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Exploring the abundance and influencing factors of antimicrobial resistance genes in manure plasmidome from swine farms

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ABSTRACT

Plasmids play a critical role in the dissemination of antimicrobial resistance genes (ARGs), however, a systematical understanding of ARGs originated from plasmids in swine production is currently lacking. Herein, quantitative polymerase chain reaction was applied to determine the prevalence of ten ARGs and the class1 integron gene *int1* of plasmid source in swine manure from 44 farms in Sichuan, Hubei and Hebei provinces, China. All assayed ARGs were observed in plasmid DNA samples, and the average absolute abundance of *aac(6)-Ib-cr*, *bla_{NDM}*, *bla_{CTX-M}*, *optrA*, *ermB*, *floR*, *mcr-1*, *qnrS*, *tetM*, *sul1* and *int1* were 7.09, 2.90, 4.67, 6.62, 7.55, 7.14, 4.08, 4.85, 7.16, 7.11 and 8.07 of 10 log copies/gram, respectively. *Int1* showed a high correlation ($r > 0.8$, $P < 0.01$) with the abundance of *aac(6)-Ib-cr* and *sul1* in swine manure. Moreover, the farm scale (i.e., herd population) and geographical location were not found to be critical factors influencing the absolute abundance of ARGs of plasmid DNA in swine farms. However, the concentrations of florfenicol, Cu, Zn, Fe, total phosphorus (TP) and total potassium (TK) demonstrated a significant correlation with the abundance of several ARGs. Particularly, Cu and Zn had high correlations with *optrA* and *bla_{CTX-M}*, respectively. Our results demonstrated that antibiotics, heavy metals and environmental nutrients are likely jointly contributing to the long-term persistence of ARGs in swine production. This study provides insights into the abundance and influencing factors of ARGs from swine manure, which is of significance for assessing and reducing the public health risks in livestock production.

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Introduction

Infections caused by antibiotic-resistant bacteria (ARB) are increasing worldwide and pose a significant threat to pub-

lic health (Ventola, 2015), and antimicrobial resistance genes (ARGs) are becoming recognized as environmental pollutants (Zhu et al., 2013). Antibiotics are extensively used in livestock and poultry industries worldwide, mainly for bacterial infection treatment and disease prevention (Checcucci et al., 2020; Zhang et al., 2015). As predicted, the global consumption of all antibiotics in livestock sector is estimated to be 131,109

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tons in 2013, and it is expected to reach 22,235 tons by 2030 (Van Boeckel et al., 2017; Zhang et al., 2015). Most antibiotics are poorly absorbed in animals and cannot be completely metabolized (Liu et al., 2010; Zhang et al., 2014). Swine manure has become a reservoir of antibiotic resistance as it contains various ARGs and antibiotic residues (He et al., 2019). Also, ARGs and antibiotic residues can be transmitted from animal manures to agricultural soil through manure application (Liu et al., 2021). Previous studies have shown that high abundance of ARGs and antibiotic residues are detected in agricultural environments, such as rivers and soil (Li et al., 2012; Muurinen et al., 2017). In addition, ARGs may be transmitted to human pathogens from agricultural environments through the food chain or horizontal gene transfer (HGT), leading to serious clinical treatment crisis (Barton, 2014; Zhu et al., 2013). Therefore, characterizing the prevalence, abundance and possible mobilization of ARGs in livestock manure is important for evaluating their public health risk and facilitating the subsequent development of control measures.

Plasmids contribute to the transmission of ARGs, promoting HGT across bacterial species (Carattoli, 2013; von Wintersdorff et al., 2016). They play a key role in the evolution of bacterial resistance, among which conjugative plasmids are undoubtedly the most important driver of the spread of ARGs in bacterial families (Rozwandowicz et al., 2018). Crucially, some of these plasmid-conjugated bacteria become 'superbugs' that disseminate uncontrollably in clinical settings (San Millan, 2018). Consequently, it is of great significance to investigate plasmid-mediated ARGs. Several studies have characterized the profile of ARGs in the plasmidome of cultivable bacteria in municipal wastewater treatment plants (WWTPs), in which they found that plasmids from bacteria of WWTP source encoded resistance to all major classes of antimicrobial drugs (Shi et al., 2018; Szczepanowski et al., 2009). However, these plasmids used for metagenome sequencing were based on culture methods, which may amplify the advantages of fast-growing culturable bacteria, and can't directly evaluate the abundance of plasmidome mediated ARGs. Swine manure, which is commonly used as a fertilizer in agriculture, served as a reservoir of ARGs, but we have limited knowledge of the plasmidome-related ARGs in there. Although most studies have investigated the diversity and the abundance of ARGs in total DNA in farm feces and receiving environments (Chen et al., 2019; He et al., 2016), which may ignore the importance of plasmid-mediated resistance genes. Therefore, further studies are needed to investigate the characteristic of ARGs in the swine farm environment via plasmidome.

