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Antibiotic Resistance Genes (ARGs) in Soil Receiving Swine Wastes in Surin Province, Thailand

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Abstract

Swine wastes are commonly used to maintain soil fertility in crop production; however, there are growing concerns over the impact on antibiotics contamination in agriculture that stores and transmits antibiotic resistance genes (ARGs). This study was conducted to investigate the dissemination of 6 ARGs in soil receiving swine wastes from the traditional and the commercial farms in Surin province, Thailand using polymerase chain reaction (PCR) method and analyze soil properties. The results were found that the soil receiving swine wastes, i.e. wastewater, sludge and manure, from the commercial farming causing higher contents of nitrogen (N), phosphorus (P) and organic matter (OM) when compared to the soil receiving manure from the traditional farming. These nutrients and OM increased soil fertility for microorganism growth, and then these microorganisms could create ARGs more than those soil with lower fertility. In this study, tetracycline resistance genes (*tet*O and *tet*M) were dominantly detected in all soil samples, and quinolone resistance gene (*qnr*B) was found in the soil receiving from the commercial farming having the history in using quinolone continuously. Besides, *tet*O and *tet*M were found in both of traditional and commercial farming, and ARGs from the soil receiving swine wastes from the commercial farming were found more than those from the soil receiving swing manure from the traditional farming. These results showed that ARGs occurred in soil samples were definitely found from both traditional and commercial farming; thus, agriculturalists should pay more attention and have awareness in using antibiotics for applying swine wastes and manure for agricultural purpose.

Keywords: Antibiotic resistance genes; Soil receiving swine wastes; Swine manure

1. Introduction

The discovery of antibiotics has been recognized as one of the greatest advances in the history of medicine, which began the era of antibiotics. Antibiotics are chemical substances produced by microorganisms to inhibit the growth of and even to destroy bacteria and other microorganisms (Sanchez and Demain, 2015). Therefore, antibiotics are widely used to treat and prevent infectious diseases in human, animal, agricultural practices, and swine farm in many countries around the world (Landers *et al.,* 2012; Adedeji, 2016) including Thailand. Antibiotics administered to swine can result in selection of antibiotic resistant bacteria and enrichment of antibiotic resistance genes (ARGs), causing their releases to soil and water environments (He *et al.,* 2020). In addition, due to the increasing use of antibiotics in swine production, antibiotic resistance has become increasingly severe (Mathew *et al.,* 2007). Swine wastes are a spreading source of ARGs via the manure carrying resistant bacteria (Udikovic-Kolic *et al.,* 2014; Chang *et al.,* 2015; Shterzer and Mizrahi, 2015) and dispersed in soil when

the manure is spread as fertilizer (Smith *et al.,* 2019). After the agricultural field application of antibiotic - polluted manure, the horizontal transfer of ARGs from fecal microorganisms to indigenous environmental bacteria is an important factor in resistance distribution (Bello-López *et al.,* 2019) with a potential for further movement in drainage water, which may contribute to an increase in ARGs in non-agricultural settings (Luby *et al.*, 2016). Consequently, there are ample opportunities for antibiotics to be released into natural ecosystems (Grenni *et al.,* 2018) where they impact the structure and activity of microbial inhabitants in the environment (Kraemer *et al.*, 2019).

In Thailand, swine wastes comprising of wastewater, sludge and manure are commonly applied to agricultural land to recycle their plant nutrients. Because of the wastes were harbored greater microbial activity and was used to maintain soil fertility in crop production. However, swine waste could be a source of antibiotics contamination in agricultural soil receiving swine waste that stores and transmits ARGs. In the environment, due to the application of the swine waste in agricultural practice, the increasing abundance of ARGs poses a potential threat to human health and environmental safety (Zhou and Yao, 2020). However, to our knowledge there are few studies investigating ARGs in agricultural soil in Thailand. Then, our study aimed: (i) to investigate the occurrence of ARGs in agricultural soils receiving swine manure during soil preparation before planting from the traditional farming; (ii) to investigate the occurrence of ARGs in agricultural soils receiving swine wastes, i.e., wastewater, sludge and manure during soil preparation before planting and during the plants growing from the commercial farming; and (iii) to analyze physicochemical properties of soil samples.

