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A New Evaluation Method for Antibiotic-Resistant Bacterial Groups in Environment

Katsuji Watanabe[1*], Naoto Horinishi[1], Kunimasa Matsumoto[1], Akihiro Tanaka[2], Kenichi Yakushido[3]

[1]Department of Life, Environment and Materials Science, Fukuoka Institute of technology, Fukuoka, Japan
[2]National Agricultural Research Center for Kyushu-Okinawa Region, National Agriculture and Food Research Organization, Kumamoto, Japan
[3]National Agricultural Research Center, National Agriculture and Food Research Organization, Tsukuba, Japan
Email: *k-watanabe@fit.ac.jp

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Abstract

In the present manuscript it was presented whether spreading of antibiotic resistant bacterial groups in environment could be monitored by our newly developed method by enumerating antibiotic resistant bacterial groups in various biological wastes and composts. Although the numbers were not so high, diverse kinds of colistin resistant bacteria (25 mg·L^{-1}) were included in row cattle feces (1.78 × 10^4 MPN g^{-1}) and cattle feces manure (>3.84 × 10^4 MPN g^{-1}). Compost originated from leftover food (>44.8 × 10^4 MPN g^{-1}) and shochu lee (>320 × 10^4 MPN g^{-1}) included higher numbers of chlortetracycline resistant *Pseudomonas* sp., (25 mg·L^{-1}), and row cattle feces included higher numbers of chlortetracycline resistant Enterobacteriacea (15.7 × 10^4 MPN g^{-1}), which mostly consisted from *Pantoea* sp. or *Xenorhobdus doucetiae*. Numbers of multi drug resistant bacteria, resistant to 25 mg·L^{-1} of ciprofloxacin, streptomycin, chloramphenicol, and ampicillin, were the highest in row cattle feces (>143.6 × 10^4 MPN g^{-1}), followed by cattle feces manure (4.19 × 10^4 MPN g^{-1}), and shochu lee (0.36 × 10^4 MPN g^{-1}), which included diverse kinds of bacterial group. The present results indicated that higher numbers of multi drug resistant bacteria were typically found in row cattle feces, and the method was found suitable to enumerate and identify them. These results suggested that the method might become their environmental risk evaluation method.

Keywords

Colistin Resistant Bacteria, Chlortetracycline Resistant Bacteria, Multi Drug Resistant Bacteria, Multiple Enzyme Restriction Fragment Length Polymorphism Analysis, The Most Probable

*Corresponding author.

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Number Method, Microchip Electrophoresis System

1. Introduction

At present, spreading of antibiotic resistant bacteria is becoming a major public health problem in all over the world [1]. As the susceptibility tests using bacterial isolates, with respect to specific nosocomial bacteria, were indispensable not only to search effective antibiotic for patient but also to prevent their nosocomial infection, the method has been used as a standard surveillance method for their risk assessment [2]-[4]. However their spreading area was recently expanding over various environments, such as drinking water [5]-[7], or vegetables [8]-[10], or daily products [11]-[13], due to their overuse not only as therapeutic agent in human and veterinary medicine but also as growth promotor in animal husbandry [14] [15], and the susceptibility tests and taxonomy determinations must be broadly expanded over a large numbers of environmentally important bacterial groups in order to know what kinds of antibiotic bacteria will be numerically dominant and then has a higher environmental risk [5]-[11] [13] [15]-[18].

With respect to the antibiotic bacteria, their taxonomic positions had no relation to the antibiotic resistance which was irregularly evolved by acquiring diverse kinds of resistant genes. Therefore molecular-based analysis method could not be used for their risk assessment because bacterial phylogenetic positions estimated by the unculture-based community analysis methods, such as DGGE or t-RFLP or clone library sequencing or pyrosequencing [19], had no-relation to those of the resistant bacterial groups. Although sequence-based metagenomics [20] [21] and real time PCR [22]-[25] targeting resistant gene afforded the information of the diversity or copy numbers of the resistant gene, they had no relation to the phylogenetic positions nor phenotypic properties of the resistant bacterial groups.

As prime feature of resistant bacteria distinguished from susceptible one was an ability to survive and proliferate under antibiotic, which was also concerned with one of their risk, we thought that environmental risk of antibiotic resistant bacteria in might be evaluated by identifying and quantifying bacteria grown under application of antibiotic. Until now, we had presented a new method to provide numbers of each taxonomically different bacterial groups in the former papers [26] [27]. By the method, sample having simple microbial diversity such as food and aquatic sample could be analyzed without cultivation, that having the huge microbial diversity such as soil and manure required cultivation before analysis for exact phylogenetic estimation [26] [27]. In this manuscript, bacterial groups resistant to colistin, chlortetracycline, and multi drugs, in row cattle feces, cattle feces manure, shochu lee, and compost originated from leftover food were identified and enumerated by the method adding these antibiotics.

2. Materials and Methods

2.1. Samples

Row cattle feces (R), which would be converted into cattle feces manure, was collected from a cow barn for daily cattle in National Agricultural Research Center for Kyushu-Okinawa Region, where daily cattle were breeding in Japanese standard method [28]. Cattle feces manure (M) was provided from farmer around National Agricultural Research Center for Kyushu-Okinawa Region. Shochu lee (S), residual aqueous solution of sweet potato after fermentation and distillation of shochu, was obtained from Akashi Shuzo Co. Ltd. (Miyazaki, Japan), which would be converted into compost. Compost originated from leftover food (L) was collected in composting facility in Kumamoto prefecture (Kumamoto, Japan).

2.2. MPN and Used Antibiotics

The number of resistant bacteria was estimated by MPN using lactose broth (LB medium Difco, Sparks MD) by adding colistin (25 $mg \cdot L^{-1}$) (P; polymyxin E), which was not used as therapeutic agent in human nor as animal growth promoter (AGP) in Japan, or chlortetracycline (T) (25 $mg \cdot L^{-1}$), which was used widely as therapeutic agent in human and veterinary medicine but also used as AGP. For multi drug resistant bacteria (X) the following antimicrobial compounds were co-applied; ciprofloxacin (25 $mg \cdot L^{-1}$), streptomycin (25 $mg \cdot L^{-1}$), chloramphenicol (25 $mg \cdot L^{-1}$), and ampicillin (25 $mg \cdot L^{-1}$). Serial 10-fold dilutions (10^{-2} to 10^{-5}) prepared from samples (1g fresh wt.) were inoculated to test vials (5 replicates) including LB medium and the antibiotics. After 5 days

incubation at 30˚C, bacterial DNA in each vial was extracted as described previously and purified by conventional methods [26] [27].

2.3. Maintaining the Integrity of the Specifications

Using the V2 forward primer (41f; 5 'GCTCAGATTGAACGCTGGCG3'), and the V6 reverse primer (1066r; 3 'GTCGAGCACAACACTTTACA5') [29], 16S rDNA of about 1070 bp length was amplified as described previously [30] [31]. Their restriction fragment lengths were measured by microchip electrophoresis systems (Cosmo-i SV1200; Hitachi Electronics Engineering Co., Ltd. Tokyo Japan, or MCE-202 MultiNA; Shimadzu Co., Ltd. Kyoto Japan) after digestion of the PCR product (10 µl) using each restriction enzyme, *Hae*III or *Hha*I or *Rsa* I (10 units, Takara Bio Co. Ltd. Shiga Japan) in buffer solution (10× Low salt buffer, Takara Bio Co. Ltd.) and 5 folds dilution by de-ionized water as described previously [30] [31].

2.4. Reference Database Used for the Phylogenetic Estimation

The reference database used for this research included 30,844 post-amplification sequence files for the 41f/1066r primers, which were mainly re-edited from small subunit rRNA files in RDP II release 9_61 [32] under 5 - bases mismatches in the both in primer annealing sites and were consisted from 1379 bacterial genera, including uncultured and unidentified bacteria.

2.5. Data Processing for Multi-Template DNA and Phylogenetic Estimation

As each MPN vials included multi-template DNAs originated from heterogeneous bacteria, the measured MERFL digested from the homogeneous 16S rDNA was selected among the mixed MERFLs digested from the heterogeneous 16S rDNA as described previously [26]. Because all the reference MERFLs were originated from the homogeneous 16S rDNA sequence. The major RFs (represented as H in **Tables 1-3**) were those with the highest relative mole concentration (ratio of fluorescent intensity to fragment size). After subtraction of the major RFs from the mixed heterogeneous RFs, the 2nd major RFs were similarly selected (represented as M in **Tables 1-3**). After subtraction of the 2nd major RFs from the remained heterogeneous RFs, the 3rd major RFs were similarly selected (represented as L in **Tables 1-3**). The similarity between the measured RFLP (A) and the reference RFLP (B) was calculated as described previously [30] [31] based on the pairwise distance (D_{AB}) according to Nei and Li [33]. The pairwise distance of the MERFLPs (D_{ABME}) was an average of all the D_{ABs} for used restriction enzymes. Similarity (%) was (1-D_{ABME}) × 100 (**Tables 1-3**). In the phylogenetic estimation, combinations of the 2 restriction enzymes was used when the identical reference MERFL (100% similarity) was not found using all of the measured MERFL for the 3 restriction enzymes. When the identical reference MERFL to the measured MERFL for 2 restriction enzymes was not found, the reference MERFL having the highest similarity (over 80%) to the measured MERFL was indicated in most cases (**Tables 1-3**) [30] [31].

2.6. Enumeration of Antibiotic Resistant Bacterial Groups by MPN

By five-tube, three-decimal-dilution experiment, MPNs of each antibiotic resistant bacterial groups (A~K) were estimated (**Tables 4-6**). Using FDA's Bacterial Analytical Manual [34], confidence limits were obtained and shown in the Tables.

3. Results

3.1. Phylogenetic Estimation of Antibiotic Resistant Bacteria

Affiliations of 67 MERFLs of colistin resistant bacteria (P) in each MPN vials were summarized in **Table 1**. All of the 67 MERFLs were divided into 67 OTUs, then ratio of total number of the OTUs to the total number of MERFLs was 100% (diversity of MERFLs), which was the highest among all the samples analyzed until now [26] [27]. Affiliations of 112 MERFLs of chlortetracycline resistant bacteria (T) in each MPN vials were summarized in **Table 2**. All of the 112 MERFLs were divided into 88 OTUs, then ratio of total number of the OTUs to the total number of MERFLs was 78.6 %, which was lower than that of P. Some *Pseudomonas* sp. (E) in shochu lee (S) (7 MERFLs) and compost originated from leftover food (L) (8 MERFLs) were placed in the same OUT (**Table 2**) and some Enterobacteriacea (F) in row cattle feces (R) (8 MERFLs) were placed in the same

Table 1. Affiliation of colistin resistant bacteria grown in serially diluted LB medium by MERFL[a].

