Antibacterial Susceptibility of Enterobacteriaceae Isolated from Raw Horsemeat Intended for Human Consumption (Basashi)

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Drug susceptibility testing was carried out using 14 antibiotics in order to identify trends in the antibiotic tolerance of 142 strains of Enterobacteriaceae isolated from horsemeat commercially available for raw consumption (basashi). A comparison of the sensitivity to the 14 antibiotics using the 90% MIC (minimum inhibitory concentration) values (MIC90) showed the strongest tolerance to ampicillin (ABPC) at a concentration of >128 µg/mL, followed by that to fosfomycin (FOM) at a concentration of 128 µg/mL. When the sensitivity to these antibiotics was examined for each individual genus of tested bacteria, Hafnia spp. exhibited relative tolerance to ceftazidime (CAZ) and ceftriaxone (CTRX) at a concentration of 4 µg/mL and 2 µg/mL, respectively, which was high in comparison to that observed for the other strains. Furthermore, Raoultella spp. and Serratia spp. were found to be highly resistant to tetracycline (TC) at a concentration of 128 µg/mL and 64 µg/mL, respectively.

Of the 142 strains of test bacteria, 140 (98.6%) demonstrated resistance to ABPC, with the exception of Hafnia alvei and Klebsiella pneumonia. In addition, a total of eight strains (5.6%), seven Serratia marcescens strains and one Raoultella terrigena strain, were found to be resistant to TC. Furthermore, one strain of Citrobacter freundii exhibited resistance to nalidixic acid (NA), while another displayed resistance to ofloxacin (OFLX) (0.7% each), and one strain (0.7%) each of Enterobacter cloacae, Serratia marcescens and Citrobacter youngae demonstrated resistance to fosfomycin (FOM), streptomycin (SM) and kanamycin (KM), respectively. A single strain of C. freundii was found to be resistant to three antibiotics, ABPC, NA and OFLX. Resistance to two antibiotics was confirmed in 11 strains, including seven strains of S. marcescens and one strain of R. terrigena (a total of eight strains) resistant to ABPC and TC, and one strain each of C. youngae, S. marcescens and E. cloacae resistant to ABPC and KM, ABPC and SM, and ABPC and FOM, respectively. In addition, 128 strains were resistant to the single antibiotic of ABPC alone.

Of the 140 strains demonstrating antibiotic resistance, 137 (97.9%) retained the conjugative R-plasmid transfer factor, excluding three strains of S. marcescens. All transfer factors were ABPC and retained by a high proportion of the bacterial groups, with one strain (100%) being resistant to three antibiotics, nine (81.8%) of the 11 strains being resistant to two antibiotics, and 127 (99.2%) of the 128 strains being resistant to a single antibiotic. In addition, we examined ESBL productivity in the 140 strains of bacteria demonstrating drug tolerance; however, no strains exhibited this characteristic. Therefore, further observation is required to ascertain trends in antibiotic-tolerant bacteria.

Key words: Enterobacteriaceae / Antibacterial susceptibility / R-plasmid / ESBLs / Raw horsemeat (basashi).

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Based on the investigative research of the authors conducted to date, *Enterobacteriaceae* has been isolated from commercially sold horsemeat for raw consumption (basashi) at a high frequency of 93.8% (Furuhata et al., 2014). In that study, although no significant differences were observed according to the place of manufacture or sale or site of the specimen in a comparison between domestic (92.6%) and imported (100%) meat, the rate of isolation in imported meat was found to be slightly higher. Among the species of *Enterobacteriaceae* identified, *Hafnia* was the most common, followed by *Klebsiella* and *Enterobacter*, which are coliforms.

Meanwhile, in the field of food hygiene, the problem of drug-resistant bacteria has long attracted attention. Regarding infectious diseases in farm animals, including cows and pigs, antibiotics are frequently used as therapeutic drugs. Moreover, antibiotics are used as a feed additive to prevent infectious diseases and promote the growth of farm animals. As observed in humans, the number of resistant bacteria increases when antibiotics are not used appropriately, leading to the possibility of resistant bacteria being transmitted to humans via meat consumption. In recent years, the generation of drug-resistant bacteria, such as *Salmonella* Typhimurium DT104 (Isakbaeva et al., 2005) and quinolone-resistant *Campylobacter* (Fitch et al., 2005), etc., has been reported.