2. Materials and methods

2.1 Study areas

Five representative soil areas were collected as the followings. Soil control sample (defined as S1) was collected from the forest area that has never been used as an animal breeding, located in Rattanaburi district, Surin province. Two soil samples (defined as S2 and S3) were collected from two rice fields receiving swine manure from the traditional farming, located in Mueang and Rattanaburi district, Surin province. The swine houses were designed for 50 - pigs small scale with open farming system.
The manure is applied to prepare soil before planting. Two soil samples (defined as S4 and S5) were collected from banana and vegetable fields receiving swine wastewater, sludge and manure from the commercial swine farming, located in Rattanaburi district, Surin province. These farms accommodated 750 - pigs medium scale with evaporative cooling system. The manure is applied to prepare soil before planting and throughout the growing stages.

2.2 Sample collection

All soil samples were collected at a depth of 15 cm below the surface layer of the soil in May, 2020. Fifteen to twenty discrete subsamples were collected depending on the size of agricultural fields. Composite samples were prepared by mixing an equal amount of discrete subsamples. About one kg of each final soil sample, selected by the quadripartite method, was taken to the laboratory then 0.5 kg of soil samples was stored at -20°C until DNA extraction and 0.5 kg was air dried at ambient temperature in the dark and homogenized by sieving through a two mm sieve, returned to the clean sealable plastic bag and storage at temperature room for soil properties analyzed.

2.3 Analysis of physical and chemical properties

Physicochemical properties were analyzed following the conventional methods. Thereafter, samples were analyzed for sand, silt, and clay fractions by the hydrometer method. Soil pH was measured with pH meter. Nitrogen (N) analysis was conducted using Kjeldahl method.

Available phosphorus (P) was conducted using a Bray II method. Total potassium (K) was conducted using flame photometer method. Soil organic matter (OM) was conducted using Walkley - Black method.

2.4 DNA extraction

Two hundred and fifty milligrams of each sample were used for DNA extraction by using GenElute™ Soil DNA Isolation Kit (Sigma - Aldrich, USA) following the manufacturer's protocol according to the manufacturer's instructions. The concentration and quality of extracted DNA were checked using 1.0% agarose gel electrophoresis and spectrophotometric analysis. Finally, the extracted DNA was preserved at -20 ◦C for further analysis.

2.5 Antibiotic Resistance Genes (ARGs) and Primer

Primers used for PCR amplification of 6 different genes and were either selected based on the published sequences available in Genbank or from published primer sequences. The target genes included two tetracycline resistance genes (*tet*M and *tet*O), two erythromycin resistance genes conferring resistances to macrolide-lincosamides-streptogramin B (MLSB genes: *erm*A and *erm*B) and two quinolone resistance genes (*qnr*A and *qnr*B). The specific primer pair and sequences were listed in Table 1.

2.6 Polymerase chain reaction (PCR) conditions

DNA was amplified using thermocycler Hybaid PCR machine in a 25 µL reaction volume. The reaction composed of 12.5 µL of PCR master mix (GeneDireX), one µL of each primer, one µL of DNA template and 9.5 µL DNase RNase free water, with the following PCR cycling conditions were pre-denaturing at 94°C for five min, followed by 35 cycles of 94°C for 1.5 min, 55°C for one min, 72 °C for one min, followed by a final extension at 72°C for five min for *tet*O, *tet*M, *erm*A and *erm*B detection. The temperature program of *qrn*A and *qrn*B were as follow: initial pre-denaturation at 94°C for two min, followed by 30 cycles of 94°C for 45 sec, 53°C for 45 sec, 72°C for one min and a final extension step of 72°C for five min.

2.7 Agarose gel electrophoresis

The PCR products were electrophoresed on TAE agarose gel buffer. Agarose gel prepared by adding 0.4 gm agarose powder to 40 ml of 1x TBE (Tris-Borate-EDTA) buffer, then carefully dissolved by microwave oven until all the particles were dissolved. After the solution cooled down, 0.5 µl RedSafe™ was added and mixed gently. The gel was poured into to the tray of which the comb inside it and sterilized tip was used to remove the bubbles around the comb tips and on the gel surface. After the gel had set, the tape around the tray and the comb was carefully removed to leave the well behind it to allow appropriate loading of PCR products inside it. Later, gently 10 µl volume of each sample were loaded onto a submerged gel that consisted of a 1 % w/v concentration of agarose. Appropriate size marker, orange DNA loading dye was loaded in the first well of the gel by micro pipette and sterile water was used as the negative control in every run, then the gel was run at 100 V for approximately 35 hours and examined under UV light. The photo was taken using digital camera.