	Vial No.[b]	Restriction enzymes[c]	Similarity (%)[d]	Name (Accession number)[e]
A	PR^{-4}1M	Ha, R	83	*Corynebacterium glutamicum* (BX9217148, BX927150, BX927152, BX927156)
	PM^{-4}4H	Ha, Hh	92.9	uncultured *Corynebacterium* (AM420211)
	PM^{-5}1H	Ha, R, Hh	88.9	*Nocardiopsis* sp. (AF361322)
	PL^{-2}1H	R, Hh	92.9	*Rhodococcus erythropolis* (AB177886, AY168580)
B	PR^{-3}3H	Ha, R, Hh	95	*Bacillus* sp. (AY461745, AY461746, AY461756), *B. thuringiensis* (AY461762)
	PR^{-3}3M	Ha, Hh	87.5	*B.oleronius* (AF393508)
	PR^{-3}5H	Ha, R, Hh	95	*Bacillus* sp. (AY461742, AY461750)
	PR^{-5}1H	R, Hh	100	*Bacillus* sp. (AF326359)
	PM^{-5}4H	Ha, R, Hh	90.5	*B. oleronius* (AY988598, X82492) *Exguobacterium* sp. (DQ246625)
	PM^{-5}3H	R, Hh	100	*Bacillus* sp. (AY461742, AY461750, AJ878858)
	PC^{-3}1M	Ha, R, Hh	100	*Bacillus* sp. (AY566219, AY583458), *B. pallidus* (Z26930)
	PL^{-3}2M	Ha, R	83.3	*B. laevolacticus* (B.lvolact3), *B. racemilacticus* (D16278), *Gracilibacillus halotolerans* (Grb. haltol)
C	PR^{-2}2M	Ha, Hh	100	*Clostridium* sp. (AY957603), *C. malenominatum* (M59099), *Anaerococcus hydrogenalis* (D14140), *Lactobacillus aviaries* (M58808)
	PS^{-4}5M	Ha, R	100	*C. collagenovorans* (C. colgenvo), *C. grantii* (C. grantii), *C. kluyveri* (M59092, CP000673), *Anaeroplasma bactoclasticum* (M25049) ([f]J)
	PL^{-3}1L	Ha, Hh	100	*C.butyricum* (M59085), *Ureaplasma diversum* (D78650), *U. canigenitalium* (D78648)
	PR^{-3}4H	Ha, R, Hh	86	*Alicyclobacillus hesperidum* (AB059678, AB059679), *A. sacchari* (AB262020)
	PR^{-2}3H	Ha, Hh	100	*A. pomorum* (AB089840), *Streptococcus mutans* (AF139601), *Enterococcus faecalis* (AY94256)
	PR^{-2}2H	Ha, R	100	*Syntrophomonas wolfei* (CP000448)
	PL^{-2}4H	Ha, R	100	*S. erecta* (DQ86234), *S. sporosyntrop* (DQ112186)
	PR^{-2}4L	Ha, Hh	100	*Paenibacillus peoriae* (D78476), *Bacillus brevis* (X60612), *B. edaphicus* (AB045093)
	PL^{-3}3L	Ha, R, Hh	82.2	*P. lautus* (D85394, D85609)
	PM^{-4}3L	Ha, Hh	85.6	*Ruminococcus albus* (AY445592),
	PL^{-4}4M	Ha, R	100	*R. productus* (AY937379), *Bacillus edaphicus* (B. edaphicu) ([f]B)
	PR^{-2}1H	Ha, R, Hh	85.6	*Desulfotomaculum nigrificans* (AB026550)
	PR^{-2}4M	Ha, R, Hh	93.3	*Desulfosporosinus orientis* (Ds.orient2)
	PR^{-4}5H	R, Hh	100	*Leuconostoc gelidum* (AB004661)
	PM^{-4}4L	Ha, R	100	*Lactobacillus vaccinostercus* (AB218801), *L. bifermentans* (M58809), *L. coryniformis* (M58813)
	PR^{-3}2H	Ha, R, Hh	100	*Slenomonas ruminantium* (AB198430, AB198432, AB198433, AB198438), M*egamonas hypermegale* (AJ420107)
	PL^{-3}1H[g]	Ha, R	100	*Staphylococcus aureus* (CP000730), *S. cohnii* (Stp.cohni3)
D	PR^{-4}1H	Ha, R	100	*Sphingomonas* sp. (Y12803)
	PL^{-3}1H[g]	R, Hh	100	*S. mali* (Y09638), *S. pruni* (Y09637), *S. asaccharolyticas* (Y09639)
	PR^{-5}4H	R, Hh	94.4	*Methylobacrerium* sp. (Mlb.sp.PK1, Mlb.sp.PR6), *Methylosporovibrio methanic*a (Mls.methan)
	PM^{-5}2H	Ha, R, Hh	84	*Orientia tsutsugamuchi* (AM494475)
	PM^{-4}3H	Ha, R	100	*Acidosphaera rubrifaciens* (D86512), *Erythrobacter longus* (Erb. longus)
	PL^{-4}4H	Ha, R, Hh	93.3	*Rhodopseudomonas acidophilas* (M34128)
	PR^{-2}1M	Ha, R, Hh	86.7	uncultured beta proteobacterium (AB294945)
	PS^{-4}5L	Ha, Hh	83.3	*Telluria mixta* (X65589)
	PL^{-3}2H	Ha, R, Hh	93.3	*Ralstonia solanacearum* (AY642432)

Continued

E	PR^{-3}4M	Ha, Hh	83.3	*Pseudomonas* sp. (DQ279343), *P. stutzeri* (U26262),
	PR^{-5}5H	Ha, R, Hh	86.9	*Pseudomonas sp.* (AM410901), *Hahella chejuensis* (CP000155)
	PS^{-4}4H	Ha, R, Hh	91.7	*Pseudomonas sp.* (AM111028)
	PS^{-4}5H	Ha, R	100	*P. putida* (DQ232745)
	PL^{-3}3M	Ha, R, Hh	94.8	*P. caricapapayae* (D84010)
F	PR^{-4}4H	Ha, R, Hh	85	*Vibrio* sp. (DQ173039), *Methylobacillus flagellates* (CP000284)
	PM^{-4}3M	R, Hh	100	*Vibrio* sp. (DQ146975), *V. harveyi* (AY911396,AY911387), *V. carchariae* (X74693),
G	PR^{-3}2M	Ha, Hh	100	*Xenorhabdus indica* (AM040494)
	PR^{-5}2H	Ha, R	93	*Photobacterium profundum* (CR378665, CR378680), *Thermoanaebacterium therm* (Tbm. thslf) ([f]C), *Eubacterium yurii* (Eub. yurii) ([f]C).
	PR^{-5}3H	R, Hh	92.9	uncultured gamma proteobacteria (AF445671)
	PM^{-4}4M	Ha, R	100	*Marinobacter* sp. (AB089803), *Pseudomonas* sp. (AM110949), *Sporolactobacillus laevis* (D16287) ([f]C)
	PL^{-3}5M	Ha, R, Hh	88.9	*Nitrococcus mobilis* (L35510,)
	PL^{-2}4M	Ha, R	100	*Nitrococcus mobilis* (Nc. mobilis), *Legionella hackeliae* (Leg. hackel)
H	PM^{-5}1M	Ha, Hh	100	*Desulfovibrio marrakechensis* (AM947130)
	PL^{-3}3H	Ha, R, Hh	91.7	uncultured delta proteobacteria (AY771945)
	PL^{-3}4H	Ha, R, Hh	90.5	*Desulfobacterium* sp. (DQ146482)
	PL^{-3}5H	Ha, R, Hh	85.7	*Stigmatella erecta* (AJ233933), *S. aurantiaca* (AJ233936, AJ233937)
	PL^{-3}5L	Ha, R	92.9	*Desulfomonile tiedjei* (M26635)
	PL^{-2}1M	Ha, R	100	*Corallococcus coralloides* (AY072739)
J	PR^{-3}1H	R, Hh	100	uncultured Gemmatimonadetes (AY9211783, AY921939, AY921994, AY922110)
	PM^{-4}1H	Ha, R, Hh	90.5	uncultured Acidobacteria bacterium (AY922163)
	PL^{-3}4M	Ha, R, Hh	84.1	*Deinococcus murrayi* (Y13042)
K	PR^{-2}4H	Ha, R, Hh	93.3	uncultured rumen bacterium (AB034009, AB185580)
	PR^{-3}5M	Ha, Hh	100	uncultured bacterium (AB240503)
	PM^{-5}3M	R, Hh	83.3	uncultured bacterium (AY854278)
	PM^{-5}4M	Ha, Hh	92.9	uncultured bacterium (AY768822, DQ251791)
	PS^{-4}4M	R, Hh	100	halophilic bacterium(AB042504)
	PR^{-2}3M	R, Hh	83.3	*Adiantum pedatum* (AF244549)
	PM^{-4}2M	Ha, R	94.4	Olavius loisae endosymbiont (AF104475)
	PM^{-4}2L	Ha, R	90	marine psychrophile IC079 (U85854)

[a]Grouping was based on affiliation by MERFL; Actinobacteria (A), *Bacillus* spp. (B), the other Firmicutes (C), *α*, *β*-Proteobacteria (D), *Pseudomonas* sp. (E), Enterobacteriacea (F), the other *γ*-Proteobacteria (GG), *δ*, *ε*-Proteobacteria (H), Cytophaga (I), the other bacteria (J), and unidentified or uncultured bacterial group (K). [b]The 1st letter in vial indicates used antibiotics; "P" stands for colistin (polymyxin E). The 2nd letter in vial indicates samples; "R" stands for row cattle feces, "M" stands for cattle feces manure, "S" stands for shochu lee, and "L" stands for compost originated from leftover food. Exponential of vial number represents the decimal dilution of the vial. The 2nd number of vial number (1 - 5) represents number in 5 replicates for the each decimal dilution. "H" of last letter represents MERFL originating from the major 16S rDNA, "M" represents from the 2nd major 16S rDNA, and "L" represents from the 3rd major 16S rDNA. [c]Restriction enzymes used for similarity search; "Ha", "R", and "Hh" stand for *Hae* III, *Rsa* I, and *Hha* I. For the measured MERFLP which had no completely identical theoretical MERFLP, the theoretical MERFLP having the highest similarity using all the RFLPs was presented with the similarity as described in the materials and method. [d]The similarity between the measured RFLP (A) and the reference RFLP (B) was calculated based on the pairwise distance (D_{AB}) according to Nei and Li [33]. [e]Species name (accession number) of the theoretical MERFL having the highest similarity with the measured MERFL. [f]The theoretical MERFL (accession number) having the same MERFL belonged to different group in parenthesis. [g]The MERFL falling into different groups by using the different restriction enzymes.

