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Tetracycline-resistant bacteria and ribosomal protection protein genes in soils from selected agricultural fields and livestock farms

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Abstract

Antibiotic resistance in soil environment has eminently been compared and studied between agricultural and pristine soils, and the role of concentrated animal feeding operations has markedly been recognized as one of the major sources of antibiotic resistance. This study described the tetracycline resistance in small-scale farms in pursuit of presenting its possible role and contribution to the persistence of antibiotic resistance in the environment. Results of the study would render additional information on the occurrence of the ribosomal protection protein (RPP) *tet* genes among the isolated bacteria from the selected agricultural soils. Four tetracycline resistance and RPP genes were determined in two different agricultural soil settings. Both the culture and molecular method were used to determine and measure tetracycline resistance in soils from arable land and animal house. Results revealed a significantly higher number of culturable antibiotic-resistant bacteria in animal houses than arable lands which was suggestive of higher antibiotic resistance in areas where there was direct administration of the antibiotics. However, quantification of the gene copy numbers in the agricultural soils indicated a different result. Higher gene copy number of *tet*O was determined in one animal house (IAH-3), while the two other *tet* genes *tet*Q and *tet*W were found to be higher in arable lands. Of the total 110 bacterial isolates, *tet*W gene was frequently detected, while *tet*O gene was absent in any of the culturable bacterial isolates. Principal component analysis of occurrence and gene copy number of RPP *tet* genes *tet*O, *tet*Q, and *tet*W also revealed highest abundance of RPP *tet* genes in the manure and arable soils. Another important highlight of this study was the similarity of the RPP *tet* genes detected in the isolated bacteria from the agricultural soils to the identified RPP *tet* genes among pathogenic bacteria. Some of the tetracycline-resistant bacterial isolates were also multidrug resistant as it displayed resistance to tetracycline, erythromycin, and streptomycin using disk diffusion testing.

Keywords: RPP-*tet* genes, Agricultural soil, Antibiotics-resistance

Introduction

Livestock farming is one of the agricultural sectors identified to have high demand for antibiotics [1]. According to Granados-Chinchilla and Rodriguez [2], antibiotics utilized in livestock farming alone amount to approximately two-thirds of the antibiotics produced worldwide. In South Korea, more than 50% of the veterinary antibiotics consumed belong to tetracycline class of antibiotics [3]. As a result, concern on the release of antibiotics and most importantly the proliferation of antibiotic resistance genes and antibiotic resistant bacteria to the environment has been raised. Antimicrobials are considered as emerging contaminants and gaining the spotlight of research at the present time, since they are products of intensive uses of antimicrobials that incite significant threat to the effectivity of currently available antibiotics against pathogenic microorganisms [4–7]. There are

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four known mechanisms of resistance to tetracycline, and a total of 59 described tetracycline resistance gene (*tet*) determinants [8, 9]. Ribosomal protection proteins (RPPs), which are second to efflux proteins in terms of the number of *tet* gene determinants, are tetracycline resistance determinants frequently detected in the environment [10–13] and have a total of 12 gene determinants [14, 15]. Substantially, RPPs also provide bacteria with additional protection from second generation tetracyclines like minocycline and doxycycline [8, 16].

Dispersion of antibiotic resistance is rapidly occurring; thus, its mitigation is urgently needed. One potential solution is to conduct surveillance programs; however, one of the challenges is that surveillance done for antibiotic resistant bacteria and antibiotic resistance in the environment is still scant [17–19]. Determining which antibiotic resistance genes is present and which bacteria carries the genes is very crucial in monitoring and performing risk assessment as well as in planning the imperative measures to attenuate dispersion of antibiotic resistance [20, 21]. Now, the culture-independent methods which are basically gene-based have convincingly offered wider scope of coverage of studying antibiotic resistance in the environment. With molecular tools, insights on the ecology of antibiotic resistance genes in agroecosystems and quantitative data could convey efficiencies of the interventions administered [22, 23]. Quantitative data could also be used to serve as background or baseline data during assessment and evaluation especially in relation to potential human health risks [18, 24]. To characterize ecology of tetracycline resistance in small-scale farms, the objective of the study was to describe tetracycline resistance in arable lands and animal houses by determining antibiotics-resistant bacteria and RPP *tet* genes and quantifying the tetracycline resistance genes from the three study areas.

