Environmental Pollution 229 (2017) 102–110

Contents lists available at ScienceDirect

Environmental Pollution

journal homepage: www.elsevier.com/locate/envpol

# Characterization and quantification of antibiotic resistance genes in manure of piglets and adult pigs fed on different diets☆

Xiao-Ming Lu [a, *], Wen-Feng Li [a], Chao-Ben Li [b]

[a] Institute for Eco-Environmental Sciences, Wenzhou Vocational College of Science and Technology, Wenzhou 325006, China
[b] College of Resources and Environment, Fujian Agriculture and Forestry University, Fuzhou 350002, China

ARTICLE INFO

*Article history:*
Received 14 March 2017
Received in revised form
28 May 2017
Accepted 28 May 2017
Available online 2 June 2017

*Keywords:*
Pig manure
ARGs
High-throughput fluorescence quantitative PCR
Antibiotic
Heavy metal

ABSTRACT

Studies have shown that pig manure is a reservoir of antibiotic resistance genes (ARGs). However, little is known about the characteristics of ARGs in the manure of piglets and adult pigs fed on different diets. In the present study, the ARG characteristics of the manure of piglets and adult pigs fed on different diets (feed, grain) were analyzed using high-throughput fluorescence quantitative PCR. Correlations between heavy metals, antibiotics, and ARGs in pig manure were analyzed. The results showed that the heavy metal and antibiotic contents in the manure of pigs receiving feed significantly exceeded those in the manure of pigs receiving grain. The heavy metal and antibiotic contents were higher in manure of piglets than in that of adult pigs. Feed significantly increased the ARG diversity in the pig manure. The ARG diversity was higher in manure of piglets than in that of adult pigs. In the manure of pigs receiving feed, 25 (from piglets), 12 (from adult pigs) ARGs were enriched significantly compared with pig fed with grain. In particular, *sat4* (in piglets) and *vatE-01* (in adult pigs) showed the highest enrichment, being increased by 59 and 19-fold, respectively. The ARG diversity correlated positively with the concentrations of antibiotics and heavy metals in the manure.



## 1. Introduction

Antibiotic resistance genes (ARGs) are a new type of environmental pollutant (Su et al., 2014). ARGs are associated with the spread of pathogenic drug resistance (Zhang et al., 2009). Antibiotics prevent diseases and stimulate growth; therefore, subtreatment doses are added to animal feed in a wide range of the applications (Chee-Sanford et al., 2009; Pan et al., 2011; Hvistendahl, 2012). In parallel with the intensive development of animal husbandry, antibiotics have been used in the breeding industry for about 50 years (Looft et al., 2012). Tetracyclines, quinolones, sulfonamides, macrolides, and β-lactam antibiotics are used commonly in Chinese animal husbandry (Zhang et al., 2015). Antibiotics in pig feed will change the intestinal microbial community structures, thereby increasing the intestinal microbial communities containing ARGs, leading to ARG enrichment. In addition to the antibiotics and heavy metals that are applied to the soil with manure, ARGs are also spread into the environment via feces (Zhu et al., 2013). The excretion of intestinal antibiotic resistant bacteria carrying antibiotic resistance genetic information could be transferred to microorganisms in the external environment (Akhtar et al., 2009; El Salabi et al., 2013). Once ARGs enter pathogens, they pose a potentially significant risk to human health. Therefore, pig manure is an important reservoir of ARGs, and it is necessary to fully understand the characteristics of ARGs pollution. Usually, the use of feeds is significantly different between piglets and adult pigs (Lu et al., 2013), and the application of antibiotics is also different between them: more antibiotics are used in piglet breeding than are used in breeding adult pigs, which could result in their fecal ARGs being different. However, there have been no reports about different feeds affecting ARGs in piglet and adult pig manure.

ARG abundance correlated significantly and positively with antibiotic and heavy metal concentrations in the soil after pig manure application (Zhu et al., 2013). Wu et al. (2015) also found that ARG levels in landfill leachate correlated significantly and positively with heavy metal concentrations. However, to date, there have been no reports on the correlation between ARGs and different forms of heavy metals. Published studies have shown that it is not sufficient to examine the potential environmental risks of heavy metals merely by their total amount (Peruzzi et al.,

☆ This paper has been recommended for acceptance by Klaus Kummerer.

* Corresponding author.
*E-mail address:* 13736961468@163.com (X.-M. Lu).

http://dx.doi.org/10.1016/j.envpol.2017.05.080
0269-7491/

2011). The environmental behavior, ecological effects, and environmental hazards of heavy metals depend to a great extent on which types of metals are present (Peruzzi et al., 2011). Therefore, it is necessary to assess the correlation between heavy metal forms and ARGs, which will aid a more in-depth interpretation of ARG occurrence. Studies have been published concerning the distribution and diversity of ARGs in city sludge (Rizzo et al., 2013), urban sewage (Pruden, 2014), soil after the application of pig manure (Cheng et al., 2016), aquaculture water (Gao et al., 2012), estuaries (Chen et al., 2013), and other environments. However, these studies analyzed a limited number of resistance genes. Recently, the high-throughput quantitative PCR (HT-qPCR) method was developed, which can perform a comprehensive overall investigation of environmental ARGs and can identify enriched genes.

Based on the above considerations, to analyze comprehensively the characteristics of pig manure ARG pollution, we collected different typical pig manures as test samples. The abundance and diversity of the ARGs were evaluated using HT-qPCR. Enriched ARGs and correlations between ARGs, heavy metal forms, and antibiotics were analyzed. This study provided a basis for further research into methods to control of ARG pollution in livestock waste.

