を含むプレートで増殖した。このことから, 日本にも BC 耐性の Lm 株が存在することが確認された。

キーワード : 塩化ベンザルコニウム, 日本の株, *Listeria monocytogenes*, 耐性

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