Materials and methods

Enumeration of total bacteria and culturable tetracycline-resistant bacteria

The soil samples were collected from agricultural fields and livestock farms located in Gimje, Iksan, and Jangsu in the fall of 2018. In the 3 study areas, a total of 18 soil samples from 9 animal houses and 9 arable lands were collected. Additionally, 3 representative manure samples were collected as available. Soils from livestock farms were collected at approximately 1 m from the corral, while soils from arable lands were collected directly from the section where crops were planted. From each site, soils collected from three different spots were binned into a composite sample. The animal houses accommodate approximately less than 50 cattle, while the arable lands are used for cultivating food crops. Additional information on study sites is shown in Additional file 1: Table S1, and the information on the location of the sampling sites is indicated in Additional file 1: Data S1.

The total bacterial level was determined by both culture method and 16S rRNA gene quantification, while total number of tetracycline-resistant bacteria was determined by culture method only. The bacterial cultures were performed by inoculating 100 µL of serially diluted 1 g of soil in 0.1X PBS into lysogeny broth (LB) agar supplemented with or without 10 µg/mL tetracycline (Sigma-Aldrich, USA) by pour plate method. Cycloheximide (Sigma-Aldrich, USA) at 20 µg/mL was also added into the media to control the growth of fungi. After incubation for 48 h at 28 °C, the number of bacterial colonies per gram of soil (CFU/g of soil) was counted. Isolation of tetracycline-resistant bacteria was followed by spreading 50-µL volume of inoculum and incubating the plates at 28 and 35 °C. Bacterial isolates were then culture-purified and tested for cell viability [25].

Quantification of total 16S rRNA gene and RPP *tet* gene copy number

DNA extraction from soil was performed using DNeasy®PowerSoil Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. On the other hand, DNA from bacterial isolate was extracted by boiling lysis with some modification [26]. A loopful of bacterial colony was transferred to a 0.2-mL PCR tube with 100 µL of deionized sterile distilled water. The PCR tube was incubated in a thermocycler at 95 °C for 15 min. After incubation, the suspension was transferred to a sterile 1.5-mL microcentrifuge tube. The lysate was then used for molecular testing.

Detection of RPP *tet* genes from soil samples was performed following the procedures indicated by Aminov et al. [27, 28] with some modifications. The PCR amplification was performed using the Biometra TProfessional thermocycler with the following conditions: initial denaturation at 94 °C for 5 min, followed by 25 cycles of denaturation at 94 °C for 30 s, annealing at the indicated temperature for 30 s, elongation at 72 °C for 30 s, and then a final elongation at 72 °C for 7 min. The annealing temperature, primer sequence, and amplicon size are indicated in Additional file 1: Table S2. A second round PCR was carried out using 1 µL of the first-round PCR product as a template under the same conditions. The second-round PCR products were analyzed by gel electrophoresis in a 2.5% (wt/vol) agarose gel containing 0.05 mg/mL ethidium bromide and visualized using FAS-Digi gel imaging system (Nippon Genetics Europe, Germany). Since there were no positive controls for the four *tet* genes, all samples with positive results were sent to Genotech (Daejeon, South Korea) for sequencing.

Sequence analysis was confirmed by comparison with data retrieved from BLAST. The confirmed positive samples detected from soil samples were then also used as control for the screening of RPP *tet* genes among culture-purified tetracycline-resistant bacterial isolates.

Real-time quantitative PCR was performed to determine the absolute copy numbers of 16S rRNA gene, *tet*O, *tet*Q, *tet*S, and *tet*W using the CFX-Connect™ Real-Time PCR Detection System (Bio-Rad Laboratories Inc, USA). Standard quantification curves were separately established for the 16S rRNA gene and RPP *tet* genes following the procedures indicated by Nograd o et al. [29], and absolute gene copy numbers were calculated directly from the extracted plasmids [30]. The qPCR conditions for the four *tet* genes were programmed according to the conditions used by Wu et al. [10] with some modifications: initial denaturation at 95 °C for 1 min, followed by 40 cycles of denaturation at 94 °C for 10 s and annealing at 58.2 °C for *tet*O and *tet*Q, and at 63.3 °C for *tet*W for 45 s. Furthermore, the fluorescence was read at different temperatures, 81 °C for *tet*O and *tet*Q, and 83 °C for *tet*W. Product specificity was confirmed by both melt curve analysis (for 16S rRNA gene at 65–95 °C with an increment of 0.5 °C held for 30 s and for the *tet* genes at 55–95 °C with an increment of 0.5 °C held for 30 s) and if needed, gel electrophoresis.