In addition to residual antibiotics, the metals and environmental factors including Fe, Cu, Zn, As, total organic carbon (TOC) and carbon to nitrogen ratio could also promote the frequent spread of antibiotic resistance (Menz et al., 2019; Zhang et al., 2020; Zhu et al., 2013). Heavy metals (e.g., Fe, Cu, Zn and As), as feed additives, are widely used to promote growth and to improve productivity or control animal diseases for intensive cultivation of livestock and poultry (Hejna et al., 2021; Zhang et al., 2018), which are subsequently discharged into the environment through manure after animal metabolism, causing the risk of heavy metal accumulation (Hou et al., 2014; Qian et al., 2018). In addition, the pres-

ence of heavy metals (e.g., Cu and Zn) provides co-selective pressure for antibiotic resistance (Zhang et al., 2020; Zhu et al., 2013). For example, ARGs of antibiotics drugs and metals efflux mechanism showed cross- or co-resistance to many antibiotics and metals (Baker-Austin et al., 2006). Furthermore, nutrient-rich environments can positively influence the ARGs' spread and facilitate cell-cell interactions (Manaia et al., 2018). Temperature, pH, carbon-nitrogen ratio and available nutrients during composting have significant effects on the distribution of various types of ARGs (Awasthi et al., 2019; Li et al., 2017; Zhang et al., 2020). However, most studies focus on the influencing factors of ARGs in the total DNA of antibiotic contaminated environment, and the effects of residual antibiotics, heavy metals, and environmental nutrients on the ARGs in the plasmidome have not been elucidated.

In the present study, we sampled manure from 44 swine farms in Sichuan (SC), Hubei (HE) and Hebei (HB) provinces in China, and analyzed ten ARGs and *int1* using conventional quantitative polymerase chain reaction (qPCR). The objectives of this study were: (1) to characterize the diversity and abundance of ARGs in plasmidome of swine manure; (2) to explore the correlation between ARGs in plasmid DNA and physicochemical properties (including antibiotics, heavy metals and environmental nutrients), to further analyze the key influencing factors for resistome in swine manure. The results of this study will provide insights into the abundance and influencing factors of ARGs in livestock manure, which is of significance for mitigating the overall prevalence of antibiotic resistance in swine production.

1. Material and methods

1.1. Sampling

A total of 132 fecal samples were collected from 44 representative swine farms (Appendix A Table S1) in SC, HE and HB, representing three main swine production provinces in China, during July and August 2017. According to the annual output, these swine farms were defined as large-scale (more than 10,000), medium-scale (between 5000 and 10,000) and small-scale (less than 5000). We used a sterile spoon to randomly select three points from the fresh manure pile produced that day from each farm to collect fecal samples. At each point, a hole approximately 5–10 cm deep was dug into the heap, about 200 g of fecal taken from each hole. The three samples were packed in a sterile sampling bag/sampling bottle from each farm then were mixed into one sample. All of the samples were stored on dry ice during transportation and stored at -80°C for subsequent analysis.

1.2. Plasmid DNA extractions

The pretreatment used to extract the DNA sample was carried out in accordance with published protocols (Hart et al., 2015; Jitwasinkul et al., 2016; Yang et al., 2020), with minor modifications. One gram of swine feces were mixed thoroughly with 9 mL of 0.01 M phosphate-buffered saline (PBS, pH 7.4), and were further centrifuged at $1000 \times g$ for 5 min. The supernatant was collected and centrifuged at $10,000 \times g$ for another 10 min. The

sediment was resuspended with PBS, and repeated the previous centrifugation step ($10,000 \times g$ for 10 min). Finally, the sediments were collected which were used for the extraction of plasmid DNA by EndoFree Mini Plasmid Kit II (TIANGEN, China). The resulting plasmid DNA was treated with Plasmid-Safe™ ATP-Dependent DNase (Epicentre, USA) to eliminate genomic DNA contamination. The concentration and quality were assessed by a NanoDrop spectrophotometer (Thermo Scientific, Wilmington, DE, USA). Three DNA replicates were stored at -20°C until further analysis.