2.8 Gel purification and sequence analysis

Gel purification, the target DNA bands were cut out from the TAE agarose gel and purified them using a PCR Clean-up & Gel extraction kit, GeneDirex (Bio-Helix, Keelung City, Taiwan). All purify DNA bands of melting gel were directly sequenced and measured of DNA concentration using a NanoDropTM spectro photometer prior to sequence analysis by Macrogen Inc. (Seoul, Korea). The results were compared against those in the GenBank nucleotide database via the BLAST web page (http://www.ncbi.nlm.nih.gov/BLAST/).

Table 1. Primers employed in the present study for PCR

Antibiotic agent	Primer	Sequences (5'→3')	Temp. (°C)	Size (bp)	References
Tetracycline	*tet*M	(F) GTG GAC AAA GGT ACA ACG AG (R) CGG TAA AGT TCG TCA CAC AC	55	406	Ng *et al.* (2001)
	*tet*O	(F) AAC TTA GGC ATT CTG GCT CAC (R) TCC CAC TGT TCC ATA TCG TCA	55	515	Ng *et al.* (2001)
Erythromycin	*erm*A	(F) CCC GAA AAA TAC GCA AAA TTT CAT (R) CCC TGT TTA CCC ATT TAT AAA CG	55	590	Kumar *et al.* (2005)
	*erm*B	(F) TGG TAT TCC AAA TGC GTA ATG (R) CTG TGG TAT GGC GGG TAA GT	55	745	Kumar *et al.* (2005)
Quinolone	*qnr*A	(F) CAG CAA GAG GAT TTC TCA CG (R) AAT CCG GCA GCA CTA TTA CTC	53	630	Ciesielczuk *et al.* (2013)
	*qnr*B	(F) GGC TGT CAG TTC TAT GAT CG (R) GAG CAA CGA TGC CTG GTA G	53	488	Ciesielczuk *et al.* (2013)

3. Results and Discussion

3.1 Physicochemical Properties of Soil

The obtained results for selected physiochemical properties of soil samples were summarized in Table 2. In fact, most crops were assigned for a suitable pH in moderately acidic to slightly acidic condition and ranged from 6.0 to 6.5 (Miller, 2016). The exception to this finding was in S1 and S4 samples. The soil pH was acidic in S4 sample (pH 5.36), which bananas were grown. Bananas could tolerate some soil acidity; however, a pH below 5.0 means that elements such as aluminium and manganese become very soluble in the soil and their toxicity can reduce plant growth (Rahman *et al.,* 2018). The soil texture was sandy loam in all samples while total N from 0.1 - 0.3%, available P from 5.55 - 828.88 %, exchangeable K from 0.04 - 0.38 % and OM from 1.73 - 12.08%. The total N in all samples were high compared with the critical levels of 0.1%. Available P in S2, S3, S4 and S5 were high compared with the critical levels of < 10 mg kg^{-1}. Using the critical levels of < 60 mg kg^{-1} exchangeable K was low.

From the physicochemical properties of soil samples, S4 and S5 collected from the waste area of the commercial farming caused higher contents of N, P and OM when compared to S2 and S3 collected from the manure area of the traditional farming. This is because of the swine house domesticating a lot of pigs (up to 750 pigs) then excreting high amount of swine wastes including manure with plenty of OM (Edmeades, 2003), of nutrients especially N and P (Choudhary *et al.,* 1996 and Loss *et al.,* 2019). When these swine wastes were applied to fertilize soil, the soil was full of nutrients for plaiting. However, if soil received nutrients exceeding the usage of plants or the carrying capacity of soil they could leak to the environment and cause a toxic problem (Antoneli *et al.,* 2019). When considering for selecting kinds of cultivated plants, S4 and S5 were used to plant bananas and vegetables and received the swine wastes from the commercial framing, and the plants are grown continuously without soil preparation every year. Then, the farmers can apply the swine wastes continuously starting from soil preparation throughout growing stages and the soil is received nutrients constantly. Partly, S2 and S3 were paddy fields received the swine manure from the traditional farming with low number of pig (up to 50 pigs), then the manure was not much and the farmers usually apply the manure when plowing in the soil preparation stage. Therefore, the nutrients gathered in S2 and S3 are lower than S4 and S5.