Susceptibility testing and identification of tetracycline-resistant bacterial isolates

The tetracycline-resistant bacterial isolates were identified by analyzing 16S rRNA genes amplified using the 27F and 1492R primer set. The PCR products were then purified and sent for sequencing to Genotech (Daejeon, South Korea). Sequence confirmation was done by comparison with data retrieved from BLAST. The antibiotic susceptibility by disk diffusion was performed following the procedures indicated by Ortez [31].

Data analysis

One-way analysis of variance (ANOVA) was performed to assess the homogeneity of variance among the average of the total culturable bacteria, total culturable tetracycline-resistant bacteria, and total bacterial level by 16S rRNA gene copy in two agricultural areas with a 95% confidence interval. While standard deviation on absolute copy number of RPP *tet* gene in each site was determined. Using the DNA sequences of the amplified RPP *tet* genes from the total extracted DNA from soil samples, a neighbor-joining tree was constructed using the MEGA-7 to determine the maximum likelihood of the *tet* genes detected in the two agricultural settings. Principal component analysis (PCA) was also performed using R to analyze the distribution and absolute *tet* gene copy number per gram of soil in each collection site.

Results and discussion

Total and tetracycline-resistant bacteria

Using ANOVA with 95% confidence interval, Fig. 1 shows a statistically significant higher bacterial level in arable land than animal houses using both culture and molecular methods. On the other hand, density of the culturable tetracycline-resistant bacteria was higher in the animal houses ($3.13 \times 10^4 \pm 5.20 \times 10^3$ CFU/g) than in the arable lands ($2.89 \times 10^4 \pm 5.43 \times 10^3$ CFU/g). The higher bacterial level in arable land could be influenced by the tillage activities that affect the soil texture and soil moisture content which influence the soil bacterial abundance [32]. Comparison of percentages of the culturable tetracycline-resistant bacteria in each agricultural site per study area revealed consistently higher ratio in animal house than in arable land (Additional file 1: Table S3). The concentration of tetracycline used for culturing the tetracycline-resistant bacteria in this study followed the methods used by Popowska et al. [33]. The percentage of the total culturable tetracycline-resistant bacteria obtained in this study was higher compared to the results obtained by Kim et al. [13]. This could be attributed to the lower concentration of tetracycline used in this study at 10 μg/L. A lower concentration than the minimum inhibitory concentration was used since in reality, microbes in the environment are exposed to a much lower concentration and that lower antibiotic concentration also enriches resistant microorganisms in the actual environment [34]. In addition to this, although the concentration of tetracycline used is lower than the breakpoint for tetracycline resistance, resistance is dictated more by the cost of fitness exhibited by the microorganism than by the concentration of the antibiotics [35].

Tetracycline-resistant genes

Looking at the prevalence of four RPP *tet* genes in the agricultural soils, the results of conventional PCR revealed that the most frequently detected RPP *tet* gene was *tet*W followed by *tet*Q and *tet*S, while the least detected was *tet*O which was present in only two sites (Additional file 1: Table S4). Manure samples, on the other hand, showed that all three manure samples indicated the presence of *tet*O, *tet*Q, and *tet*W except the Gimje manure sample which was positive only for *tet*W. There was no positive control for the RPP *tet* genes detected, and so the PCR products were sent for DNA sequencing for confirmation. After confirmation, a phylogenetic tree was constructed by neighbor-joining method (Fig. 2). The figure shows a high similarity of RPP *tet* genes detected in the two agricultural soil