In particular, extended-spectrum beta-lactamase (ESBL)-producing strains are becoming problematic among drug-resistant bacteria (Mendonça et al., 2007). *Escherichia coli* (Brinas et al., 2005) and *Klebsiella pneumoniae* (Jemima and Verghese, 2008) have been reported as representative strains of ESBL-producing coliforms. Moreover, it has long been known that drug resistance is transmitted via plasmids in Gram-negative bacilli, a phenomenon that is also greatly involved in the increase in drug-resistant bacteria.

Against this background, we carried out drug susceptibility testing among bacterial strains isolated from horsemeat for raw consumption (basashi) with the objective of understanding the current drug resistance status of *Enterobacteriaceae*. In addition, an investigation was conducted regarding the presence of R-plasmid and ESBL productivity.

**MATERIALS AND METHODS**

**Bacterial strains and cultivation**

A total of 142 strains previously isolated from raw edible horsemeat (basashi) samples in Japan were studied (TABLE 1). The breakdown of these strains with regard to the genus was: *Enterobacter* spp.: 36 strains; *Hafnia* sp.: 33 strains; *Klebsiella* spp.: 32 strains; *Serratia* spp.: 16 strains; *Citrobacter* spp.: 10 strains; *Raoultella* sp.: 9 strains; *Escherichia coli* sp. and *Proteus mirabilis* sp.: 3 strains each.

All of the isolates were confirmed to be *Enterobacteriaceae* based on a characteristic test using the API20E system (Sysmex bioMerieux co., Ltd.), with an identification percentage of 85% or greater. The test strains were suspended in 10% skim milk and stored at −80°C. The strains were cultivated in Nutrient agar (Becton, Dickinson and Company) at 36°C for 20 h before use in the susceptibility tests.

**Susceptibility testing**

A total of 14 antibiotics, including ampicillin (ABPC: Sigma-Aldrich Co. LLC), ceftaxime (CTX: Chugai Pharmaceutical Co., Ltd.), cefazidine (CAZ: Nichi-Iko Pharmaceutical Co., Ltd.), ceftriaxone (CTRX: Nichi-Iko Pharmaceutical Co., Ltd.), aztreonam (AZT: Eisai Co., Ltd.), gentamicin (GM: Fuji Pharma Co., Ltd.), kanamycin (KM: Sigma-Aldrich Co. LLC), streptomycin (SM: Wako Pure Chemical Industries, Ltd.), tetracycline (TC: Wako Pure Chemical Industries, Ltd.), chloramphenicol (CP: Sigma-Aldrich Co. LLC), nalidixic acid (NA: Sigma-Aldrich Co. LLC), norfloxacin (NFLX: Sigma-Aldrich Co. LLC), ofl oxacin (OFLX: Sigma-Aldrich Co. LLC) and fosfomycin (FOM: Nichi-Iko Pharmaceutical Co., Ltd.), were used as test antibiotics, and the minimum inhibition concentration (MIC) was measured according to the method of the CLSI (2006). The results were interpreted using the CLSI (formerly National Committee for Clinical Laboratory Standards) criteria (CLSI, 2007).

The testing isolates were smeared in streaks on

**TABLE 1. Test strains.**

| Species                        | No. of strains |
|-------------------------------|---------------|
| *Enterobacter cloacae*        | 26            |
| *E. amnigenus*                | 5             |
| *E. gergoviae*                | 5             |
| *Hafnia alvei*                | 33            |
| *Klebsiella pneumoniae*       | 27            |
| *K. oxytoca*                  | 5             |
| *Serratia marcescens*         | 9             |
| *S. liquefaciens*             | 7             |
| *Citrobacter freundii*        | 7             |
| *C. youngae*                  | 3             |
| *Raoultella terrigena*        | 9             |
| *Escherichia coli*            | 3             |
| *Proteus mirabilis*           | 3             |
| Total                         | 142           |

strains: *Serratia* spp.: 16 strains; *Citrobacter* spp.: 10 strains; *Raoultella* sp.: 9 strains; *Escherichia* sp. and *Proteus* sp.: 3 strains each.
was used to identify NA-resistant bacteria.

**Extended-spectrum β-lactamase (ESBL) producing test**

Double disk synergy testing was carried out in accordance with Jarler’s method (Jarler et al., 1988). That is, after smearing a specific amount of test bacteria solution on the Müeller-Hinton agar, disks for antibiotic sensitivity testing (Japan BD) of amoxicillin/clavulanic acid (AMPC/CVA, 20/10 µg) were placed in the center, with disks of cefotaxime (CTX, 30 µg), ceftriaxone (CTRX, 30 µg), ceftazidime (CAZ, 30 µg), aztreonam (AZT, 30 µg), and cefpodoxime (CDPX, 10 µg) placed radially, each separated by a distance of 25 mm, then cultured at 36°C for 20 h. Subsequently, the bacterial strains observed to display an increase in the inhibition ring between each antibiotic, including AMPC/CVA, CTX, CTRX, CAZ, AZT and CDPX, were classified as ESBL-producing strains.