Table 2. Physiochemical properties of soil

Parameter	Soil sample				
	S1 Control	S2 rice	S3 rice	S4 banana	S5 Vegetable
pH	5.33	6.00	6.50	5.36	6.30
Organic matter (%)	1.94	0.73	1.45	11.71	12.08
Total N (%)	0.2	0.1	0.1	0.3	0.3
Available P (mg kg^{-1})	5.55	419.69	516.79	766.63	828.28
Exchangeable K (mg kg^{-1})	0.05	0.04	0.16	0.08	0.38
Sand	59.7	60.7	61.7	59.8	61.5
Silt	27.3	25.5	24.8	29.5	25.7
Clay	13.2	13.8	13.5	10.8	12.7
Textural class	sandy loam	sandy loam	sandy loam	sandy loam	sandy loam

Note: S1, soil control sample; S2 and S3, paddy soil receiving swine manure from open farming; S4, banana field soil receiving swine wastes from evaporative cooling farming and S5, vegetable field soil receiving swine wastes with evaporative cooling farming

3.2 Antibiotic resistance genes in agricultural soil

The results showed that the agricultural soil samples receiving swine manure found tetracycline resistance genes (*tet*O and *tet*O) in S2, S3, S4 and S5 samples, and quinolone resistance gene (*qnr*B) was found in S4 and S5 samples, while ARGs were not found in the control in this study (Table 3), indicating their dominance and persistence in swine manure. Tetracycline genes were the most common ARGs in soil, swine manure and animal fecal samples (Zhu *et al.,* 2013). The results also indicated that tetracycline resistance genes were most frequently detected ARGs in agricultural soil samples receiving swine manure while in the S4 and S5 samples were detected type of ARGs more than the other samples. The data for the similarity DNA analysis were obtained from sequences contained in the BLAST databases available from NCBI. Besides, the sequence from S2, S3, S4 and S5 sample was found 98 - 100% similarly to *tet*M and *tet*O (GenBank accession no. CP008813.1, CP015626.1, NG_048218.1, FM202720.1, KF845961.1, NG_048254.1, NG_048267.1 and JN086171.1). Also, the sequence from S4 and S5 sample was found 99% similarly to *qnr*B (GenBank accession no. KJ850486.1 and CP017293.1). In large scale swine farms, animals generate huge amounts of animal wastes, which contain high levels of organic substances, nutrients and diverse microorganisms. On - farm waste treatment systems such as lagoon and digester are designed to reduce these traditional contaminants such as nitrogen and phosphorus, but not the emerging contaminants like ARGs (Pruden *et al.,* 2006).

Table 3. Detection of ARGs in the soil samples by PCR.

Sample	Strain(s) showing presence of genes encoding					
	*tet*M	*tet*O	*erm*A	*erm*B	*qnr*A	*qnr*B
S1	-	-	-	-	-	-
S2	+	+	-	-	-	-
S3	+	+	-	-	-	-
S4	+	+	-	-	-	+
S5	+	+	-	-	-	+

Note: S1, soil control sample; S2 and S3, paddy soil receiving swine manure from traditional farming; S4, banana field soil receiving swine waste from commercial farming and S5, vegetable field soil receiving swine waste commercial farming

Based on interviews with farmers, tetracycline, gentamycin, penicillin G, amoxicillin, novalcin, enrofloxacin and tylosin were used via feed, water drinking and injection for growth promotion and disease prevention in commercial farming. In contrast, the traditional farming rarely uses antibiotics except sickness of pigs on case by case, and the domesticating pigs in the traditional farming are stronger than those in the commercial farming. However, some antibiotics could not be absorbed completely in pig's body then an excess would excrete via urine and manure in the form of parent compound (Boxall *et al*., 2004), contaminating with wastewater, sludge and soil from swine houses (Chuanpit *et al.,* 2018). As a result, an accumulation of antibiotics in the environment causes microorganism adaption to produce ARGs.