1.3. Detection and quantification of ARGs

We examined the abundance of 10 ARGs (*aac(6′)-Ib-cr*, *bla_{NDM}*, *bla_{CTX-M}*, *optrA*, *ermB*, *floR*, *mcr-1*, *qnrS*, *tetM*, *sul1*) and the class I Integron-integrase gene *int11* (Appendix A Table S2). The main reason for selecting these resistance genes was that beta-lactams, aminoglycosides, sulfonamides, tetracyclines, macrolides, quinolones, and florfenicol are commonly used in livestock production (McEwen and Fedorka-Cray, 2002), and such use is perceived to contribute to the development of antibiotic resistance (van den Bogaard and Stobberingh, 2000). Moreover, *int11* gene, which can carry a variety of ARGs for HGT between microorganisms, was included in the assessment of the presence of MGEs (Gillings et al., 2015).

To investigate the gene copy (copies) numbers of ARGs and *int11* in plasmid DNA, the selected ARGs and *int11* were quantified by qPCR and each DNA sample was normalized to the same concentration for subsequent analysis. The ARGs copy number were compared with the standard samples prepared from plasmids containing these specific genes, as previously described (Chen and Zhang, 2013). The standard samples were diluted to produce a series of 10-fold concentrations from 10^8 to 10^2 copy numbers for generating qPCR standard curves. Three replicates were set for each concentration template. The R^2 values of all standard curves were higher than 0.990. The PCR was performed in $20 \mu\text{L}$ reaction volume and protocol followed as described previously (Tuo et al., 2018). The thermal cycle consisted of 3 sec at 95°C , followed by 40 cycles of denaturation at 95°C for 30 sec, at the annealing temperature for 30 sec and extension at 72°C for another 30 sec. Melting profile analysis was used to verify the specificity of the qPCR products amplified. The primers used for qPCR and the annealing temperatures were listed in Appendix A Table S3. Negative controls containing no template DNA were run following experimental samples to exclude any possible contaminations. The density of each target gene in the DNA sample was determined by the standard curve method and all qPCR reactions were tested in triplicate. Genes with amplification efficiencies ranged from 90% to 110.0%. And positive samples were checked by electrophoresis in agarose gel (2% agarose, 80 V, 30 min, PowerPac200, Bio-Rad Laboratories Inc., USA).

1.4. Measurement of fecal samples physicochemical properties

Samples from six large-scale farms in Sichuan Province were used to determine physicochemical factors. The pH and TOC concentration were measured with a pH meter (PHS-3C, Inesa, China) and a TOC analyzer (TOC-VCPH, Thermo, China),

respectively. TN, TP, TK and heavy metals including Cu, Zn, As, Fe concentrations were determined by inductively coupled plasma mass spectrometry (ICP-MS, Optima, 2000 DV, Perkin Elmer, USA) (Yuan et al., 2004). The residual concentrations of doxycycline (DO), oxytetracycline (OTC), enrofloxacin (ENR), and florfenicol (FFC) were determined using ultra-high-performance liquid chromatography-tandem mass spectrometry (UHPLC-MS) (Wang et al., 2020b). The physicochemical properties of each sample were subjected to 3 parallel experiments.

1.5. Statistical analysis

One-way ANOVA, fixed effects model and Tukey's honestly significant difference (HSD) test in SPSS version 17.0 (SPSS, Chicago, IL, USA) were used to analyze the results and values of $P < 0.05$ were determined statistically significant. Principal Component Analysis (PCA) was used to cluster the different farm samples, which was performed in R 3.2.2 with vegan package 2.3-1 (Qiao et al., 2021). Network analysis was used for exploration of the underlying associations among ARGs, between physicochemical properties and ARGs by calculating all pairwise Pearson's correlation at P -value < 0.05 , which was performed in R environment using "Hmisc" package and visualized via Gephi 0.9.2 (Li et al., 2015). The heatmap was drawn in R 3.2.2.