**RESULTS**

**MIC distribution of basashi-derived Enterobacteriaceae**

TABLE 2 shows the ranges of the MIC, 50% MIC (MIC50) and 90% MIC (MIC90) values of the 14 antibiotics for the test strains. The range of MIC for CAZ, GM, KM, SM, TC, CP, NA, NFLX and OFLX was wide with a unimodal distribution. In contrast, FOM showed a bimodal MIC distribution, with the widest range.

### TABLE 2. Susceptibility of Enterobacteriaceae from raw edible horsemeat (basashi) to 14 antibiotics.

| Antibiotic | Range (µg/mL) | MIC50 (µg/mL) | MIC90 (µg/mL) | Break point* (µg/mL) | No. of resistant strains (%) | n=142 |
|------------|---------------|---------------|---------------|----------------------|------------------------------|-------|
| ABPC       | 8             | >128          | >128          | 8                    | 32                           | 140 (98.6) |
| CTX        | ≤0.031        | 1             | 0.125         | 8                    | 64                           | 0     |
| CAZ        | ≤0.031        | 4             | 0.25          | 8                    | 32                           | 0     |
| CTRX       | ≤0.031        | 4             | 0.125         | 8                    | 64                           | 0     |
| AZT        | ≤0.031        | 0.25          | 0.063         | 8                    | 32                           | 0     |
| GM         | 0.125         | 1             | 0.25          | 4                    | 16                           | 0     |
| KM         | 0.5           | 64            | 1             | 16                   | 64                           | 1 (0.7) |
| SM         | 1             | 32            | 2             | 8                    | 16                           | 1 (0.7) |
| TC         | 0.5           | >128          | 2             | 4                    | 16                           | 8 (5.6) |
| CP         | 1             | 16            | 4             | 8                    | 32                           | 0     |
| NA         | 0.25          | >128          | 2             | 4                    | 16                           | 32 (1) |
| NFLX       | ≤0.031        | 8             | 0.125         | 8                    | 16                           | 0     |
| OFLX       | ≤0.031        | 8             | 0.125         | 2                    | 8                            | 1 (0.7) |
| FOM        | 0.25          | 256           | 8             | 128                  | 64                           | 256 (1) |

*: S=susceptible, R=resistant
According to the susceptibility testing of ABPC, the number of strains with the highest concentration of MIC tested was 74 (>128 µg/mL, 52.1%), indicating a high level of resistance. In contrast, with respect to the susceptibility to CTX, CTRX and AZT, the number of strains with the lowest concentration of MIC tested was 49 (≤0.031 µg/mL, 34.5%), 44 (≤0.031 µg/mL, 30.0%) and 44 (≤0.031 µg/mL, 30.0%), respectively, thus reflecting a high level of susceptibility. In the comparison of the MIC\textsubscript{90} values of the tested antibiotics, ABPC showed the lowest level of antibacterial activity (>128 µg/mL) among the 14 antibiotics, followed by FOM (128 µg/mL). In contrast, the MIC\textsubscript{90} values of the other antibiotics ranged from 0.125 µg/mL to 8 µg/mL, indicating no resistance.

**TABLE 3** shows the MIC\textsubscript{90} values of *Enterobacteriaceae* according to the main genus. When the sensitivity to these antibiotics was examined for each individual genus of the tested bacteria, *Hafnia* spp. was found to be relatively tolerant to ceftazidime (CAZ) and ceftriaxone (CTRX) at concentrations of 4 µg/mL and 2 µg/mL, respectively, which was high in comparison to that observed for the other strains. Furthermore, *Raoultella* spp. and *Serratia* spp. were found to be highly resistant to tetracycline (TC) at concentrations of 128 µg/mL and 64 µg/mL, respectively. At the same time, *Serratia* spp. and *Citrobacter* spp. were found to be sensitive to FOM at a comparatively low level of 4 µg/mL and 2 µg/mL, respectively. Meanwhile, *Enterobacter* spp. and *Klebsiella* spp. showed no significant differences with the other strains of bacteria with respect to any of the antibiotics.