Table 2. Affiliation of chlortetracycline resistant bacteria grown in serially diluted LB medium by MERFL[a].

	Vial No.[b]	Restriction enzymes[c]	Similarity (%)[d]	Name (Accession number)[e]
A	TM^{-3}2M	R, Hh	90	*Streptomyces* sp. (DQ250003), *S. filamentosus* (DQ026632), *S. bikiniensis* (Stm. bikini)
	TR^{-4}1M	R, Hh	100	*Streptomyces* sp. (U93336, U93338), *Streptoverticillium baldaccii* (X53164), *S. abikoense* (X53168)
	TM^{-5}1H[g]	Ha, R	100	*Microbacterium flavescens* (Mbm. Flaves), *M. hominis* (Mbm. homini), *Stomatococcus mucilaginosus* (Stt. muclag) ([f]F)
	TM^{-5}3H	Ha, Hh	100	*Gordonia defluvii* (AY650267)
	TM^{-4}1M	Ha, R	100	uncultured actinobacterium (AY792227)
	TR^{-2}1H	Ha, R, Hh	95.2	uncultured actinobacterium (AY792228)
	TM^{-3}1L[g]	Ha, Hh	83.3	*Bifidobacterium* sp. (AF321296), *B. subtile* (D89378, D89379), *B. gallicum* (D86189), *B.magnum* (D86193)
	TS^{-3}1L	R, Hh	100	*Kutzneria kofuensis* (AF114801)
	TS^{-3}2H	R, Hh	100	*Propionibacteriacease bacterium* (AB298731), *Mycobacteriaceae bacterim* (AB298730)
	TL^{-3}4H	R, Hh	92.9	*Streptacidiphilus neutrinimicu* (AF074409), *Lactobacillus catenaformis* (L. catenofo) ([f]F), *Geodermatophilus* sp. (X92358, X92361)
B	TM^{-4}5H	Ha, R, Hh	100	*Bacillus funiculus* (AB271136), *B. cereus* (CP000764, AY920248, DQ207729, DQ209210),
	TM^{-3}1H	Ha, R, Hh	100	*B. cereus* (AY920248, DQ207729, DQ209210), *B. mycoides* (B. mycoides), *B. weihenstephanensis* (CP000903)
	TM^{-3}5L	Ha, R	100	*B. anthracis* (X55059)
	TM^{-4}2M	R, Hh	92.9	*B. cohnii* (DQ166855)
	TM^{-3}2H	Ha, R	100	*B. sphaericus* (L14010, L14011, L14012), *B. pumilus* (DQ275671), *Clostridium botulinum* (CP000726) ([f]C)
	TM^{-3}4M	Ha, R, Hh	88.6	*B. badius* (AB098575)
	TS^{-5}3L	R, Hh	90	*B. pantothenticus* (D16275), *Virgibacillus marismortui* (DQ010162), *B. olivae* (Dq139839)
	TL^{-4}3M	Ha, Hh	83.3	*B. edaphicus* (AB045093, AF006076), *B. brevis* (X60612)
	TL^{-4}3L	Ha, R, Hh	85.7	*B. vortex* (AM039409)
C	TS^{-3}2M	Ha, R	100	*Clostridium collagenovorans* (C. colgenvo), *C. kluyveri* (CP000673, M59092), *C. grantii* (C. grantii), *Mycoplasma meleagridis* (L24106), *M. fermentans* (M. fermenta), *Acholoeplasma modicum* (Acp. modicu)
	TL^{-4}2M	Ha, R	92.9	
	TS^{-4}3M	Ha, Hh	92.9	*P. terrigena*(AB248087)
	TL^{-5}1M	Ha, R	100	*P. thiaminolyticus* (D88513), *P. alvei* (Pae. alvei, Pae. alvei3), *P. azotofixans* (Pae. azofi2), *Staphylococcus capitis* (L37599)
	TL^{-5}3L	Ha, Hh	83.3	*P. lautus* (D85609, D85394), *Clostridium proteoclasticum* (U37378), *Leuconostoc fallax* (Lc. fallax), *Mycoplasms penetrans* (BA000026) ([f]I)
	TS^{-5}1H	Ha, R, Hh	91.7	*Panibacillus* sp (AB043866, AB23867, AB43869), *P. campinasensis* (DQ232773),
	TR^{-5}4L	R, Hh	100	*Lactobacillus fermentum* (AF522394),
	TR^{-5}5H	Ha, R	100	*L. plantarum* (DQ239695, DQ239699), *L. casei* (L. casei1), *L. mali* (M58824)
	TS^{-4}3H	R, Hh	100	*L. sanfranciscensis* (L. sanfranc), *Vagococcus salmoninarum* (Vag. salmon), *Brevibacterium incertum* (Y14650) ([f]A)
	TL^{-3}1H	R, Hh	100	*L. delbrueckii* (CR954253)
	TR^{-3}1M	Ha, R, Hh	100	*Eubacterium* sp. (AF385552), *E. subrreum*-like (AF2887776), *Streoptococcus constellatus* (Stc.const3)
	TS^{-3}2L	Ha, R	82.9	*E.cylindroides* (AB018186)
	TM^{-4}3H	Ha, R, Hh	93.3	*Streptococcus* sp. (AF084833)
	TM^{-4}4H	Ha, R, Hh	91.7	*Pullulanibacillus naganoensis* (AB021193), *P. mentitum* (AM931441)
	TR^{-2}1M	R, Hh	92.9	*Thermoanaerobacter celluloly* (Tab. cllul, Tab. cellu2)
	TS^{-5}1L	Ha, Hh	92.9	*Facalibacterium prausnitzii* (AJ270469), S*elenomonas sputigena* (Slm. sputig)
	TS^{-4}1L	Ha, Hh	100	*Pediocuccus urinaeequi* (D87677)
	TS^{-4}2M	R, Hh	100	*Desulfosporosinus orientis* (Ds.orient2)
	TS^{-3}4M	R, Hh	92.9	*Desulfotomaculum luciae* (AF069293)
	TS^{-3}5L	R, Hh	90	*Sporohalobacter lortetii* (M59122)

Continued

	TL^{-5}3M	Ha, R, Hh	88.9	*Leuconostoc fallax* (AB362604, AF218797), *Nitrosospira multiformis* (Nss.multi2) (^{t}D)
	TL^{-3}2H	Ha, R, Hh	95.2	*Staphylococcus aureus* (BA000033, CP000255, CP000703, CP000730), *Macrococcus carouselicus* (Y15713)
	TL^{-3}3H	Ha, R, Hh	95.2	*Halobacillus* sp. (AY966034, DQ089675), *Oceanobacillus* sp. (DQ190427), *O. iheyensis* (BA000028)
D	TM^{-4}2H	Ha, R, Hh	95.2	*Rhizobium* sp. (AJ294417), *R. gallicum* (AF417559), *R. mangolense* (U89818)
	TM^{-3}4H	Ha, R, Hh	95.2	*R. leguminosarum* (AF417563), *Ochrobactrum* sp. (DQ133574)
	TS^{-5}5H	Ha, R	92.9	*Rhodopseudomonas acidophila* (M34128)
	TM^{-4}1H	Ha, R, Hh	93.3	
	TM^{-5}1H^{g}	Ha, Ha	100	*Methylobacterium fujisawaensis* (AJ250801)
	TM^{-3}1M	Ha, Hh	100	*Acidocella aluminiidurans* (AB362219)
	TR^{-3}3M	Ha, Hh	100	*Neisseria animalis* (Nis.animal), *N. flavescens* (L06168), *N. denitrificans* (Nis. dentri), *N. elongata* (Nis. elong2), *Kingella oralis* (L06164)
	TS^{-5}3M	Ha, R	94.4	*Pandoraea sputorum* (AM921627)
	TS^{-3}1M	Ha, Hh	100	*Chromobacterium violaceum* (AE016825, M25510), *C. subtsugae* (AY344056)
	TC^{-5}4L	Ha, Hh	90	*Matsuebacter* sp. (AB024305)
	TC^{-4}1M	R, Hh	100	*Bordetella* sp. (AB039335)
E	TS^{-5}4H	R, Hh	100	
	TS^{-4}4H	Ha, R, Hh	81	
	TS^{-4}1H	Ha, R, Hh	90.5	
	TS^{-4}2H	Ha, R, Hh	90.5	
	TS^{-4}5H	Ha, R, Hh	88.6	
	TS^{-3}4H	Ha, R, Hh	93.3	
	TS^{-3}5H	Ha, R, Hh	93.3	
	TL^{-5}1H	Ha, R, Hh	93.3	*Pseudomonas* sp. (AF456214, AJ391194, AM111052, AY014824, DQ200851), *P. amygdali* (AB021378), *P. cichorii* (Z76658), *P. corrugata* (D84012), *P. stutzeri* (AJ288148), *P. entomophila* (CT573326), *Alkalilimnicola halodurans* (AJ404972) (^{t}G)
	TL^{-5}4H	Ha, R, Hh	93.3	
	TL^{-5}5H	Ha, R, Hh	93.3	
	TL^{-4}1H	R, Hh	100	
	TL^{-4}2H	Ha, R, Hh	93.3	
	TL^{-4}3H	Ha, R, Hh	95.2	
	TL^{-4}4H	Ha, R, Hh	93.3	
	TL^{-4}5H	Ha, R, Hh	88.9	
	TM^{-3}3L	Ha, Hh	90	*Pseudomonas* sp. (D87346), *P. otitidis* (AY953247)
	TS^{-5}2H	Ha, R	100	*Pseudomonas* sp. (AY998984), *P. syringae* (CP000058, CP000075), *P. fluorescens* (CP000094), *P. stutzeri* (U65012)
	TS^{-3}3H	R, Hh	100	*P. putida* (AY958233), *Pseudomonas* sp. (DQ205299, DQ227388)
	TS^{-3}5M	R, Hh	100	*Pseudomonas* sp. (AM934700), *Advenella* sp. (AY569461) (^{t}D)
	TL^{-5}2H	R, Hh	100	*Pseudomonas* sp. (AM184269, AY573031), *P. aureofaciens* (D84008), *P. chlororaphis* (D84011)
F	TR^{-5}2H	Ha, R, Hh	100	
	TR^{-5}3H	Ha, R, Hh	100	
	TR^{-4}1H	Ha, R, Hh	100	
	TR^{-4}2H	Ha, R, Hh	100	
	TR^{-3}1H	Ha, R, Hh	100	*Pantoea* sp. (DQ094146), *Xenorhobdus doucetiae* (DQ211702)
	TR^{-3}2H	Ha, R, Hh	100	
	TR^{-3}4H	Ha, R, Hh	100	
	TR^{-3}5H	Ha, R, Hh	93.3	