2. Results

2.1. Prevalence of ARGs in plasmid DNA

Ten ARGs and *int11* were detected in plasmid DNA, of which *aac(6′)-Ib-cr*, *qnrS*, *floR*, *tetM*, *sul1*, *ermB*, *optrA* and *int11* genes were present in all assessed samples (100%), and *bla_{CTX-M}* were detected at relatively high rates of 97.8%. In particular, the detection rate of *bla_{NDM}* genes was 47.7%. Besides, the detection rate of *mcr-1* in swine manure plasmid DNA reached 79.6%.

2.2. The abundance of ARGs in plasmid DNA in swine farms

The average absolute abundance of ARGs ranged from 2.29 to $8.07 \log_{10}$ copies/g, of which *int11*, *ermB* and *tetM* showed relatively high abundance levels with 8.07, 7.55 and $7.16 \log_{10}$ copies/g, respectively (Fig. 1). *bla_{NDM}*, *mcr-1*, *bla_{CTX-M}* and *qnrS* genes had relatively low abundances with 2.29, 4.08, 4.67 and $4.85 \log_{10}$ copies/g, respectively (Fig. 1). Of note, the average abundance of *optrA* was relatively high, reaching $6.62 \log_{10}$ copies/g (Fig. 1). The gene copy numbers of different genes in all sample sites were summarized in Fig. 2. The concentration for all ARGs in 7 farms (i.e., HESZC5, SCSZC7, SCSZC17, SCSZC11, HBSZC2, HBSZC1 and SCSZC8) were higher than the average absolute abundance ($P < 0.05$), the *int11* gene abundance was above $8.55 \log_{10}$ copies/g. Among them, the highest concentrations of *aac(6′)-Ib-cr*, *bla_{CTX-M}*, *int11*, *mcr-1* and *qnrS* were 9.07, 8.45, 10.05, 7.24 and $7.95 \log_{10}$ copies/g, respectively, on farm SCSZC11. The highest concentrations of the *bla_{NDM}* genes were detected on farm HESZC5 as high as 6.54

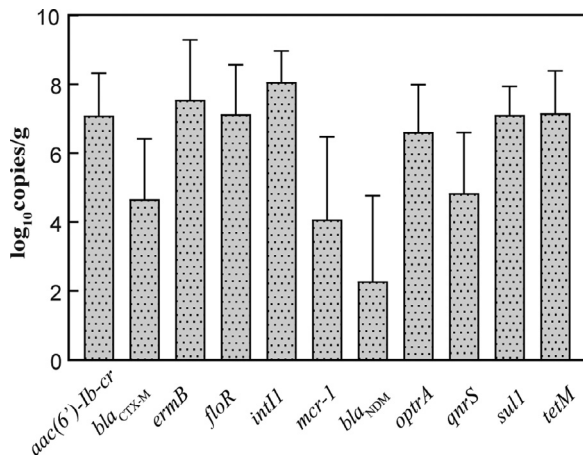


Fig. 1 – Average absolute abundance of antimicrobial resistance genes and *int1* gene in plasmid DNA from swine manure.

log₁₀copies/g. Moreover, the absolute abundance of the *oprA* gene in farms SCSZC4, SCSZC6, HBSZC2, HBSZC1, HESZC11 and HESZC12 was over 8.06 log₁₀copies/g.

Next, we systematically assessed the prevalence variations of ARGs across farms at distinct scales from different provinces in plasmid DNA. Our results showed that the absolute abundance of ARGs in fecal plasmid DNA among farms with distinct scales was not significantly different ($P > 0.05$) (Appendix A Fig. S1a). Although the overall absolute abundance in Hebei was slightly higher than that in Sichuan and Hubei, there was no significant difference in the absolute abundance of ARGs in fecal plasmidome DNA among the three provinces ($P > 0.05$) (Appendix A Fig. S1b). The PCA results showed that some farms (i.e., SCSZC19, SCSZC20, SCSZC21, SCSZC22, SCSZC23 and SCSZC24) came with a larger loading (above 2.0) on the PC1 (Appendix A Fig. S2a). Also, swine farms in Hubei Province have formed an obvious cluster on PC1 and PC2 (Appendix A Fig. S2b).