**Antibiotic resistance trends**

Of the 142 strains of test bacteria, 140 (98.6%) demonstrated resistance to ABPC, with the exception of *Hafnia alvei* and *Klebsiella pneumonia*. In addition, a total of eight strains (5.6%), including seven *Serratia marcescens* strains and one *Raoultella terrigena* strain, were resistant to TC. Furthermore, one strain of *Citrobacter freundii* demonstrated resistance to nalidixic acid (NA), while another demonstrated resistance to ofloxacin (OFLX) (0.7% each), and one strain (0.7%) each of *Enterobacter cloacae*, *S. marcescens* and *Citrobacter youngae* demonstrated resistance respectively to fosfomycin (FOM), streptomycin (SM) and kanamycin (KM) (**TABLE 2**).

**TABLE 4** shows the antimicrobial resistance patterns of the *Enterobacteriaceae* strains isolated from raw horsemeat. A single strain of *C. freundii* was found to be resistant to three antibiotics, ABPC, NA and OFLX. Resistance to two antibiotics was confirmed in 11 strains, including seven strains of *S. marcescens* and one strain of *R. terrigena* (for a total of eight strains) resistant to ABPC and TC and one strain each of *C. youngae*, *S. marcescens* and *E. cloacae* resistant to ABPC and KM, ABPC and SM, and ABPC and FOM, respectively. In addition, 128 strains were resistant to the single antibiotic of ABPC alone.

**TABLE 4** shows the frequency of conjugative R-plasmids. Of the 140 strains demonstrating antibiotic resistance, 137 (97.9%) retained conjugative R-
been published concerning drug tolerance among bacteria that cause food poisoning and are found in meat (Van et al., 2007; Ahmed et al., 2009; Robin et al., 2009), although there are few reports related to the drug susceptibility of Enterobacteriaceae, an indicator bacteria for contamination. Therefore, analyzing the distribution of drug resistant bacteria in food provides beneficial information for maintaining food hygiene.

In this study, we tested the drug susceptibility of 142 strains of Enterobacteriaceae isolated from commercially horsemeat intended for raw consumption (basashi) to 14 antibiotics, noting the greatest tolerance to ABPC at a MIC90 of \(128 \mu g/mL\) among 140 strains \(98.6\%\), regardless of the bacterial species. Ishizaki et al. (2011) studied the drug susceptibility of Enterobacteriaceae isolated from commercially meat in Japan and found ABPC resistance in 43 of 67 strains \(64.2\%\), a lower rate of tolerance than that found in the present report. The subject matter used in their study included chicken, pork and beef, while we used only horsemeat, suggesting that the rate of tolerance may differ according to the subject matter.

Hammad et al. (2009) reported a high level \(93\%\) of ABPC tolerance in Enterobacteriaceae isolated from 80 Egyptian cheese samples during drug receptivity testing. Their group studied the mechanisms underlying plasmid transfer factor, with the exception of three strains of S. marcescens. All transfer factors were ABPC and retained by a high proportion of the bacterial groups, with one strain \(100\%\) being resistant to three antibiotics, nine \(81.8\%\) of the 11 strains being resistant to two antibiotics and 127 \(99.2\%\) of the 128 strains being resistant to a single antibiotic (TABLE 4).

**Extended-spectrum \(\beta\)-lactamase (ESBL) production characteristics**

We screened for ESBL productivity using the double disk synergy method among the 140 strains demonstrating tolerance to any one of the test antibiotics and found no augmentation in the inhibition zone in any strain. As a result, no ESBL productive strains were identified.

**DISCUSSION**

The contamination of food by drug-resistant bacteria has recently been reported in the USA and Europe, as well as worldwide (Tadesse et al., 2012; Hiroi et al., 2012). Such contamination can result in serious problems among groups that tend to become infected, including infants, the elderly, hospital inpatients, etc. (Cernela et al., 2014). Furthermore, many reports have

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**TABLE 4.** Antimicrobial resistance patterns of Enterobacteriaceae isolates and the presence of conjugative R-plasmids.