Continued

	TR^{-4}2M	Ha, R, Hh	95.2	*Vibrio wodanis* (AJ132227), *V. logei* (AY292928), *V. fischeri* (X74702), *Enterobacter* sp. (AY941832)
	TR^{-3}3H	Ha, R, Hh	100	*V. fischeri* (CP00020), *Klebsiella* sp. (DQ229100)
	TM^{-3}5H	Ha, R, Hh	95.2	*Yersinia aldovae* (Yer. aldova), *Y. intermedia* (Yer.intme2), *Serratia* sp. (AM050059)
G	TR^{-5}2M	Ha, R	83.3	uncultured gamma proteobacteria (AB294936)
	TR^{-5}4H	Ha, R, Hh	100	*Moritella* sp. (AB183497)
	TR^{-3}4M	Ha, R	90	*Frateuria aurantia* (Frt. aurant)
	TS^{-5}3H	R, Hh	100	*Halomonas campisalis* (DQ077910)
	TS^{-3}1H	R, Hh	100	*Thicapsa roseopersicina* (AF113000), *Rheinheimera baltica* (AJ002006)
H	TL^{-4}4M	R, Hh	100	uncultured *Enrotheonella* sp. (AY897125)
	TL^{-4}5M	Ha, Hh	94.4	
	TR^{-5}5M	Ha, R	100	uncultured delta proteobacteria (AY921877)
	TL^{-3}3M	R, Hh	100	uncultured delta proteobacteria (AB425060)
	TL^{-5}2M	Ha, Hh	90	*Arcobacter* sp. (AJ271654)
	TL^{-5}3H	Ha, R,Hh	82.2	*Helicobacter pullorum* (AF047850)
I	TS^{-5}5M	Ha, R	92.9	*Bacteroidetes bacterium* (AM932279)
	TL^{-5}5M	Ha, R	100	
	TR^{-3}2L	Ha, Hh	100	uncultured Bacteroidetes (AY921921)
	TM^{-5}1M	Ha, Hh	100	uncultured Cytophaga sp. (DQ070792)
	TR^{-3}3L	R, Hh	100	*Prevotella* sp. (AF385558), *Sulfurivirga caldicularium* (AB245479, AB245480) ([f]G)
	TL^{-3}1M	Ha, R	92.9	*Hymenobacter* sp. (AB251884)
J	TS^{-5}4M	R, Hh	100	*Mycoplasma imitans* (L24103)
	TS^{-5}4L	Ha, R	90	*M. gallinaceum* (L24104), *M. pullorum* (M. pollorum)
	TS^{-4}5M	Ha, R, Hh	95.2	*M. penetrans* (BA000026)
	TM^{-3}5M	Ha, R	100	*Ureaplasma canigenitalium* (D78648)
	TS^{-4}1M	Ha, R, Hh	100	*Acholeplasma modicum* (Acp. modicu)
	TL^{-5}4M	Ha, R	100	*A. polakii* (AF031479)
	TM^{-5}1H[g]	R, Hh	100	*Fusobacterium mecrophorum* (AF044948)
	TM^{-3}1L[g]	Ha, R	83.3	*Spirochaeta bajacaliformiensis* (M71239), *Leptospira fainei* (U60594), *L. inadai* (Z21634), *Holospora obtusa* (X58198) ([f]G)
	TR^{-5}3M	Ha, Hh	92.9	*Verrucomicrobic bacterium* (AB331888)
	TR^{-3}2M	Ha, R	90	*Leptospira fainei* (U65094), *L. inadai* (Z21634)
K	TM^{-4}4M	Ha, R	100	uncultured bacterium (AF382142, AY344400)
	TR^{-3}5M	Ha, R, Hh	84.1	uncultured bacterium (AB294747, AJ48807)
	TS^{-3}3M	R, Hh	100	uncultured bacterium (AM777948)
	TS^{-4}3L	Ha, Hh	90	unidentified bacterium (AY796034)
	TL^{-3}3L	R, Hh	100	*Oenothera berteriana* (Oeno ber_M)
	TL^{-3}5H	R, Hh	100	Uncultured Green Bay ferroma (AF293008)

[a]Grouping was based on affiliation by MERFL; Actinobacteria (A), *Bacillus* spp. (B), the other Firmicutes (C), *α*, *β*-Proteobacteria (D), *Pseudomonas* sp. (E), Enterobacteriacea (F), the other *γ*-Proteobacteria (GG), *δ*, *ε*-Proteobacteria (H), Cytophaga (I), the other bacteria (J), and unidentified or uncultured bacterial group (K). [b]The 1st letter in vial indicates used antibiotics; "T" stands for chlortetracycline. The 2nd letter in vial indicates samples; "R" stands for row cattle feces, "M" stands for cattle feces manure, "S" stands for shochu lee, and "L" stands for compost originated from leftover food. Exponential of vial number represents the decimal dilution of the vial. The 2nd number of vial number (1 - 5) represents number in 5 replicates for the each decimal dilution. "H" of last letter represents MERFL originating from the major 16S rDNA, "M" represents from the 2nd major 16S rDNA, and "L" represents from the 3rd major 16S rDNA. [c]Restriction enzymes used for similarity search; "Ha", "R", and "Hh" stand for *Hae* III, *Rsa* I, and *Hha* I. For the measured MERFLP which had no completely identical theoretical MERFLP, the theoretical MERFLP having the highest similarity using all the RFLPs was presented with the similarity as described in the materials and method. [d]The similarity between the measured RFLP (A) and the reference RFLP (B) was calculated based on the pairwise distance (D_{AB}) according to Nei and Li [33]. [e]Species name (accession number) of the theoretical MERFL having the highest similarity with the measured MERFL. [f]The theoretical MERFL (accession number) having the same MERFL belonged to different group in parenthesis. [g]The MERFL falling into different groups by using the different restriction enzymes.

Table 3. Affiliation of multi drug resistant bacteria grown in serially diluted LB medium by MERFL[a].

	Vial No.[b]	Restriction enzymes[c]	Similarity (%)[d]	Name (Accession number)[e]
A	MM10^{-5}1L	Ha, R	100	*S. aureus* (Stp. aureu4, Stp. sureu5, Stpaureus), *S. arlettae* (Stp.arlet2), *S. haemolyticus* (Stp. haemo2)
	MR10^{-4}1H	Ha, Hh	90	*S. aureus* (L37598), *S. camosusu* (Stp.carno2), *S. condimenti* (Y15750), *S. piscifermenta* (Y15754)
	MR10^{-5}1M	Ha, Hh	90	*Catellatospora citrea* (D85477)
	MR10^{-4}3H	R, Hh	94	*Rhodococcus* sp. (AY864653)
	MR10^{-3}3M	Ra, Hh	100	*Bifidobacterium choerinum* (D86186), *B. gallicum* (D86189), *B. inhantis* (M58738), *B. longum* (M58739), *B. suis* (M58743)
B	MM10^{-2}1M	Ha, R, Hh	82	*Bacillus edaphicus* (AF006076), *B. brevis* (X60612)
C	MM10^{-3}3M	Ha, R	100	*Clostridium botulinum* (C.botuliC2, C.botuliD2), *C. novyi* (L37594), *Peptostreptococcus anaerobiu* (D14150, L04168)
	MR10^{-5}2H	Ha, R	93	*C.tyrobutyricum* (C.tyrobu51, C. tyobut, M59113),
	MR10^{-3}2H	Ha, R, Hh	81	Clostridiaceae bacterium (DQ270662), *Deinococcus grandis* (Y11329) ([f]I)
	MR10^{-4}5H	Ha, R	100	*Streptococcus* sp. (AY923140)
	MR10^{-2}3H	Ha, R	100	*S. sanguinis* (Stc. angui)
	MM10^{-4}3L	Ha, Hh	100	uncultured *Streptococcus* sp. (DQ016726)
	MM10^{-5}5M	Ha, R	100	*Eubacterium* sp. (AF385498)
	MR10^{-4}3M	Ha, Hh	90	uncultured eubacterium (AF018186, AF018192, AF018194)
	MR10^{-3}1L	R, Hh	88	uncultured eubacterium (AM422271)
	MM10^{-4}2M	Ha, R	83	*Tissierella praecuta* (Tss. praea2, Tss. praeac), *Melittangium lichenicola* (Mel. lichen) ([f]H), *Myxococcus coralloides* (Myx. corall) ([f]H)
	MM10^{-2}3M[g]	R, Hh	93	*Aicyclobacillus acidoterre* (AB042057, AB042058, AB059676, AY573797)
	MM10^{-2}5L	Ha, Hh	90	*Enterococcus saccharolyticus* (Eco. saclyt), *Clostridium butyricum* (M59085), *Eubacterium rectale* (Eub. rectal), *Roseburia cecicola* (L14676)
	MR10^{-4}4L[g]	Ha, Hh	100	*Atopostipes suicloacalis* (AF445248)
	MR10^{-4}5M	Ha, Hh	83	*Pseudoramibacter alactolyticus* (AB036759, AB036760, AN036761)
	MR10^{-4}5L	Ha, R	100	*Paenibacillus* sp. (AB043868, AM162327)
D	MM10^{-4}2L	R, Hh	90	uncultured Rhodobacteraceae (AF543930)
	MR10^{-4}4H[g]	Ha, R	100	*Rhodopseudomonas acidophila* (M34128)
	MR10^{-3}3H	R, Hh	88	*Azospirillum amazonense* (AY741146)
	MR10^{-2}4L	Ha, Hh	93	Sphingomonas roseiflava (D84520)
	MM10^{-3}2L	Ha, R, Hh	84	*Burkholderia* sp. (AJ551104)
	MM10^{-3}3L	R, Hh	100	uncultured beta proteobacterium (AY221606)
	MR10^{-5}5M	Ha, Hh	83	*Telluria mixta* (X65589), *Janthinobacterium agaricidam* (Y08845)
E	MM10^{-5}4H	Ha, R, Hh	100	*Pseudomonas* sp. (AM111052, AJ574911)
	MM10^{-5}5H	Ha, R, Hh	100	
	MM10^{-4}2H	Ha, R, Hh	100	
	MM10^{-3}5H	Ha, R, Hh	100	
	MM10^{-5}2H	Ha, R	100	
	MM10^{-3}3H	Ha, R, Hh	93.3	*Pseudomonas* sp. (AM111035, AM110993)
	MM10^{-3}2H	Ha, R, Hh	85	
	MM10^{-3}4H	Ha, R, Hh	85	*Pseudomonas* sp. (DQ200857)
	MR10^{-2}5M	Ha, Hh	82.9	*Pseudomonas* sp. (AY646430)
F	MS10^{-5}2H	Ha,R	100	*Yersinia frederiksenii* (Yer. Friksn)
	MM10^{-5}4M	R,Hh	100	*Salmonella chingola* (U92192)
	MR10^{-2}4M[g]	Ha,Hh	83	*Vibrio fischeri* (V. fischer4)
G	XM10^{-2}3H	Ha, R, Hh	95	*Thiomicrospira crunogena* (AF069959, CP000109)
	XM10^{-2}5H	Ha, R, Hh	89	
	XM10^{-2}1H	Ha, R, Hh	92	*Cycloclasticus* sp. (AB080112), *Rhodovulum imhoffii* (AM180953) ([f]D)

