NOTE

Bacteriology

Prevalence of 16S rRNA methylases in Gram-negative bacteria derived from companion animals and livestock in Japan

Masaru USUI1)*, Akari KAJINO1), Michiha KON1), Akira FUKUDA1), Tomomi SATO1), Takahiro SHIRAKAWA2), Michiko KAWANISHI2), Kazuki HARADA3), Chie NAKAJIMA4), Yasuhiko SUZUKI4) and Yutaka TAMURA1)

1)Laboratory of Food Microbiology and Food Safety, School of Veterinary Medicine, Rakuno Gakuen University, Ebetsu, Hokkaido 069-8501, Japan
2)National Veterinary Assay Laboratory, Ministry of Agriculture, Forestry and Fisheries, 1-15-1 Tokura, Kokubunji, Tokyo 185-8511, Japan
3)Department of Veterinary Internal Medicine, Tottori University, Minami 4-101, Koyama-cho, Tottori-Shi, Tottori 680-8553, Japan
4)Division of Bioresources, Hokkaido University Research Center for Zoonosis Control, Sapporo, Hokkaido 001-0020, Japan

ABSTRACT. The emergence and spread of aminoglycoside-resistant bacteria are a public health concern. The acquisition of the genes encoding 16S rRNA methylases, such as armA, rmtA, and rmtB, confers high-level resistance to aminoglycosides. However, the prevalence has not been well investigated in Japanese veterinary fields. To determine the prevalence of 16S rRNA methylases in animals, we detected 16S rRNA methylases genes in Gram-negative bacteria from animals. Here, we report the isolation of rmtB and armA from two of the 446 Escherichia coli (0.5%) and one of the 103 Klebsiella spp. isolates (1.0%) from companion animals, respectively. However, none of the isolations were observed from 2445 E. coli isolates derived from livestock in Japan. The prevalence of 16S rRNA methylases in animals, especially in companion animals, should be carefully monitored in Japanese veterinary fields to avoid the spreading of the genes.

KEY WORDS: aminoglycoside resistance, companion animals, livestock, 16S rRNA methylases

The spread of antibiotic-resistant bacteria and resistance genes in clinical settings and veterinary fields is a global public health concern [23]. Therefore, the WHO recommends the monitoring of antibiotic resistance in each country [25]. In response to this recommendation, the trend in antibiotic resistance in Japanese livestock has been continually monitored since 1999, and this system is known as the Japanese Veterinary Antimicrobial Resistance Monitoring System (JV ARM) [16].

On the other hand, the prevalence of antibiotic-resistant bacteria and resistance genes in companion animals has not been monitored in Japan, and several local and intermittent studies have been reported [7, 18]. In 2016, the government of Japan proposed the “National Action Plan on Antimicrobial Resistance 2016–2020” to address these antimicrobial problems. The importance of antimicrobial surveillance in veterinary fields is also documented from the view of One-Health approach in that plan.

Aminoglycosides are used for severe bacterial infectious diseases in both clinical settings and veterinary fields [13], and emergence of aminoglycoside-resistant bacteria is problematic. In Japan, the amounts of aminoglycosides usage were lower than those of some antibiotics in livestock and companion animals [17]. In aminoglycosides, streptomycin is relatively used in pigs and streptomycin resistance in E. coli are highly isolated from pigs [16, 17]. Most aminoglycosides bind to the decoding aminoacyl-tRNA recognition site of the 16S rRNA that is part of the bacterial 30S ribosome, and subsequently interfere with bacterial growth through inhibition of protein synthesis [15]. Bacteria acquire resistance against aminoglycosides through several mechanisms. Aminoglycoside-modifying enzymes [aminoglycoside acetyltransferase (AAC), aminoglycoside phosphotransferase (APH), and aminoglycoside nucleotidyltranferases (ANT)], which inactivate specific aminoglycosides, are the most prevalent mechanisms [21]. In addition, 16S rRNA methylases has been reported as a novel aminoglycoside resistance mechanism [24]. Differently from aminoglycoside-modifying enzymes, 16S rRNA methylases can confer high-level aminoglycoside resistance by modifying specific nucleotides in the aminoglycoside binding site of 16S rRNA [24].