Pearson correlation analysis indicated that the abundance of ARG in plasmid DNA were positively correlated with each other (Fig. 3). In particular, *int1* had a moderate correlation ($r > 0.5$, $P < 0.01$) with *bla_{CTX-M}*, *ermB*, *floR*, *bla_{NDM}*, *qnrS* and *tetM*, and a strong correlation ($r > 0.8$, $P < 0.01$) with *aac(6)-Ib-cr* and *sul1* ($r = 0.86$ and 0.9 , respectively).

2.3. Associations of antimicrobial resistance genes with physicochemical factors

To explore influencing factors of ARGs in plasmidome, the physicochemical factors in 6 large-scale farms in Sichuan were measured. The correlation analyses were conducted to identify the relationship between ARGs and physicochemical factors (including antibiotics, heavy metals, and environmental nutrients) (Appendix A Table S4). The pH of all samples was relatively high indicating a weakly alkaline condition (7.20–7.60), and had a weak correlation with ARGs. Network analysis revealing the co-occurrence between ARGs and physicochemical factors in swine manure (Fig. 4) ($r > 0.5$, $P < 0.05$). For antibiotics, the OTC and DO had no correlations with ARGs.

However, the FFC was significantly positively correlated with *aac(6)-Ib-cr*, *qnrS*, *oprA*, *tetM*, *floR* and *ermB* ($r > 0.5$, $P < 0.05$). For heavy metals, Cu and Zn had a significant positive correlation with multiple ARGs ($r > 0.5$, $P < 0.05$), except for *sul1* and *mcr-1*. As only has a significant correlation with *aac(6)-Ib-cr* and *ermB* ($r > 0.5$, $P < 0.05$). In addition, the TOC was only correlated with *sul1*. However, the TN, TP and TK were positively correlated with the abundance of many ARGs in plasmid DNA. Particularly, *oprA* were significantly positively correlated with Cu, Zn, and Fe respectively ($r > 0.9$, $P < 0.05$). Moreover, *ermB*, *tetM*, *aac(6)-Ib-cr*, *floR* and *oprA* had significant co-occurring relationship with multiple physicochemical factors, among which *ermB* had co-occurrence with 9 physicochemical factors.

3. Discussion

A systematical investigation was performed using qPCR to specifically evaluate the prevalence and abundance of several clinically relevant ARGs in plasmidome from swine fecal samples in China. Our results showed that the *aac(6)-Ib-cr*, *qnrS*, *floR*, *tetM*, *sul1*, *ermB* and *int1* genes have been detected in all samples, which are likely a result of the continuous use of relevant antibiotics in swine farms. The results partly agreed with another survey about antibiotic resistance in swine farm environments (He et al., 2019). Previous studies indicate that tetracyclines, aminoglycosides, sulfonamides, macrolides and penicillins are the largest antimicrobial classes by weight used in swine production (Krishnasamy et al., 2015). The detection rate of *mcr-1* in swine manure plasmid DNA reached 79.6%. Moreover, Gurnessa et al. (2021) showed that the greater prevalence and abundance of AMR genes in cattle manure compared to poultry litter. Therefore, using livestock manure for pastureland fertilization may pose a greater risk for AMR dissemination to the environment than poultry litter.

The qPCR results showed that the macrolide resistance genes *ermB* and the tetracycline resistance genes *tetM* were the most abundant ARGs in plasmid DNA. Our results partly agreed with another survey about typical ARGs in swine farm sewage (Sui et al., 2017). Zhang et al. (2017) reported the abundance of tetracycline and macrolide resistance genes in the soil is the highest after applying swine manure on farmland. These results show that the ARGs contamination of tetracycline and macrolide antibiotics still exist in a large amount in swine farm environment. The highest concentrations of the *bla_{NDM}* genes were detected on farm HESZC5. This might be explained by the fact that beta-lactam antibiotics were constantly used on this farm. In addition, Wang et al. (2017) showed that *bla_{NDM}* can be disseminated via breeders, dogs, people, flies, and wild birds. Therefore, it may also be caused by the farm environment and related animal transmission.