Continued

	XM10^{-2}2H	R, Hh	88	*Haemophilus influenza* (AF224305, AF224306, AY613568, AY613580)
	XR10^{-5}1H	Ha, R, Hh	89	*Stenotrophomonas maltophilia* (AB180661, AB294554, AB294555, DQ141193)
	XR10^{-5}4M[g]	Ha, Hh	93	*Oceanospirillum maris* (AB006771), *Pelagicoccus mobilis* (AB286015) ([f]J)
	XR10^{-4}2H	Ha, Hh	86	uncultured gamma proteobacteria (AJ567535, AJ567542)
	XR10^{-4}4H[g]	R, Hh	100	*Halorhodospira halophile* (CP000544)
	XR10^{-3}1H	Ha, R	100	*Buchnera aphidicola* (AJ296759)
H	XM10^{-2}5M	Ha, R, Hh	82	
	XR10^{-4}4M	Ha, Hh	83	*Angiococcus disciformis (*Ang. discif)
	XR10^{-2}5H	Ha, Hh	93	
	XM10^{-5}1M	R, Hh	83	*Desulfovibrio africanus* (M37315)
	XR10^{-5}2M	R, Hh	83	*D. salexigens* (Dsv. salexi)
	XM10^{-4}3H	Ha, Hh	90	uncultured delta proteobacteria (AF154094)
	XR10^{-2}4H	Ha, R, Hh	81	*Pelobacter propionicus* (CP000482, X70954)
	XS10^{-5}2M	Ha, R, Hh	86	*Arcobacter* sp. (AJ271654)
	XR10^{-4}1M	Ha, Hh	100	uncultured epsilon proteobacteria (AB235370, AM712353)
I	XM10^{-2}3L	Ha, R, Hh	93	*Bacteroides* sp. (AF139525), *B. thetaiotaomicron* (AE016936, AE016937, M58763),
	XR10^{-5}1L	Ha, R	83	*Colwellia* sp. (DQ027051) ([f]G)
	XM10^{-5}3H	Ha, R, Hh	84	*Flavobacterium* sp. (AM934661)
	XR10^{-5}2L	Ha, R	88	uncultured Cytophagales bacteria (AF361196)
	XR10^{-2}4M[g]	Ha, R	83	*Prevotella* sp. (AB166777)
J	XM10^{-4}3M	Ha, R	100	*Entomoplasma freundtii* (AF036954), *Encarsia pergandiella* (AF319783), *Bacteroidetes*
	XM10^{-3}5M	Ha, R	100	*endocymbiont* (AY753170) ([f]I)), *Cardinium endosymbiont* (AY327472)([f]I), *Bacillus tipchiralis*
	XR10^{-2}2H	Ha, R	100	(AF039408) ([f]B)
	XM10^{-2}2M	Ha, R	90	*Spiroplasma leptinotarsae* (AY189305)
	XR10^{-4}4L[g]	R, Hh	100	*S. mirum* (M24662), *S. citri* (Spp. cit2HP), *S. poulsonii* (Spp. poulsn)
	XR10^{-2}5L	R, Hh	90	
	XR10^{-3}2L	Ha, R	90	*S.linguale* (M62789),
	XM10^{-5}2L	Ha, Hh	90	*Ureaplasma cati* (D78649), *U. felinum* (D78651)
	XS10^{-5}2L	Ha, Hh	83	uncultured planctomycetes (AM040106)
	XR10^{-5}5H	Ha, R	93	
	XM10^{-2}3M[g]	Ha, R	93	Aquificales str. (AF255598, AF255597)
	XM10^{-2}1L	Ha, R	83	*Heroetosiphan aurantiacus* (M34117), *Chlamydomonas reinhardtii* (BK000554) ([f]K), *Leptospira fainei* (U60594)
	XR10^{-5}4M[g]	Ha, R	93	uncultured cyanobacterium (AY874085, AJ431339), uncultured eubacterium (AF018194)
	XR10^{-4}2L	Ha, Hh	93	*Arthrospira platensi*s (DQ279767, DQ279768, DQ279769, DQ279770)
K	XR10^{-5}4H	Ha, Hh	100	uncultured bacterium (AY869688)
	XR10^{-3}5H	Ha, Hh	100	
	XM10^{-5}1H	Ha, Hh	100	
	XM10^{-5}2M	Ha, R, Hh	100	uncultured bacterium (AF072927, AJ867657, AY661977, AY571416)
	XM10^{-3}4M	Ha, R, Hh	100	
	XR10^{-3}2M	Ha, Hh	100	
	XM10^{-3}4L	Ha, R	83	*Melosira varians* (AJ536464), *Phaeodactylum tricomutum* (DQ174248)
	XR10^{-3}5M	Ha, R	100	*Plantago sericea* (AJ389621)

[a]Grouping was based on affiliation by MERFL; Actinobacteria (A), *Bacillus* spp. (B), the other Firmicutes (C), *α*, *β*-Proteobacteria (D), *Pseudomonas* sp. (E), Enterobacteriacea (F), the other *γ*-Proteobacteria (GG), *δ*, *ε*-Proteobacteria (H), Cytophaga (I), the other bacteria (J), and unidentified or uncultured bacterial group (K). [b]The 1st letter in vial indicates used antibiotics; "X" stands for multi drugs, ciprofloxacin, streptomycin, chloramphenicol, and ampicillin. The 2nd letter in vial indicates samples; "R" stands for row cattle feces, "M" stands for cattle feces manure, "S" stands for shochu lee, and "L" stands for compost originated from leftover food. Exponential of vial number represents the decimal dilution of the vial. The 2nd number of vial number (1 - 5) represents number in 5 replicates for the each decimal dilution. "H" of last letter represents MERFL originating from the major 16S rDNA, "M" represents from the 2nd major 16S rDNA, and "L" represents from the 3rd major 16S rDNA. [c]Restriction enzymes used for similarity search; "Ha", "R", and "Hh" stand for *Hae* III, *Rsa* I, and *Hha* I. For the measured MERFLP which had no completely identical theoretical MERFLP, the theoretical MERFLP having the highest similarity using all the RFLPs was presented with the similarity as described in the materials and method. [d]The similarity between the measured RFLP (A) and the reference RFLP (B) was calculated based on the pairwise distance (D_{AB}) according to Nei and Li [33]. [e]Species name (accession number) of the theoretical MERFL having the highest similarity with the measured MERFL. [f]The theoretical MERFL (accession number) having the same MERFL belonged to different group in parenthesis. [g]The MERFL falling into different groups by using the different restriction enzyme.

Table 4. Most probable numbers of colistin (P) resistant bacterial groups (A~K) of organic wastes and composts and 5% confidence limits obtained using FDA's Bacterial Analytical Manual [34]. *Under estimated number due to lack of dilution vials higher than 10^{-6}. **Total bacteria.

Groups	cattle feces manure (M)				row cattle feces (R)				shochu lee (S)				compost originated from leftover food (L)			
	Three Score dilutions		×10³MPN g⁻¹	5% limits Low/High	Three Score dilutions		×10³MPN g⁻¹	5% limits Low/High	Three Score dilutions		×10³MPN g⁻¹	5% limits Low/High	Three Score dilutions		×10³MPN g⁻¹	5% limits Low/High
A	10^{-3} 10^{-4} 10^{-5}	0-1-1	0.50	0.025/1.90	10^{-3} 10^{-4} 10^{-5}	0-1-0	1.99	0.1/7.51					10^{-1} 10^{-2} 10^{-3}	0-1-0	0.005	0.0003/0.02
B	10^{-4} 10^{-5} 10^{-6}	0-2-0	>10.40*	1.96/-	10^{-3} 10^{-4} 10^{-5}	3-0-1	12.2	3.87/25.4					10^{-2} 10^{-3} 10^{-4}	0-1-0	0.05	0.003/0.19
C	10^{-3} 10^{-4} 10^{-5}	0-2-0	1.04	0.196/2.80	10^{-3} 10^{-4} 10^{-5}	2-1-0	7.5	1.99/18.8	10^{-3} 10^{-4} 10^{-5}	0-1-0	3.6	0.18/13.6	10^{-3} 10^{-4} 10^{-5}	3-1-0	3.08	0.98/7.23
D	10^{-4} 10^{-5} 10^{-6}	0-2-0	>10.40*	1.96/-	10^{-3} 10^{-4} 10^{-5}	1-1-0	3.97	0.77/11.1	10^{-3} 10^{-4} 10^{-5}	0-1-0	3.6	0.18/13.6	10^{-3} 10^{-4} 10^{-5}	2-1-0	1.90	0.50/4.76
E					10^{-3} 10^{-4} 10^{-5}	1-0-1	4.42	0.77/11.1	10^{-3} 10^{-4} 10^{-5}	0-2-0	7.4	1.4/20.0	10^{-2} 10^{-3} 10^{-4}	0-1-0	0.05	0.003/0.19
F	10^{-3} 10^{-4} 10^{-5}	0-1-0	0.50	0.025/1.90	10^{-3} 10^{-4} 10^{-5}	0-1-0	1.99	0.1/7.51								
G	10^{-3} 10^{-4} 10^{-5}	0-1-0	0.50	0.025/1.90	10^{-3} 10^{-4} 10^{-5}	1-0-2	6.63	1.99/16.6					10^{-2} 10^{-3} 10^{-4}	1-1-0	0.11	0.02/0.34
H	10^{-3} 10^{-4} 10^{-5}	0-1-0	0.50	0.025/1.90									10^{-2} 10^{-3} 10^{-4}	1-4-0	0.31	0.098/0.61
I																
J	10^{-3} 10^{-4} 10^{-5}	0-1-0	0.50	0.025/1.90	10^{-2} 10^{-3} 10^{-4}	0-1-0	0,2	0.01/0.76					10^{-2} 10^{-3} 10^{-4}	0-1-0	0.05	0.003/0.19
K	10^{-4} 10^{-5} 10^{-6}	2-2-0	>26.0*	9.51/-	10^{-2} 10^{-3} 10^{-4}	2-1-0	0.75	0.2/1.9	10^{-3} 10^{-4} 10^{-5}	0-1-0	3.6	0.2/13.6				
T**		3-4-0	>58.7*	19.0/-	10^{-3} 10^{-4} 10^{-5}	5-4-5	475.1	166/1216	10^{-3} 10^{-4} 10^{-5}	0-2-0	7.4	1.4/20	10^{-3} 10^{-4} 10^{-5}	5-1-0	9.23	2.80/28.0