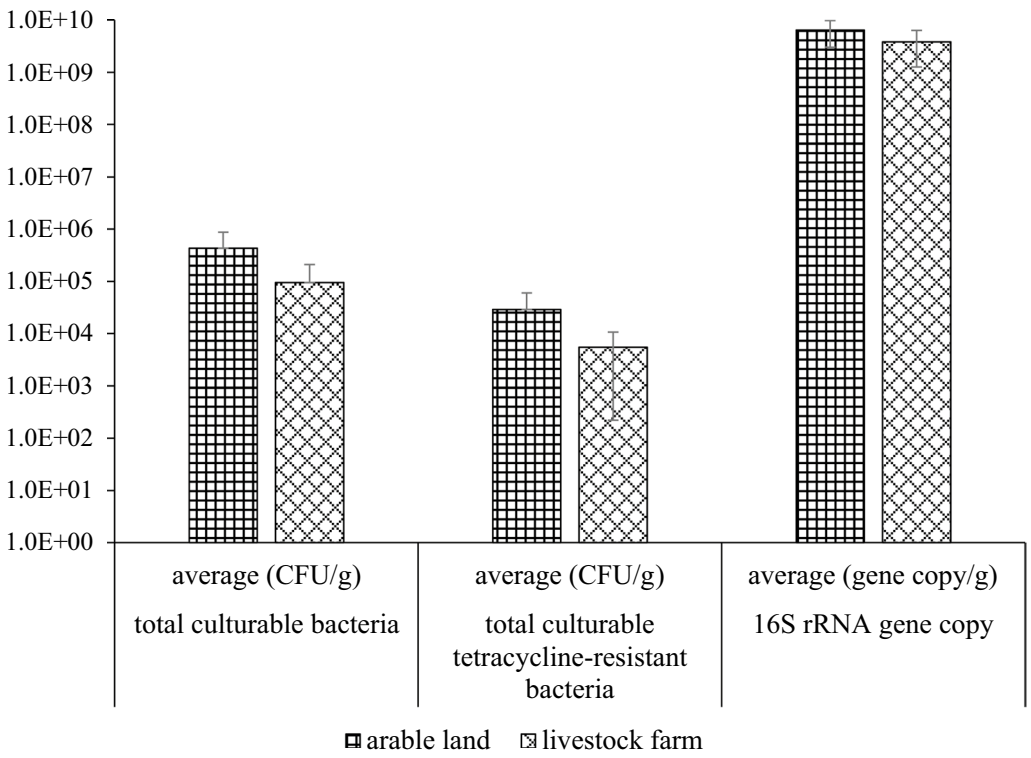

Fig. 1 Comparison of the average of total culturable bacterial level by culture and molecular method and total culturable tetracycline-resistant bacteria in arable land and animal house. ANOVA was used to determine the significance of difference in the average values of total culturable bacterial level and total culturable tetracycline-resistant bacteria in the two areas at 95% confidence interval