It was paramount to note that the average abundance of *oprA* was relatively high, reaching 6.62 log₁₀copies/g. *oprA*, as a multi-drug resistance gene, can also cause bacteria to develop resistance to oxazolidinones (linezolid and tedizolid) and phenicols (chloramphenicol and florfenicol). There were two possible reasons for the high abundance of *oprA* in plasmid DNA of swine farms. Firstly, the co-selection of multi-drug resistant bacteria may be the reason for the high abundance

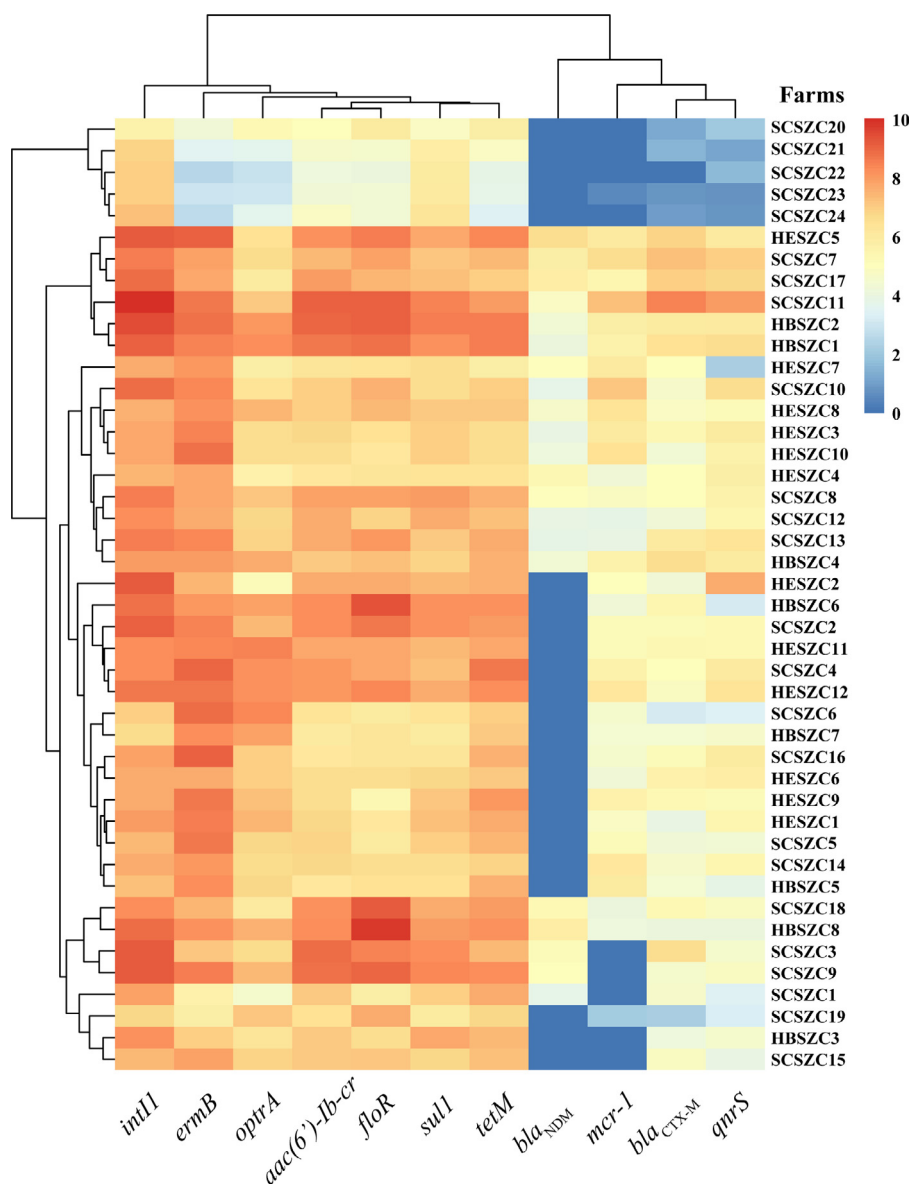


Fig. 2 – Heat map showing the abundance of ARGs detected in different swine manure. Each column is the results from a single primer set, and each row is labeled with the sample name. The first two letters, SC, HB and HE, stand for Sichuan, Hebei and Hubei Province respectively. SZC stands for swine farm sample and the last number stands for the sample number. Values plotted are the absolute abundance of the target ARGs for all samples. The legend denotes corresponding fold change values, which is a log scale.