In general, 16S rRNA methylases genes are located in transferable plasmids [24]. In addition, 16S rRNA methylases-harboring...
plasmids frequently contain other classes of antibiotic resistance genes such as β-lactamase genes [24]. Therefore, these observations warn the increase of multi-drug resistant bacteria by an acquisition of 16S rRNA methylase-harborong plasmid. Although 16S RNA methylates possessing Gram-negative bacteria has been found in livestock and companion animals in foreign countries [5, 24, 27], the isolation and prevalence have not been reported in Japanese animals. In this study, we investigated the prevalence of 16S rRNA methylase genes in Gram-negative bacteria isolated from companion animals and livestock in Japan.

A total of 212 and 234 Escherichia coli isolated from feces samples of dogs that visited 13 veterinary hospitals (all these hospitals are located in Ebetsu city, Japan) in 2005 and 2015–2016, respectively. A total of 1,029 and 1,418 E. coli isolates derived from livestock (cattle, pigs, and chicken feaces) were collected by the JVARM in 2004–2005 and 2013–2014, respectively [16]. In addition, a total of 103 Klebsiella spp. [9], 60 Enterobacter spp. [8], and 81 Acinetobacter spp. isolates, obtained from clinical specimens collected from dogs and cats between 2003 and 2015, were used for this study. Bacterial identification was conducted using matrix-assisted laser desorption/ionization—time of light mass spectrometry (MALDI-TOF MS) with the Bruker MALDI Biotyper system (Bruker Daltonics, Bremen, Germany) [3].

To screen aminoglycoside-resistant isolates, minimum inhibitory concentrations (MICs) of gentamicin (Sigma-Aldrich, St. Louis, MO, U.S.A.) were determined using the agar dilution method according to Clinical Laboratory Standards Institute (CLSI) guidelines [2, 24]. E. coli ATCC25922, Staphylococcus aureus ATCC29213, Enterococcus faecalis ATCC29212, and Pseudomonas aeruginosa ATCC27853 were used as quality control strains. In gentamicin-resistant isolates, MICs of several types of aminoglycosides [amilacin, neomycin, and apramycin (all obtained from Sigma-Aldrich)] were determined to screen putative 16S rRNA methylase positive isolates according to a previous study [24]. Interpretations for neomycin and apramycin resistance were defined according to a previous report [10], because of the no definition in CLSI guidelines. MIC of arbekacin (Sigma-Aldrich), which is not modified by most aminoglycoside-modifying enzymes, was also determined in the putative 16S rRNA methylase positive isolates [4]. The breakpoint for arbekacin was similar to that for amikacin as defined by CLSI guidelines (≥32 µg/ml) [2].

Some E. coli isolates derived from canine feces were resistant to gentamicin (MIC ≥16 µg/ml; 15.6 and 29.5% in 2005 and 2015–2016, respectively; Table 1). In contrast, E. coli isolates derived from livestock feces were rarely resistant to gentamicin (1.9 and 1.0% in 2004–2005 and 2013–2014, respectively; Table 1). Gentamicin-resistant Klebsiella, Enterobacter, and Acinetobacter spp. isolates derived from companion animals in 2003–2015 were 31.1, 26.2, and 6.2% of the total, respectively (Table 1). From the MIC values of aminoglycosides, three putative 16S rRNA methylases-positive isolates were found in two E. coli isolates (RGU-60 and RGU-78) and one Klebsiella pneumoniae isolate (KL39) (Table 2). In contrast, none of the putative 16S rRNA methylase positive isolates were found in Enterobacter or Acinetobacter spp. derived from companion animals.

Three of the putative 16S rRNA methylase-positive isolates were screened for 16S rRNA methylases genes (i.e., armA, rmtA, rmtB, rmtC, rmtD, rmtE, and npmA) using PCR and DNA sequencing [4, 6, 29]. The rmtB gene was found in two E. coli isolates from dogs in 2005 (0.9% of total isolates in 2005 and 0.5% of E. coli isolates from canine feces), and armA gene was found in one K. pneumoniae isolate (1.0%) derived from canine feces in 2015 (Tables 1 and 2).