environments, and most notably these RPP *tet* genes bear resemblance to previously identified RPP *tet* genes among pathogenic microorganisms. The abundance of the RPP *tet* genes was then determined by quantitative real-time PCR and was reported as ratio (or relative gene copy number) between *tet* gene copy and total 16S rRNA gene copy per gram of soil (Fig. 3). Among the three genes quantified, *tet*W was found to have the highest relative gene copy number than the other two *tet* genes. The *tet*W ratio was found to be highest in JAF-2 and GAH-2 sites with $1.84 \times 10^{-4} \pm 4.32 \times 10^{-5}$ and $1.42 \times 10^{-4} \pm 5.64 \times 10^{-5}$, respectively. Subsequently, *tet*W was followed by the gene copy number of *tet*O which was higher in IAH-3 at $1.64 \times 10^{-5} \pm 5.07 \times 10^{-6}$ than in GAF-1 at $3.65 \times 10^{-6} \pm 9.87 \times 10^{-7}$. Lastly, the relative copy number of *tet*Q was found to be higher in GAF-1 ($6.26 \times 10^{-6} \pm 8.46 \times 10^{-6}$) compared to that in IAH-1 ($2.39 \times 10^{-6} \pm 1.12 \times 10^{-6}$). The results demonstrated that *tet*W and *tet*Q were more abundant in arable land, while *tet*O was more in livestock farms. The abundance of the RPP *tet* genes in manure samples followed the same pattern to that of the soil samples. Additionally, among manure samples, manure from Jangsu had the highest relative gene copy number of the *tet* genes compared to Gimje and Iksan (Fig. 3). Figure 4 shows the principal component analysis (PCA) of the gene abundance in each site showing clustering of sites in terms of the abundance and presence of the RPP *tet* genes. It revealed that *tet* genes were the most abundant in the following samples J-manure > I-compost > GAF-1. The figure also shows that *tet*O and *tet*W were strongly correlated to each other, which could be explained by its high similarity of amino acid sequences between TetO and TetW, which were categorized into the same group based on the amino acid sequence similarity [8]. The results of this study in terms of the frequency of detection of the RPP *tet* genes showed that *tet*O and *tet*Q, were not as prevalent in the agricultural sites evaluated compared to previous agricultural sites evaluated in previous studies [10, 13, 36]. The widespread detection of *tet*W in both arable land and livestock farms was in agreement with other studies [11, 13, 36], while the infrequent detection of *tet*S in the agricultural areas of study coincided with the results obtained by Wu et al. [10]. It is important to note that one animal house in Iksan was found to be negative for the presence of any RPP *tet* genes. This is possibly not truly negative as this could be attributed to PCR inhibitors present in the soil samples from this area [11].