Table 5. Most probable numbers of chlortetracycline (T) resistant bacterial groups (A~K) of organic wastes and composts and 5% confidence limits obtained using FDA's Bacterial Analytical Manual [34]. *Under estimated number due to lack of dilution vials higher than 10^{-6}. **Total bacteria.

Groups	cattle feces manure (M)				row cattle feces (R)				shochu lee (S)				compost originated from leftover food (L)			
	Three Score dilutions	×10³ MPN g⁻¹		5% limits Low/High	Three Score dilutions	×10³ MPN g⁻¹		5% limits Low/High	Three Score dilutions	×10³ MPN g⁻¹		5% limits Low/High	Three Score dilutions	×10³ MPN g⁻¹		5% limits Low/High
A	10^{-3} 10^{-4} 10^{-5}	2-1-2	3.36	1.15/7.27	10^{-2} 10^{-3} 10^{-4}	1-0-1	0.44	0.08/1.11	10^{-2} 10^{-3} 10^{-4}	0-2-0	0.74	0.14/2.0	10^{-2} 10^{-3} 10^{-4}	0-1-0	0.05	0.003/0.19
B	10^{-3} 10^{-4} 10^{-5}	4-2-0	6.15	1.90/14.0					10^{-3} 10^{-4} 10^{-5}	0-0-1	3.6	0.18/13.6	10^{-3} 10^{-4} 10^{-5}	0-2-0	1.03	0.2/2.80
C	10^{-3} 10^{-4} 10^{-5}	0-2-0	1.03	0.196/2.80	10^{-3} 10^{-4} 10^{-5}	1-0-2	6.63	1.99/16.58	10^{-3} 10^{-4} 10^{-5}	4-4-2	94.0	30.0/240.0	10^{-4} 10^{-5} 10^{-6}	1-3-0	>23.2*	9.51/-
D	10^{-3} 10^{-4} 10^{-5}	2-2-1	6.15	1.90/14.5	10^{-2} 10^{-3} 10^{-4}	0-1-0	0.2	0.01/0.76	10^{-3} 10^{-4} 10^{-5}	1-0-2	12.0	3.6/30.0	10^{-3} 10^{-4} 10^{-5}	0-1-1	1.07	0.2/2.80
E	10^{-2} 10^{-3} 10^{-4}	1-0-1	0.12	0.020/0.28					10^{-3} 10^{-4} 10^{-5}	4-4-2	94.0	30.0/240.0	10^{-4} 10^{-5} 10^{-6}	5-4-0	>363.6*	100.7/-
F	10^{-2} 10^{-3} 10^{-4}	0-1-0	0.05	0.003/0.19	10^{-3} 10^{-4} 10^{-5}	5-3-2	157	57.5/442.0								
G					10^{-3} 10^{-4} 10^{-5}	1-0-2	6.63	1.99/16.58	10^{-3} 10^{-4} 10^{-5}	1-0-1	8.0	1.4/20.0				
H					$10^{-3}10^{-4}$ 10^{-5}	0-0-1	1.99	0.1/7.51					10^{-3} 10^{-4} 10^{-5}	2-1-2	3.36	1.15/7.27
I	10^{-3} 10^{-4} 10^{-5}	0-0-1	0.50	0.03/1.9	10^{-3} 10^{-4} 10^{-5}	0-2-0	4.09	0.77/11.05	10^{-3} 10^{-4} 10^{-5}	0-0-1	3.6	0.18/13.6	10^{-3} 10^{-4} 10^{-5}	1-0-1	1.12	0.2/2.80
J	10^{-3} 10^{-4} 10^{-5}	2-1-0	1.90	0.50/4.2	10^{-3} 10^{-4} 10^{-5}	1-0-1	4.42	0.77/11.05	10^{-4} 10^{-5} 10^{-6}	2-2-0	>186.0*	68.0/-	10^{-3} 10^{-4} 10^{-5}	0-0-1	0.50	0.03/1.90
K	10^{-3} 10^{-4} 10^{-5}	0-1-0	0.50	0.03/1.9	10^{-2} 10^{-3} 10^{-4}	0-1-0	1.99	0.1/0.75	10^{-3} 10^{-4} 10^{-5}	1-1-0	3.6	0.18/13.6	10^{-2} 10^{-3} 10^{-4}	0-2-0	0.10	0.02/0.28
T**	10^{-4} 10^{-5} 10^{-6}	5-2-0	>137.0*	42.0/ -	10^{-4} 10^{-5} 10^{-6}	2-4-0	>166*	65.2/-	10^{-3} 10^{-4} 10^{-5}	5-5-5	>3200.0*	1400/-	10^{-3} 10^{-4} 10^{-5}	5-5-5	>447.6	195.8/-

Table 6. Most probable numbers of multi drug (X) resistant bacterial groups (A~K) of organic wastes and composts and 5% confidence limits obtained using FDA's Bacterial Analytical Manual (34). *Under estimated number due to an absence of dilution vials higher than 10^{-6}. **Total bacteria.

Groups	cattle feces manure (M)				row cattle feces (R)				shochu lee (S)				compost originated from leftover food (L)			
	Three Score dilutions	$\times 10^3$ MPN g^{-1}		5% limits Low/High	Three Score dilutions		$\times 10^3$ MPN g^{-1}	5% limits Low/High	Three Score dilutions	$\times 10^3$ MPN g^{-1}		5% limits Low/High	Three Score dilutions	$\times 10^3$ MPN g^{-1}		5% limits Low/High
A	10^{-3} 10^{-4} 10^{-5}	0-0-1	0.50	0.03/1.90	10^{-3} 10^{-4} 10^{-5}	1-2-1	9.28	3.76/24.30								
B	10^{-1} 10^{-2} 10^{-3}	0-1-0	0.005	0.003/0.02												
C	10^{-3} 10^{-4} 10^{-5}	1-2-1	2.94	0.95/6.15	10^{-4} 10^{-5} 10^{-6}	5-1-0	>364.4*	110.5/-								
D	10^{-3} 10^{-4} 10^{-5}	2-1-0	1.91	0.50/4.76	10^{-3} 10^{-4} 10^{-5}	1-1-1	7.74	1.88/16.57								
E	10^{-3} 10^{-4} 10^{-5}	4-1-3	8.76	2.80/19.6	10^{-1} 10^{-2} 10^{-3}	0-1-0	0.02	0.001/0.075								
F	10^{-3} 10^{-4} 10^{-5}	0-0-1	0.50	0.03/1.90	10^{-3} 10^{-4} 10^{-5}	0-0-1	1.99	0.1/7.51								
G	10^{-2} 10^{-3} 10^{-4}	4-0-0	0.04	0.01/0.1	10^{-4} 10^{-5} 10^{-6}	2-2-0	>102.8*	37.6/-								
H	10^{-3} 10^{-4} 10^{-5}	0-1-1	1.01	0.20/2.80	10^{-3} 10^{-4} 10^{-5}	0-2-1	6.08	1.99/16.57	10^{-3} 10^{-4} 10^{-5}	0-0-1	3.6	0.18/13.6				
I	10^{-3} 10^{-4} 10^{-5}	0-0-1	0.50	0.03/1.90	10^{-4} 10^{-5} 10^{-6}	0-2-0	>40.9*	7.73/-								
J	10^{-3} 10^{-4} 10^{-5}	1-1-1	1.90	0.50/4.2	10^{-4} 10^{-5} 10^{-6}	2-2-0	>102.8*	37.6/243.1	10^{-3} 10^{-4} 10^{-5}	0-0-1	3.6	0.18/13.6				
K	10^{-3} 10^{-4} 10^{-5}	2-0-2	2.54	0.86/6.15	10^{-3} 10^{-4} 10^{-5}	3-0-1	12.2	3.9/25.4								
T**	10^{-4} 10^{-5} 10^{-6}	2-4-0	>41.9*	16.5/ -	10^{-4} 10^{-5} 10^{-6}	5-4-0	>1436.4*	398.0/-	10^{-3} 10^{-4} 10^{-5}	0-0-1	3.6	0.18/13.6				

OUT (**Table 2**), which lowered the diversity of the MERFLs. Affiliations of 80 MERFLs of multi drug resistant bacteria (X) in each MPN vials were summarized in **Table 3**. All of the 80 MERFLs were divided into 62 OTUs, then ratio of total number of the OTUs to the total number of MERFLs was 77.5%, which was also lower than that of P (**Table 3**). Some MERFLs of the group E, group G, group H, group I, group J, and group K were placed in the same OUTs (**Table 3**).

The precisions of the affiliations of each MERFLs were lower than those of the former studies [26] [27]. Although, ratio of the MERFLs having 100% similarity to the reference MERFLs with respect to the major MERFL (37%; **Tables 1-3**) was lower than that of the 2nd major MERFLs (48.0%; **Tables 1-3**), that of the 3rd major MERLs (30%) was not so lower than that of the 2nd MERFLs and higher than those of the former studies [26]. The lower precision of the major MERFL and higher precision of the 3rd major MERFLs was caused from lower ratio of *Bacillus* spp. as the followings; 8 MERFLs in colistin resistant bacteria (**Table 1**), 9 MERELs in chlortetracycline resistant bacteria (**Table 2**), and 1 MERFL in multi-drug resistant bacteria (**Table 3**). Because16S rDNA of *Bacillus* spp. was preferentially amplified and increased the relative mole concentration of the major MERFL, and decreased those of the 2nd and 3rd major MERFLs [26] [27], which increased the precision of the major MERFLPs, and decreased those of the 2nd and 3rd major MERFLPs.