In this study, we found *tet*O and *tet*M swine manure from the traditional farming because tetracycline resistance genes were the most frequently detected for ARGs in animal manure and manure-amended soils in many countries (Ji *et al.,* 2012). The genes *tet*W, *tet*O and *tet*Q were very common in swine manure,

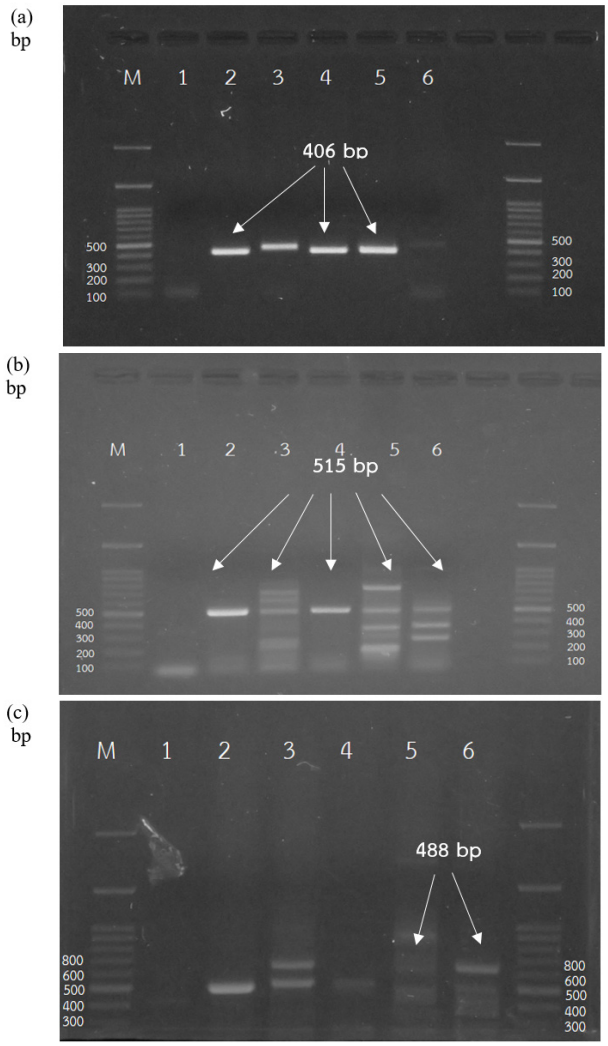

Figure 1. Agarose gels of PCR products stained with red safe for: (a) *tet*M, (b) *tet*O and (c) *qnr*B. Lanes: M, 100 bp DNA ladder; 1, negative control; 2 and 3, S2 and S3, paddy soil receiving swine manure from traditional farming; 4, S4, banana field soil receiving swine waste from commercial farming and 5, S5, vegetable field soil receiving swine waste from commercial farming

suggesting that they were stably maintained in the animals'gut microbiota (Kyselkova *et al.*, 2015). *tet*M could be detected in microcosms supplemented with swine manure. In swine manure samples from 120 farms, the genes *tet*M and *tet*O showed higher concentrations when tetracycline residues were detectable (Schwaiger *et al.,* 2009). Tetracyclines and quinolones have low potential for mobility in soil and they have been detected in manure and manured soil due to their strong sorption on soil and delayed biodegradation (Wang and Wang, 2015 and Xu *et al.,* 2015), tetracycline and quinolone resistance genes could be found in the agricultural application of swine manure (Xu *et al.,* 2015). The availability of these ARGs could be affected by various factors, such as physicochemical properties of drugs, environmental conditions (Cycoń *et al.,* 2019), soil properties (Wang *et al.,* 2020), soil types, pH, water content, soil particle adsorption, climate, microbial population, and other soil pollutants (Li *et al.*, 2012).

Moreover, our results showed that the gene target *tet*O, *tet*M and *qnr*B were more abundant in soil with high OM, N and P than soil with low OM, N and P. Soil nutrients and OM could be enhancing soil organisms, their activities and influences the increase in the prevalence of ARGs (Milanov *et al.*, 2016). Several studies reported that *tet*M, *tet*O and *qnr*B were common resistance genes in soil receiving wastewater from swine production (Zhu *et al.,* 2013; Auerbach *et al*., 2007 and Chen *et al.,* 2007) as found in S4 and S5 soil samples. In addition, numerous studies such as Robicsek *et al.,* 200; Forcella *et al.,* 2010; Cummings *et al.,* 2011) have been reported the isolation of *qnr*A and *qnr*B from the environment source and wastewater effluent in many regions.

4. Conclusions

This study showed that antibiotic resistance genes (ARGs) survived in soil receiving swine waste. The swine waste led to increase abundance of ARGs in soil, which could affect microbial ecology in soil. Importantly, inappropriate usage of antibiotics causing a mutation allowing them to survive in the environment with the development of resistance genes.

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