MICs of streptomycin, ampicillin, and tetracycline (all obtained from Sigma-Aldrich) were determined as described above. Each isolate was evaluated for the presence of genes for aminoglycoside-modifying enzymes (aadA1, aac(3), aadA2, aphA1, aphA2, strA, strB, and aac(6′)-Ib-cr) [14, 19], β-lactamases (blaTEM, blaSIM2, blaCMY-2, blaGAL, and blalDH) [12, 20, 28], and tetracycline resistance (tetA, tetB, tetC, tetD, tetE, and tetG) [11] using PCR.

Isolates RGU-60 and KL39 were resistant to neomycin and possessed the aminoglycoside phosphotransferase gene aphA1, which is related to neomycin (fradiomycin) and kanamycin resistance (Table 2). All three 16S rRNA methylases-positive isolates were resistant to streptomycin and possessed the aminoglycoside phosphotransferase genes, strA and strB, which are related to streptomycin resistance. All three 16S rRNA methylases positive isolates were resistant to ampicillin and possessed the β-lactamase gene (Table 2). In addition, isolate KL 39 possessed the β-lactamase genes blaSIM2 and blaDH, and isolates RGU-60 and KL39 were resistant to tetracycline and possessed the tetA gene.

Transferability of 16S rRNA methylases genes in E. coli was determined using previously described broth-mating methods, with slight modifications [22]. Briefly, the recipients were rifampicin-resistant E. coli K12 DH5α strain and mating was conducted at 37°C. Transconjugants were isolated on Mueller-Hinton agar supplemented with 50 µg/ml rifampicin (Sigma-Aldrich) and 4 µg/ml gentamicin. Broth-mating methods were repeated three times. Some transconjugants were randomly selected and characterized by MIC determination of tested antibiotics and possession of antibiotic resistance genes.

The rmtB genes were transferred from isolates RGU-60 and RGU-78 to the recipient strain using the broth mating method, with transfer frequencies of 1.7 × 10−5 and 2.4 × 10−3, respectively (Table 2). All transconjugants were resistant to gentamicin, amikacin, arbekacin, streptomycin, and ampicillin (Table 2) and possessed the β-lactamase gene (Table 2). In addition, isolate KL39 possessed the β-lactamase genes blaSIM2 and blalDH, and isolates RGU-60 and KL39 were resistant to tetracycline and possessed the tetA gene.

In this study, the prevalence of gentamicin resistance in E. coli isolates from Japanese livestock was found to be low (<2.0%), and no 16S rRNA methylase genes were found in these isolates. In China, amikacin-resistant E. coli and 16S rRNA methylases genes (rmtB, armA, and rmtE) were observed in 19.7 and 18.5% of isolates derived from livestock, respectively [27]. In general, usage of veterinary antibiotics appears to contribute to the appearance of antibiotic resistance in E. coli isolates from healthy livestock [1]. In addition, an increase in prevalence of 16S rRNA methylases genes is attributed to use of amikacin and/or arbekacin [24]. These antibiotics are used as growth promoters in livestock in China [27] but not in Japan. Therefore, these observations may explain the low prevalence of gentamicin-resistant E. coli in livestock in Japan.

Our results showed the higher prevalence of gentamicin-resistant Gram-negative bacteria in companion animals than in
Table 1. Prevalence of gentamicin-resistant bacteria and 16S rRNA methylases genes in Japanese animals

| Origin Bacterial species                                                        | Year       | n    | Gentamicin resistance | 16S rRNA methylases |
|---------------------------------------------------------------------------------|------------|------|-----------------------|---------------------|
| Canine fecal samples *Escherichia coli*                                         | 2005       | 212  | 33 (15.6%)            | rmtB 2 (0.9%)       |
| E. coli                                                                        | 2015–2016  | 234  | 69 (29.5%)            | 0                   |
| Cattle, Pig, Chicken fecal samples *E. coli*                                     | 2004–2005  | 1,029| 20 (1.9%)             | 0                   |
|                                                                                   | 2013–2014  | 1,418| 14 (1.0%)             | 0                   |
| Canine, Feline clinical specimens *Klebsiella spp.*                             | 2003–2015  | 103  | 32 (31.1%)            | armA 1 (1.0%)       |
| *Enterobacter spp.*                                                              | 2003–2015  | 65   | 17 (26.2%)            | 0                   |
| *Acinetobacter spp.*                                                             | 2003–2015  | 81   | 5 (6.2%)              | 0                   |

a) Gentamicin resistance indicates a minimum inhibitory concentration ≥16 µg/ml.