3.2. Enumeration of Each Antibiotic Resistant Bacterial Groups by Mpn

As colistin was bactericidal to gram-negative bacteria and little to no effect on gram-positive bacteria, gram positive bacterial groups (A to C) were eliminated from antibiotic resistant bacteria (**Table 4**, **Figure 1**). Numbers of the resistant bacteria was the highest in cattle feces manure (M; >3.84 × 10^4 MPN g^{-1} dry matter), followed by row cattle feces (R; 1.78 × 10^4 MPN g^{-1}), shochu lee (S; 1.46 × 10^4 MPN g^{-1}), and compost originated from leftover food (L; 0.24 × 10^4 MPN g^{-1}) (**Figure 1**). *α*, *β*-proteobacteia (D; 1.04 × 10^4 MPN g^{-1}) was the numerically dominant resistant bacteria in M, *γ*-proteobacteria (*Pseudomonas* spp. (E);0.44 ×10^4 MPN g^{-1}, Enterobacteriaceae (F); 0.19 × 10^4 MPN g^{-1}, the other *γ*-Proteobacteria (G); 0.66 × 10^4 MPN g^{-1}) was the numerically dominant in R, and *α*, *β*-proteobacteia (D; 0.36 × 10^4 MPN g^{-1}), and *Pseudomonas* spp. (E; 0.74 × 10^4 MPN g^{-1}) were the numerically dominant in S (**Table 4**, **Figure 1**).

Numbers of chlortetracycline resistant bacteria (T) was the highest in shochu lee (S; >320 × 10^4 MPN g^{-1}),

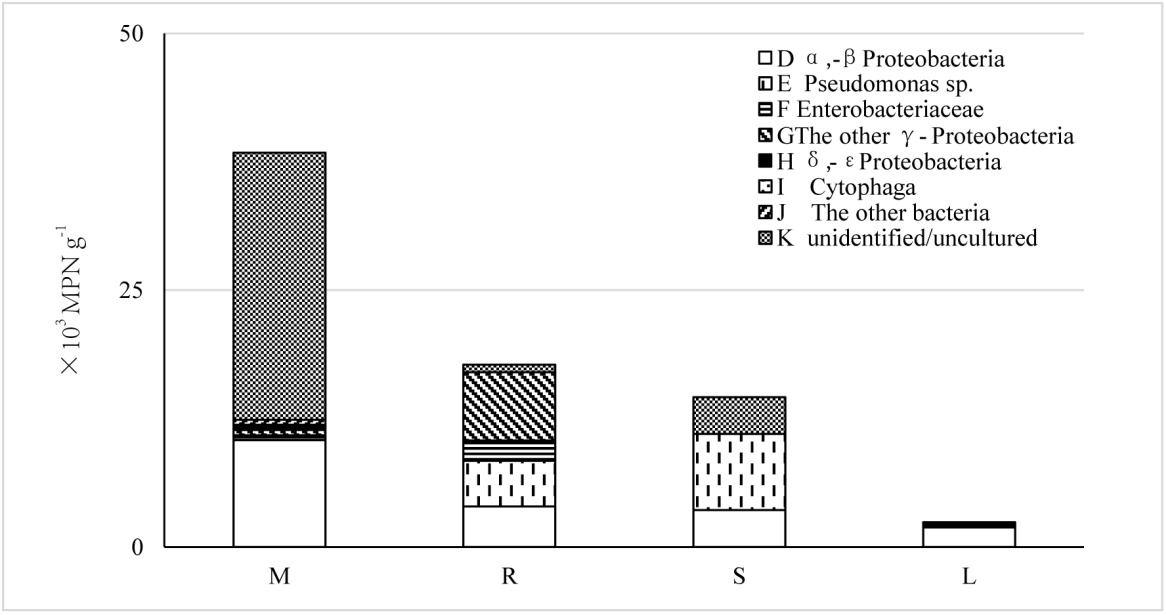

Figure 1. Numbers of colistin resistant (25 mg·L^{-1}) bacterial groups estimated by MPN and MERFLP in cattle feces manure (M), row cattle feces (R), shochu lee (S), and compost originated from leftover food (L). Number of *α*, *β*-Proteobacteria (D; □), *Pseudomonas* spp. (E; ⁙), Enterobacteriacea (F; ▤), the other *γ*-Proteobacteria (G; ▧), *δ*, *ε*-Proteobacteria (H; ■), Cytophaga (I; ⁙), the other bacteria (J; ▨), and unidentified or uncultured bacterial group (H; ▩) were presented.

followed by compost originated from leftover food (L; >44.8 × 10^4 MPN g^{-1}), row cattle feces (R; 16.6 × 10^4 MPN g^{-1}), and cattle feces manure (M; 13.7 × 10^4 MPN g^{-1}) (**Table 5**). In S, the other Firmucutes (C; 9.4 × 10^4 MPN), *Pseudomonas* spp. (E; 9.4 × 10^4 MPN g^{-1}), and the other bacterial group (J; 18.6 × 10^4 MPN, including *Mycoplasma* spp), were the numerically dominant (**Table 5**, **Figure 2**). As 7 MERELs of *Pseudomonas* spp. (E) in S having the same MERFLs, they might proliferate preferentially than the other bacterial groups in S (**Table 2**). As *Pseudomonas* spp. (E) were the numerically dominant (>36.4 × 10^4 MPN g^{-1}) in L (**Table 5**) and 8 MERFLs of them also having the same MERFLs (**Table 2**), they might also proliferate preferentially in L. As Enterobacteriacea (F; 15.7 × 10^4 MPN g^{-1}) was the numerically dominant in R (**Table 5**) and 8 MERFLs of them also having the same MERFLs (**Table 2**), they might also proliferate preferentially in R (**Table 2**).

Numbers of multi drug resistant bacteria (X) was the highest in row cattle feces (R; >143.6 × 10^4 MPN g^{-1}), followed by cattle feces manure (M; 4.19 × 10^4 MPN g^{-1}), and shochu lee (S; 0.36 × 10^4 MPN g^{-1}) (**Table 6**, **Figure 3**). In R, the other Firmicutes (C; 36.4 × 10^4 MPN g^{-1}), including *Clostridium* sp, and *Streptcoccus* sp, the other γ-proteobacteria (G; 10.3 × 10^4 MPN g^{-1}), and the other bacteria (J; 10.3 × 10^4 MPN g^{-1}), including *Spiroplasma* sp, were the numerically dominant bacterial groups (**Table 3**, **Table 6**), while there was no bacteria which proliferated preferentially in R (**Table 3**). As *Pseudomonas* sp. (E; 0.88 × 10^4 MPN g^{-1}) was the numerically dominant in M (**Table 3**, **Table 6**) and 4 MERFLs and 3 MERFLs of them had the same MERFLs, they were supposed to proliferate preferentially in M (**Table 3**).

4. Discussion

In the former studies, we had detected and enumerated polymyxin B resistant bacteria not only in field soils where liquid livestock feces had annually been applied [35] [36] but also in a paddy field soil where organic manure had annually been applied [26], although polymyxin B have mainly been used in hospitals and have never been used as AGP and there was no-report of the resistant bacteria except for nosocomial resistant bacteria in hospital [37]. Colistin (polymyxin E), which had a similar structure and the same site of action [37], had been used as AGP in Japan. The sample included diverse kinds of the resistant bacteria and no-specific numerically dominant bacterial group (**Table 1**), which might be caused from gradual proliferation of diverse kinds of bacteria having the lower resistance by a continual sub-therapeutic administration of the antibiotic as AGP. As higher

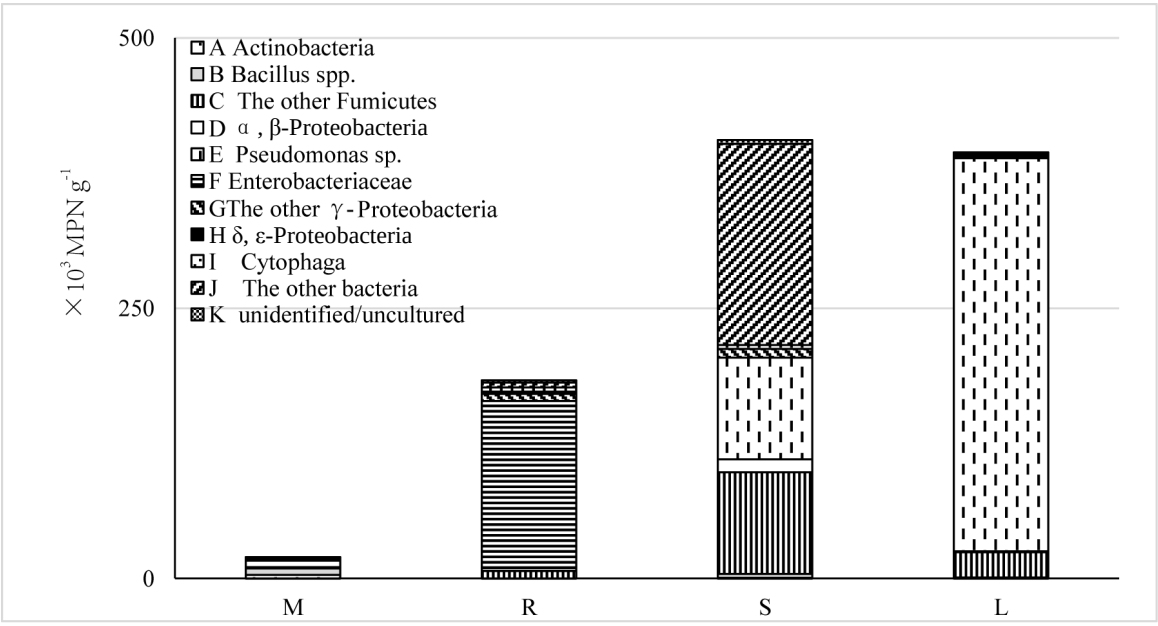

Figure 2. Numbers of chlortetracycline resistant (25 mg·L^{-1}) bacterial groups estimated by MPN and MERFLP in cattle feces manure (M), row cattle feces (R), shochu lee (S), and compost originated from leftover food (L). Number of Actinobacteria (A;), *Bacillus* spp. (B;), the other Firmicutes (C;), *α*, *β*-Proteobacteria (D;), *Pseudomonas* spp. (E;), Enterobacteriacea (F;), the other *γ*-Proteobacteria (G;), *δ*, *ε*-Proteobacteria (H;), Cytophaga (I;), the other bacteria (J;), and unidentified or uncultured bacterial group (H;) were presented.