| No. of antibiotic | Resistance pattern | Species                  | No. of isolates (%) | No. of conjugative R plasmid strains (%) | R factor |
|-------------------|--------------------|--------------------------|----------------------|----------------------------------------|----------|
| 3                 | ABPC/ NA/ OFLX     | Citrobacter freundii     | 1 (0.7)              | 1 (100)                                | ABPC     |
|                   | ABPC/ TC           | Serratia marcescens      | 7                    | 5                                      | ABPC     |
|                   |                    | Raoultella terrigena     | 1                    | 1                                      | ABPC     |
| 2                 | ABPC/ KM           | Citrobacter youngae      | 1                    | 1                                      | ABPC     |
|                   | ABPC/ SM           | Serratia marcescens      | 1                    | 1                                      | ABPC     |
|                   | ABPC/ FOM          | Enterobacter cloacae     | 1                    | 1                                      | ABPC     |
| **Subtotal**      |                    |                          | 11 (7.7)             | 9 (81.8)                               |          |
| 1                 | ABPC               | Enterobacter spp.        | 35                   | 35                                     | ABPC     |
|                   |                    | Hafnia alvei             | 32                   | 32                                     | ABPC     |
|                   |                    | Klebsiella spp.          | 31                   | 31                                     | ABPC     |
|                   |                    | Serratia spp.            | 8                    | 7                                      | ABPC     |
|                   |                    | Citrobacter spp.         | 8                    | 8                                      | ABPC     |
|                   |                    | Raoultella terrigena     | 8                    | 8                                      | ABPC     |
|                   |                    | Escherichia coli         | 3                    | 3                                      | ABPC     |
|                   |                    | Proteus mirabilis        | 3                    | 3                                      | ABPC     |
| **Subtotal**      |                    |                          | 128 (90.1)           | 127 (99.2)                             |          |
| 0                 | Hafnia alvei       |                          | 1                    |                                        |          |
|                   | Klebsiella pneumoniae |                          | 1                    |                                        |          |
| **Total**         |                    |                          | 142 (100)            | 137 (96.5)                             |          |
ABPC tolerance from a genetic perspective and found tolerance resulting from the action of TEM-1-type β-lactamase in the highest number of cases (28.3%). In our drug susceptibility study, the highest rate of tolerance was observed for ABPC, and we hope to implement a genetic analysis in order to clarify the mechanisms of ABPC resistance.

In this study, a single strain of C. freundii was found to be resistant to three antibiotics, ABPC, NA and OFLX, the largest number of drugs. Resistance to two antibiotics was confirmed in 11 strains, including seven strains of S. marcescens and one strain of R. terrigena (for a total of eight strains) resistant to ABPC and TC and one strain each of C. youngae, S. marcescens and E. cloacae resistant to ABPC and KM, ABPC and SM, and ABPC and FOM, respectively. Therefore, tolerance to two or more antibiotics was observed in only 12 strains (8.5%).

Ahmed et al. (2009) reported that, in a study of drug susceptibility in 69 strains of Escherichia coli isolated from commercially available chicken meat in Japan, 26 strains (40.6%) showed multiple tolerance, a greater proportion than that observed in our results. In addition, a high proportion (51 strains: 73.9%) showed resistance to ABPC.

The existence of R-factor in resistant bacteria has been known for a long time (Mitsuhashi et al., 1967). In particular, many studies on Escherichia coli have been conducted (Babcock et al., 1973; Bensink et al., 1981: Ishizaki et al., 2011). In this study, of the 140 strains indicating drug resistance, 137 (97.9%) carried conjugative R-plasmids, with the exception of three strains of S. marcescens. The transfer factor was ABPC in all cases and present in one strain (100%) of a triple-drug-resistant bacteria, as well as nine of 11 (81.8%) double-drug-resistant strains and 127 of 128 (99.2%) single-drug-resistant strains. Therefore, regardless of whether the Enterobacteriaceae was Enterobacter spp., Hafnia alvei, Klebsiella spp., Serratia spp., Citrobacter spp., etc., the presence of the ABPC conjugative R-plasmid indicates its role as a contributing factor to ABPC tolerance.

Substrate-specific extended-spectrum β-lactamase (ESBL)-producing bacteria have recently been acknowledged to be one of the most problematic drug-resistant bacteria, with many studies having been reported (Jemima and Verghese, 2008: Anjum et al., 2013; Tschudin-Sutter et al., 2014: Jørgensen et al., 2014). We examined ESBL productivity in the 140 strains of bacteria demonstrating drug tolerance; however, no strains exhibited this characteristic. In the study by Shimojima et al. (2011), however, 31 strains of ESBL-producing Escherichia coli were detected in 22 of 65 specimens of meat commercially available for human consumption in Japan. Although ESBL productivity is noted at a high rate in cases of Escherichia coli (Ahmed et al., 2009), the fact that only three strains of Escherichia coli were found in the samples tested in this study may explain why no ESBL productivity was identified. Further observation is required to ascertain trends in antibiotic-tolerant bacteria.

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