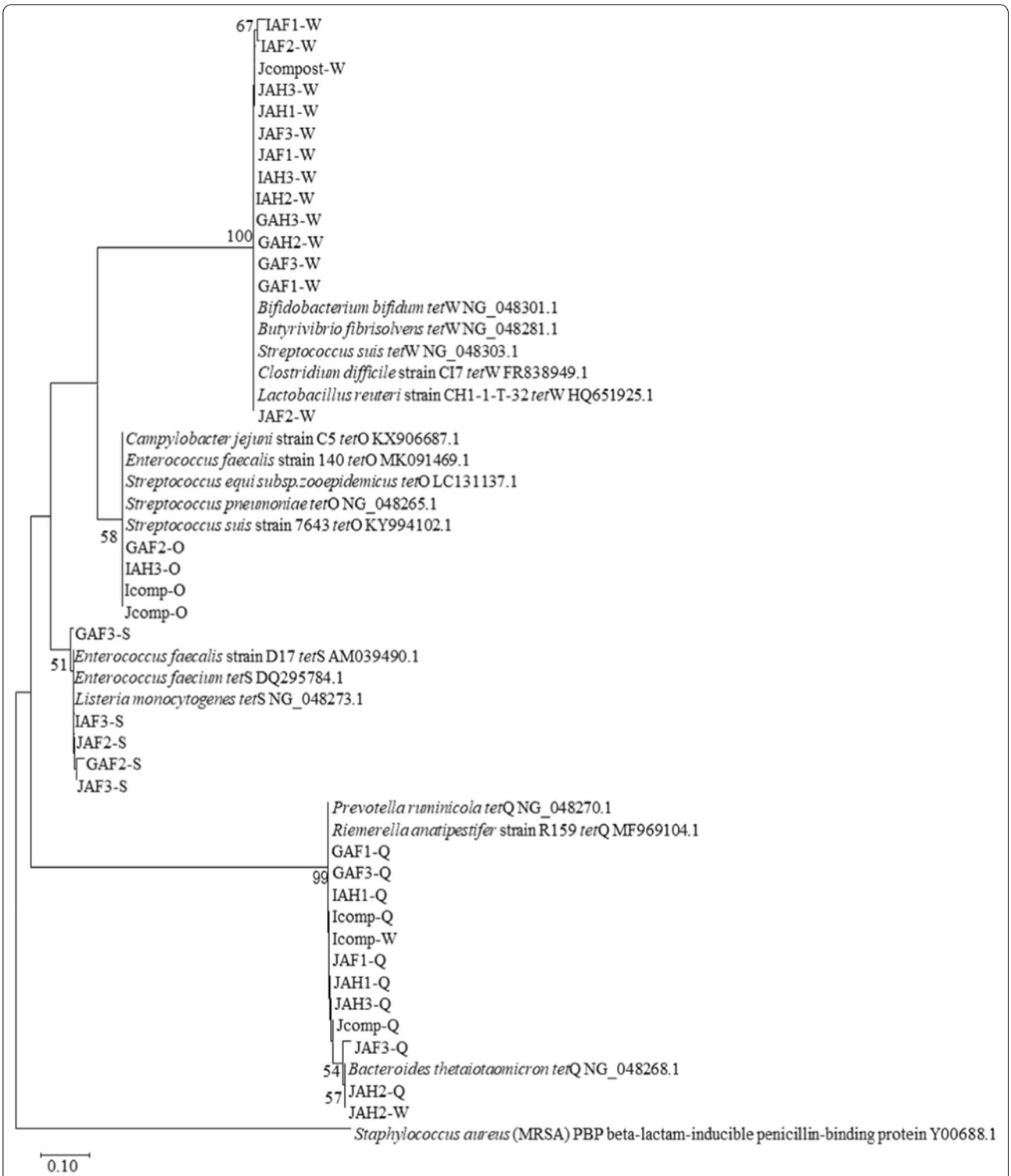

Fig. 2 Evolutionary relationships of detected Ribosomal Protective Protein (RPP) genes namely *tet*O, *tet*Q, *tet*S, and *tet*W detected in the two agricultural areas of Gimje, Iksan, and Jangsu. The evolutionary history was inferred using the Neighbor-Joining method. Evolutionary analyses were conducted in MEGA-7