## 2. Materials and methods

### 2.1. Sample collection and treatment

In July 2016, three adult pigs (weighing approximately 200 kg each), and three piglets (15 kg each) were bred with feed (containing additives) in a conventional farm in Yongjia county, Zhejiang province, China. At the same time, farmer's grain (no additives) was used to raise three adult pigs and three piglets of similar sizes to the corresponding pigs receiving feed for comparison in the farm. Thus, 12 pigs were raised for 3 months. Except for the antibiotic additives in the feed, the pigs/piglets did not receive additional antibiotics during the 3 months or before. During the breeding process, the amount and frequency of feeding between both groups of adult pigs and between both groups of piglets were consistent. In addition, according to the local farmer's feeding habits, the 12 pigs were reared in the same way. All pigs were genetically related. They were bred and fattened on the same farm. They were not kept on straw. In the present study, the pigs did not have contact with each other or with other pigs. The feed was not clearly labeled with the type of antibiotics and/or heavy metals, nor with their respective contents. The feed contained various additives. Different additives contained different antibiotics and/or heavy metals. Currently, about 14 kinds of additives are used extensively in the Chinese pig breeding industry.

In October 2016, sampling was performed once a week. Four samples were collected from each pig resulting in 12 samples per group. A 1-kg sample of fresh pig manure (manure deposited in one day) was collected from each pig using a Petite Ponar sampler (Wildlife Supply Company, Saginaw, MI), and mixed on site. These samples were stored in sterile 400-mL canning jars (Ball Corporation, Muncie, IN, USA). Samples of pig manure (FAPM = manure from an adult pig receiving a feed diet, FYPM = manure from a young pig receiving a feed diet, GAPM = manure from an adult pig fed a grain diet, GYPM = manure from a young pig fed a grain diet) were then stored in an ice box, transferred quickly to the laboratory, and kept at −20 °C, for chemical analysis and DNA extraction. The manure samples from one animal were combined to one sample for chemical analysis. The average in each group was obtained for analysis.

### 2.2. Heavy metal analysis

Heavy metal classification was performed using the continuous extraction method established by Tessier et al. (1979). The order of extraction was as follows: the exchangeable state, 1.0 mol $L^{-1}$ $MgCl_2$, pH 7.0, water bath (22 ± 3 °C); the carbonate bound state, 1 mol $L^{-1}$ NaAc, pH 5.0, room temperature; the iron and manganese oxide bound state, 0.04 mol $L^{-1}$ $NH_2OH{\cdot}HCl$, water bath (96 °C); sulfide and organic binding, 0.02 mol $L^{-1}$ $HNO_3$ + 30% $H_2O_2$ (w/w), pH 2.0, water bath (85 °C); the residual state, concentrated $HNO_3$, heating on the hot plate to near dry. Cd was analyzed using a graphite furnace - atomic absorption spectrometer (AAS Vario 6, Analytik Jena AG, Jena, Germany). Cr, Cu, Pb, and Zn were analyzed using a flame atomic absorption spectrometer (AAS Vario 6, Analytik Jena AG). As was analyzed using hydride generation atomic fluorescence spectrometry (AFS - 2202, Beijing Haiguang Instrument Co., Ltd, Beijing, China).

### 2.3. Basic physicochemical indexes and antibiotic determination

Organic carbon, total nitrogen, total phosphorus, full potassium, PH value, and water content were analyzed using the potassium dichromate method, the Kjeldahl method, the vanadium molybdenum yellow colorimetric method, the flame photometer method, the conductivity method, and the mass method, respectively. All these methods were performed as stated by Lu (2000).

Determination of antibiotics was performed as follows:

1) Reagents and equipment

Methanol and acetonitrile were pure chromatography grade (Sigma-Aldrich Co. LLC, Darmstadt, Germany); the experimental water was high purity water; other chemical reagents were of analytical grade (Sigma-Aldrich Co. LLC). The antibiotics used for the antibiotic standards were tetracycline (TTC), oxytetracycline (OTC), chlortetracycline (CTC), norfloxacin (NOR), ciprofloxacin (CIP), penicillin (PEN), cephalosporin (CEP), sulfamethoxypyrimidine (SM1), sulfamethazine (SM2), tylosin (TYL), and erythromycin (ERY). Antibiotic standards were produced in Augsburg in Germany by Dr. Ehrenstorfer Gmbh, and their purity was greater than 98%. An antibiotic standard solution was made by accurately weighing various antibiotic standards and dissolving them separately in acetonitrile to make 100 μg $mL^{-1}$ antibiotics standard stock solutions. Appropriate amounts of the various stock solutions were diluted with acetonitrile to form a mixed standard mother liquor, which was then gradually diluted with acetonitrile to prepare a calibration curve working fluid with the concentration range of 0.01–1.00%. The standard stock solution was kept in the refrigerator. EDTA-McIlvaine buffer was prepared as follows: 12.9 g of citric acid, 27.5 g disodium hydrogen phosphate, 37.2 g of ethylenediamine tetraacetic acid disodium (EDTA) were dissolved in 1 L of water (pH = 4.0).

The equipment (Shimadzu Ltd., Kyoto, Japan) used comprised a Shimadzu LC-20AT high performance liquid chromatography (UV detector); an LC solution workstation; a Visiprep™-DL solid phase extraction device (Supelco); an LC-SAX solid phase extraction cartridge (3 mL/500 mg, Supelco); and an LC-18 solid phase extraction cartridge (3 mL/500 mg, Supelco).

2) Collection and pretreatment

Pig manure was collected and mixed. The samples were then subjected to dividing the samples into quarters method. After drying, the samples were crushed over a 60-mesh sieve in the room in which they were to be tested. The samples were pretreated by