Table 2. Characterization of 16S rRNA methylases-positive isolates from dogs and their transconjugants

| Strain name | Bacterial species       | Year       | Microdilution (µg/ml)a) | Aminoglycoside-modifying enzyme | Beta-lactamase genes | Tetracycline resistance gene |
|-------------|-------------------------|------------|-------------------------|--------------------------------|----------------------|-----------------------------|
| RGU-60      | *Escherichia coli*      | 2005       | >256                    | rmtB                            | aphA1, strA, strB    | blayEM                      |
| RGU-78      | *E. coli*               | 2005       | >256                    | rmtB                            | strA, strB           | blayEM                      |
| KL39        | *Klebsiella pneumoniae* | 2015       | >256                    | armA                            | aphA1, strA, strB    | blayEM, blaySHV, blayDHA    |
| Recipient   | DH5α                    |            | 0.25                    |                                  |                      |                             |
| Transconjugants | TC-RGU-60-1             |            | >256                    |                                  | strA, strB           | blayEM                      |
| TC-RGU-60-2 | >256                    | 16         | >128                    |                                  | strA, strB           | blayEM                      |
| TC-RGU-60-3 | >256                    | 16         | >128                    |                                  | strA, strB           | blayEM                      |
| TC-RGU-78-1 | >256                    | 16         | >128                    |                                  | strA, strB           | blayEM                      |

a) Values in parentheses indicate breakpoints. Bold type indicates resistance to individual antibiotics. b) Clinical Laboratory Standards Institute (CLSI) breakpoints [2]. c) Previously reported breakpoints [10]. d) Reference from CLSI breakpoints for amikacin. ABK; arbekacin, ABPC; ampicillin, AMK; amikacin, APR; apramycin, GM; gentamicin, MIC; minimum inhibitory concentration, NEO; neomycin, SM; streptomycin, TET; tetracycline.
livestock. Although the total amount of aminoglycosides usage in companion animals were not higher than those of the other antibiotics in companion animals, the amount of gentamicin usage were more than half in aminoglycosides [17]. Gentamicin are frequently used for companion animals mainly as external medicine (Personal communication). External usage of antibiotics would affect the antibiotic resistance in Enterobacteriaceae, although the direct verification has not been reported. The high rates of gentamicin resistance in companion animals would be related to the usage of gentamicin in companion animals.

16S rRNA methylases-positive strains were resistant to not only aminoglycosides but also to other classes of antibiotics, and rmtB genes were easily transferred to other E. coli strains. In addition, some resistance genes (strA, strB, and blaTEM) were invariably transferred with 16S rRNA methylases genes. In general, 16S rRNA methylases genes are present in plasmids with other antibiotic resistance genes [24]. Although more detailed analyses are needed to characterize the 16S rRNA methylases-harboring plasmid found in this study, these plasmids contribute to multi-drug resistance.

Two types of 16S rRNA methylases genes were detected in companion animals, which are in close contact with humans. It indicates the possibility of the transmission of 16S rRNA methylase gene possessed bacteria and/or the harboring plasmids between human and companion animals, at relatively easy. According to the previous study, the sporadic spread of a specific K. pneumoniae lineage that possessed rmtB has been reported from companion animals, and this lineage (ST37) is also isolated form clinical setting in China [26]. This observation may support the possibility. On the contrast, the prevalence of 16S rRNA methylase genes in companion animals in this study was lower compared with the neighbor country, China, corresponding with the low prevalence of 16S rRNA methylase genes in human clinical settings in Japan [24]. Thus, current risk of the transmission of 16S rRNA methylase gene between human and companion animals should be estimated low in Japan. However, it should be required the continuous monitoring from the view of multidrug resistance of the isolated 16S rRNA methylase positive bacteria and the co-transferable aspect with other antimicrobial resistance.

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