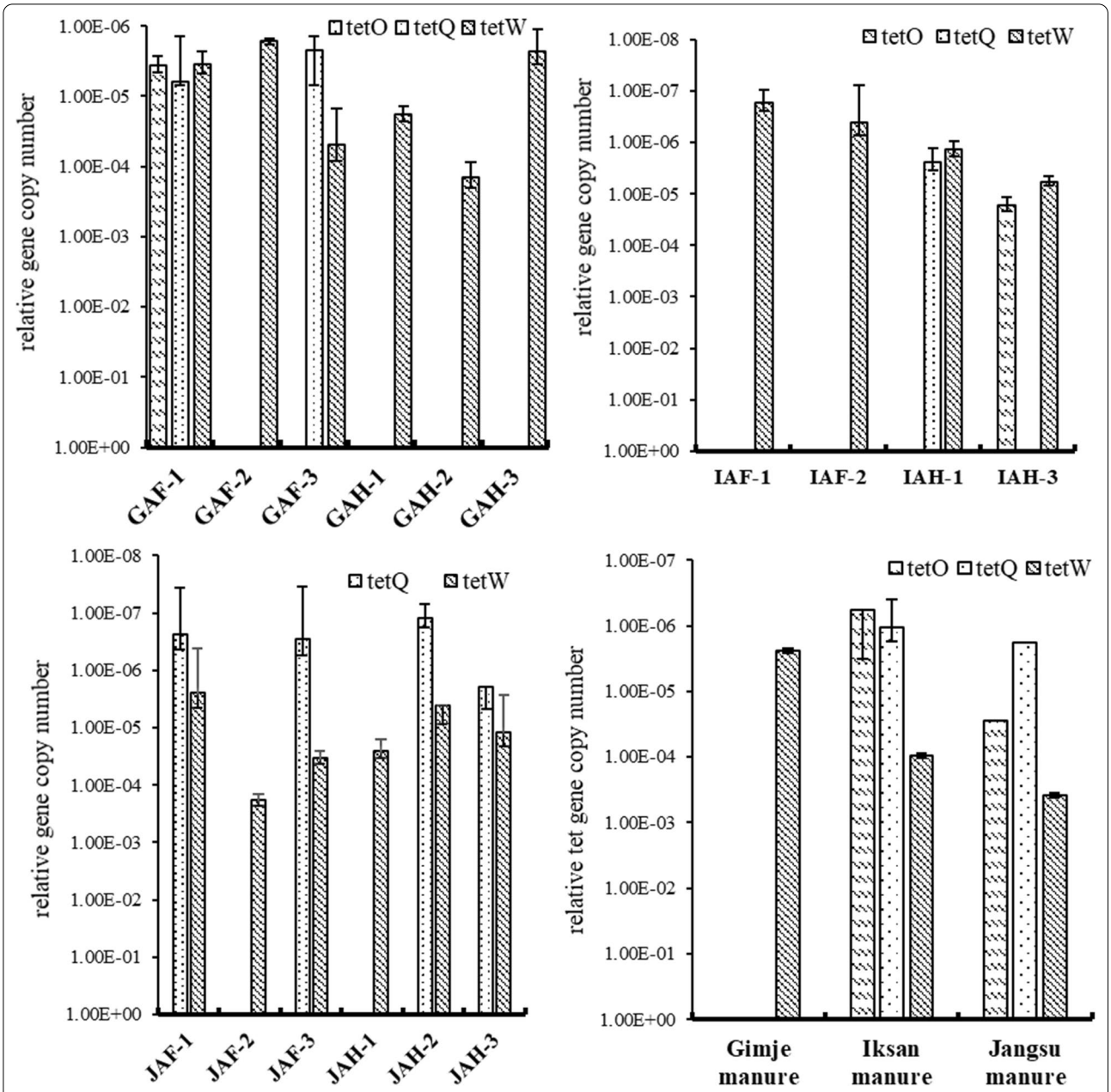

Fig. 3 Relative copy number of *tet* genes to the 16S rRNA gene in soil and compost samples collected from arable land and livestock farm in Gimje, Iksan, and Jangsu

One of the contributing factors to the enrichment of antibiotic resistance genes in the environment is the application of manure as fertilizer [37]. In this study, manure samples were only collected when available. Now, among the three manure samples, Gimje manure sample showed to have *tet*W only. This could be explained by the difference in the crudity of the three manure samples, showing that Gimje manure has already undergone composting. The effect of composting on the reduction of antibiotic resistance genes has been observed in the study by Wang et al. [38]. While antibiotic genes are acknowledged to be naturally present in the environment, mere determination of its frequency can give incomplete details whether it could be considered as background level or baseline level, and so quantification of the gene copy number was also performed to assess the magnitude of the occurrence of the RPP *tet* genes [13, 23]. Quantification of the RPP *tet* genes can be used to determine if it could be used as a

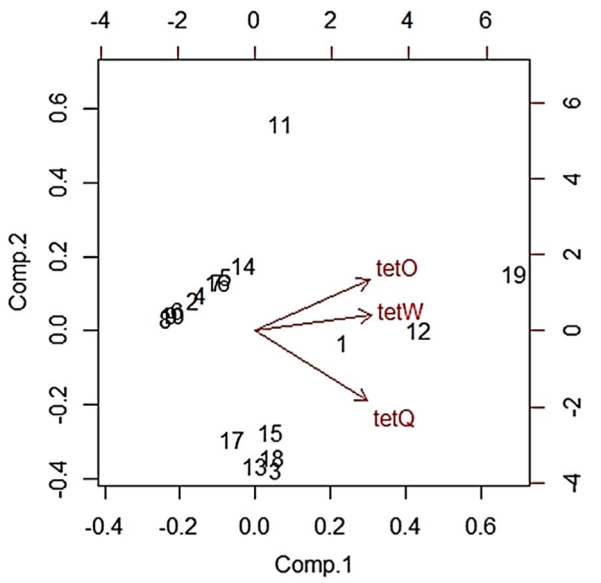

Fig. 4 PCA biplot clustering based on antibiotic resistance abundance. Each number represents an individual sample tested in this study and clustered according to the abundance of three tetracycline resistance genes. (# 1–3: GAF-1 to GAF-3; # 4–6: GAH-1 to GAH-3; # 7: Gimje-manure; # 8, 9: IAF-1 and IAF-2; # 10, 11: IAH-1 and IAH-3; # 12: Iksan-manure; # 13–15: JAF-1 to JAF-3; # 16–18: JAH-1 to JAH-3; and # 19: Jangsu-manure)

background or baseline level which is important to confirm the causes of antibiotic resistance in agro-ecosystems, to evaluate the change or variation of the antibiotic resistance level over time, and to establish relationship of the use of antibiotics in agriculture and spread of resistance [18]. It is also essential to determine myriad of antibiotic resistance genes that occur in the environment as this will aid which antibiotic resistance genes should be included for monitoring during surveillance [15]. Likewise, quantification of the antibiotic resistance genes could also be used to check the efficiency of the intervention applied to reduce antibiotic resistance [24].