dosage of the antibiotics was used in this study (25l mg·L^{-1}) than those in the former studies (5 mg·L^{-1}), estimated numbers of the resistant bacteria in cattle feces manure (M; >3.84 × 10^4 MPN), and row cattle feces (R; 1.78 × 10^4 MPN) were lower than those estimated for the manure applied field soil (3.11 × 10^6 MPN g^{-1}) [26] and those for field soils applied with liquid livestock feces (from 31.7 × 10^6 CFU g^{-1} to 258 × 10^6 CFU g^{-1}) [35] [36]. Although numerically dominant bacterial groups in M (unidentified bacterial group, *α*, *β*-proteobacteria; **Table 1**, **Table 4**) were different from those of upland field soil (*Prevotella* spp. and Cytophagales) [26], colistin resistant gram negative bacteria detected in R and M in this study were concluded to be polymyxin B resistant bacteria in the former studies [26] [35] [36]. The antibiotic bacteria detected and enumerated in this study were supposed to have higher resistance to colistin than those of the former studies [26] [35] [36], and those of the reported resistant bacteria [37] because they could proliferate higher concentration (25 mg·L^{-1}) than the reported resistance breakpoints for *Acinetobacter* spp. (>2 or 4 mg·L^{-1}), that for *Pseudomonas* spp. (>4 mg·L^{-1}), and that for Enterobacteriacea (>2 mg·L^{-1}) [14] [37].

As chlortetracycline has widely been used for the past forty years as therapeutic agent for human and veterinary medicine but also as AGP, their numbers were higher than those of colistin resistant bacteria (**Table 4**, **Table 5**, **Figure 1**, **Figure 2**), which was coincident with the other report [14]. Although the numbers were underestimated due to an absence of MPN dilution vial higher than 10^{-6}, the numbers were higher in shochu lee (S; >320 × 10^4 MPN g^{-1}) and compost originated from leftover food (L; >44.8 × 10^4 MPN g^{-1}) (**Table 5**, **Figure 2**), where *Pseudomonas* spp. was not only the numerically dominant microorganisms but also proliferated preferentially (**Table 2**, **Table 5**). In row cattle feces (R), specific resistant bacteria, *Pantoea* sp. or *Xenorhobdus doucetiae*, occupied the entire resistant bacterial group (**Table 2**, **Table 5**), which might suggest that therapeutic application of higher dosage of the antibiotic resulted in a rapid proliferation of this bacterial group in the cattle intestine. Although total number of the resistant bacteria in cattle feces manure (M; >13.7 × 10^4 MPN g^{-1}) were lower than those of the others (**Table 5**, **Figure 2**), the composition of the resistant bacterial groups (**Table 2**) were similar to those in the reported field soils, where continuous application of organic manure was supposed to cause accumulation of the resistant bacteria due to sub therapeutic use of the antibiotic as AGP [18]. As the concentration of applied chlortetracycline (25 mg·L^{-1}) was as the same level as those of the reported resistance breakpoints for *Salmonella* spp., *E. coli*, *Camphylobacter* spp., and *Enterococcus* spp. (>16 mg·L^{-1}) [14], the

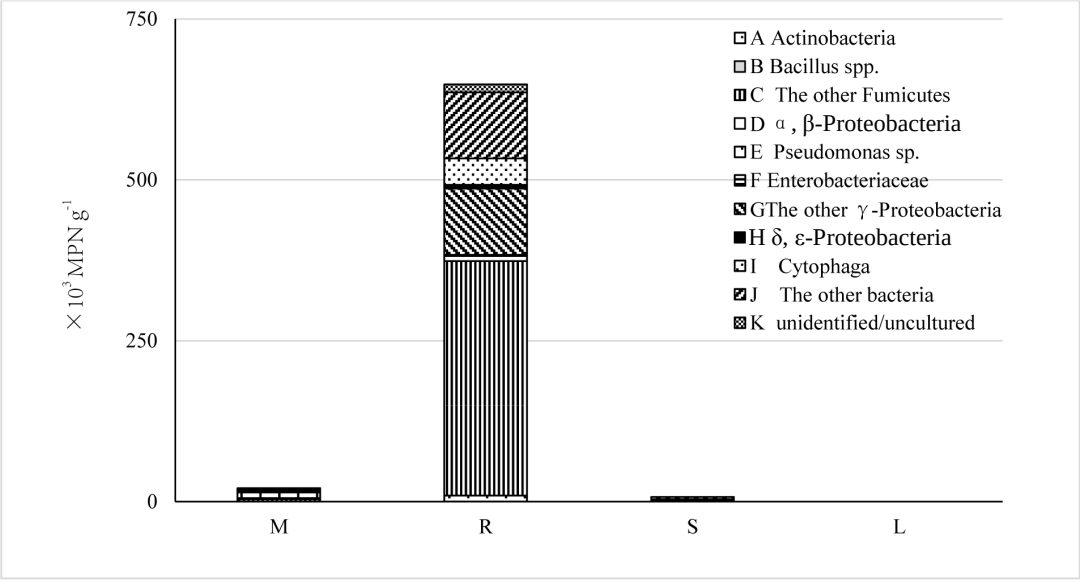

Figure 3. Numbers of multi drug, ciprofloxacin (25 mg·L^{-1}), streptomycin (25 mg·L^{-1}), chloramphenicol (25 mg·L^{-1}), and ampicillin (25 mg·L^{-1}), resistant bacterial groups estimated by MPN and MERFLP in cattle feces manure (M), row cattle feces (R), shochu lee (S), and compost originated from leftover food (L). Number of Actinobacteria (A;), *Bacillus* spp. (B;), the other Firmicutes (C;), *α*, *β*-Proteobacteria (D;), *Pseudomonas* spp. (E;), Enterobacteriacea (F;), the other *γ*-Proteobacteria (G;), *δ*, *ε*-Proteobacteria (H;), Cytophaga (I;), the other bacteria (J;), and unidentified or uncultured bacterial group (H;) were presented.

enumerated resistant bacteria was estimated to be as same as the reported chlortetracycline resistant bacteria [14] [18] [25].

Although the numbers were underestimated due to lack of MPN dilution vial higher than 10^{-6}, multi drug resistant bacteria was typically observed in row cattle feces (R) (**Table 6**, **Figure 3**). As their numbers in cattle feces manure (M) was 3% of that in R (**Table 6**), the most of the multi drug resistant bacterial groups in row feces might decrease during manuring process. Concentration of the used ampicillin (25 mg·L^{-1}) was higher than that of the reported resistance breakpoints for *Haemophilus influenzae*, *Listeria monocytogenes*, *Neisseria meningitidis*, and *Pasteurella multocida* (>1 mg·L^{-1}), those for *Streptococcus pneumoniae*, and Enterobacteriacea (> 2 mg·L^{-1}), and those for *Entrococcus* spp. (>8 mg·L^{-1}) [14] [37]. Concentration of the used chloramphenicol (25 mg·L^{-1}) was also higher than those for *Haemophilus influenzae*, and *Moraxella catarrhalis* (>2 mg·L^{-1}), those for *Neisseria meningitidis* (>4 mg·L^{-1}), those for Enterobacteriacea, *Staphylococus* spp, and *Streptococcus pneumoniae* (>8 mg·L^{-1}) [37]. Concentration of the used ciprofloxacin (25 mg·L^{-1}) was much higher than those for *Neisseria meningitidis* (>0.03 mg·L^{-1}), those for *N. gonorrhoeae*, and *Pasteurella multocida* (>0.06 mg·L^{-1}), those for *Haemophilus influenzae*, *Moraxella catarrhalis*, and *Camphylobacter jejuni*/*coli* (>0.5 mg·L^{-1}), those for Enterobacteriacea, *Pseudomonas* spp, *Acintobacter* spp, *Staphylococus* spp, and *Corynebacterium* spp. (> 1 mg·L^{-1}), those for *Streptococcus pneumoniae* (>2 mg·L^{-1}), and *Entrococcus* spp. (>4 mg·L^{-1}) [37]. While concentration of the used streptomycin (25 mg·L^{-1}) was lower than those for *Salmonella* spp. and *E. coli* (>64 mg·L^{-1}), or *Enterococcus* spp. (>1000 mg·L^{-1}) [14], the detected bacteria by the method was estimated to be one of the multi drug resistant bacteria. As the bacteria detected by this method had survived and proliferated under co-application of higher concentrations of these antibiotics, they might have higher resistance than those detected by the ordinal susceptibility tests where each antibiotic was separately applied for evaluation [3] [4] [7] [15] [17] [37] [38].

5. Conclusions

Until now the risk of antibiotic resistant bacteria has mainly been evaluated by the susceptibility tests using isolates [7]-[18] or by using selective primer of resistant gene [5] [22]-[25]. Although the susceptibility test was indispensable to search what kinds of antibiotics were effective for specific bacterial group, it was difficult to use the method for the environmental risk assessment. Because the susceptibility tests and taxonomy determinations had to be expanded broadly over a large numbers of environmentally important bacterial groups, and it was difficult to estimate numbers of the resistant bacterial group from these isolates due to the isolation bias [7]-[18]. Although the spreading of antibiotic resistant gene into various environments could be monitored by tracing resistant gene [22]-[25], the molecular-based analysis method could not be used for their environmental risk assessment because detected resistant gene had no relation to their phylogenetic positions nor phenotypic properties.

Method presented here had the following superior properties as monitoring method for the antibiotic resistant hbacteria spreading into various environment, although some bacterial groups might be underestimated as described previously [26] [27]; 1) We could easily know what kinds of antibiotics had higher risk for emergence of resistant bacteria by changing the kinds and combinations of applied antibiotics without preliminary information [26]. 2) We could easily know the environment where the number of the antibiotic resistant bacteria was high. Because false-negative results could be removed by using microbial DNA extracted after proliferation in the growth medium and decimal dilution vials of MPN where the effect PCR inhibiting substances included in various environmental samples decreased as described previously [26] [27]. 3) Because the susceptibility tests using bacterial isolates were not required for the monitoring, the risk of their community acquired infection might be evaluated safely by using it as stand-alone method.

The present results indicated that multi drug resistant bacteria might widely be spreading through animal husbandry. Their reduction method and spreading into environment will be presented in the following manuscripts by using this method.

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