Tetracycline-resistant isolates and the genes

A total of 110 bacterial isolates were subjected for antibiotic susceptibility using disk diffusion assay (Additional file 1: Data S2); however, the test was applicable only to 77 isolates as some did not grow well in Mueller Hinton Agar (MHA). Out of the 77 bacterial isolates, 69 isolates were tetracycline-resistant except for 8 bacterial isolates. However, these 8 bacterial isolates were found to be resistant to either erythromycin or streptomycin. Multidrug resistance was also observed in the following genera in each site: from Gimje, *Citrobacter*, *Bhargavaea*, and bacterial isolates classified as *Proteobacteria*; from Iksan, *Chryseobacterium*, *Paenibacillus*, *Streptomyces*, and *Bacillus*; from Jangsu, *Serratia*, *Streptomyces*, *Burkholderia*, and one classified under family *Yersiniaceae*. It is important to note that some of these genera have species that are considered pathogens. The conventional PCR among the bacterial isolates revealed that 57 isolates among 110 tested were negative for the presence of any of the four RPP *tet* genes, while the remaining 53 bacterial isolates were positive for the presence of RPP *tet* gene. The most frequently detected RPP *tet* gene was *tet*W which was present in 37/53 isolates. On the other hand, 11/53 isolates were found positive for *tet*Q, and 23/53 isolates were found positive for *tet*S. Furthermore, 14/53 isolates carried two RPP *tet* genes and 2/53 isolates had three *tet* genes namely *tet*Q, *tet*S, and *tet*W. The *tet*O was not detected in any of the 53 bacterial isolates. This could imply the possibility of its presence among nonculturable or difficult-to-culture bacteria, since the *tet*O gene was detected in the total extracted DNA from Gimje and Iksan soil samples. Another possibility is that majority of the *tet*O in the study area resides in the chromosome of resident bacteria resulting to its limitation to be transferred to other bacterial host [39]. This is in contrast to *tet*W, which is the frequently detected RPP *tet* gene and which has the highest *tet* gene relative copy number. This could be explained by its association with conjugative transposons [40]; however, further studies should be done to substantiate this presumption. Lastly, antibiotic susceptibility testing results revealed that some of the bacterial isolates were multidrug-resistant to commonly used antibiotics in agriculture. Identification of the bacterial isolates exhibiting the multidrug resistance is important in assessing the risk to human health since multidrug resistance is considered a public health crisis. Furthermore, identification of the bacterial isolates could also hint the possible source that is crucial to understanding the ecology of antibiotic resistance, which in turn help in creating measures to hinder or prevent its rapid transmission. High prevalence and high quantified gene copy number of antibiotic resistance gene is indicative of its inclusion for surveillance [41–43].

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13765-021-00613-6.

Additional file 1: **Table S1**. Soil samples collected from arable land and livestock farms located in Gimje, Iksan, and Jangsu. **Table S2**. List of PCR primers used to amplify selected Ribosomal Protection Proteins (RPP) tet genes and 16S rRNA gene using both conventional and real-time PCR (qPCR). **Table S3**. Percent of total culturable tetracycline-resistant (CFU/g) to total culturable bacteria (CFU/g) in soils collected from arable land and livestock farms in three study areas. **Table S4**. Prevalence of RPP tet gene in different agricultural sites in three study areas. **Data S1**. GPS data of soil sample collection sites. **Data S2**. Summary of KB testing to three antibiotics and molecular testing of tetO, tetQ, tetS, and tetW of bacterial isolates from the three study areas.

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Authors' contributions
KN performed and analyzed the soil antibiotics-resistant bacteria as the main contributor of the writing. TU and H-GH suggested constructive ideas and revised the manuscript. J-HL designed and supervised this research. All authors read and approved the final manuscript.

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Availability of data and materials
Supplementary Material is followed.

Declarations

Competing interests
Not applicable.

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