of *oprA*, for it was usually found on transferable plasmids carrying other resistance genes of commonly used antibiotics such as tetracycline, lincosamide and aminoglycosides (Torres et al., 2018). Secondly, florfenicol has been widely used in the process of animal husbandry since approved in 1999 (Wang et al., 2015b). The higher abundance of *oprA* may also be due to the extensive use of florfenicol in swine farms. It suggested that florfenicol should be used properly and reasonably in farms. Altogether, the fact demonstrated that the cross-selection for anti-microbial resistance (AMR) to last-resort antimicrobials can occur on swine farms.

int11 gene was detected in all samples with the highest abundance with 8.07 log₁₀ copies/g, which is usually related to

mobile genetic elements, such as conjugative plasmids, transposons, and insert sequences, leading it to potentially transfer and exchange ARGs among diverse bacteria (Deng et al., 2015; Wang et al., 2015a). The relatively high abundance of *int11* gene in plasmid DNA may contribute to frequent HGT (Guo et al., 2020). Moreover, *int11* had significant positive correlations with all detected ARGs (Fig. 3), which was agreed with the opinion that *int11* is a molecular marker of antibiotic resistance in the environment (Amos et al., 2015). Especially, *int11* was highly correlated with *aac(6)-Ib-cr* and *sul1*, and the abundance of *aac(6)-Ib-cr* and *sul1* also existed at a high level. Suhartono et al. (2018) found that the number of ARGs in *E. coli* would also increase when *int11* was present. These phenom-

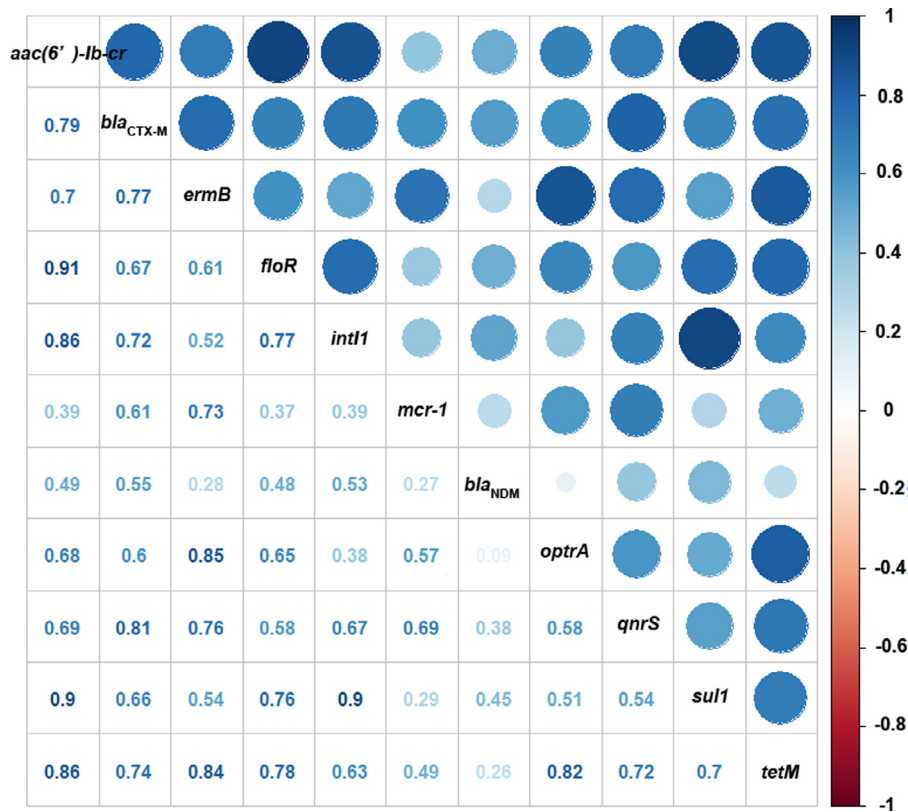


Fig. 3 – The correlation between the abundance of ARGs and *int11* in plasmid DNA. The size of the circle and the intensity of the color are proportional to the correlation. The numbers represent *r* value, a value of $|r|$ greater than 0.8 is considered a strong correlation and anything between 0.5 and 0.8 is a moderate correlation.

ena imply that the high abundance of ARGs in these farms may be due to the selection pressure of antibiotics and the promotion of resistance gene transmission by *int11*.

Simultaneously, there was no significant difference in the abundance of ARGs among the three scales and provinces of swine farms ($P > 0.05$), which partly agreed with another survey about antibiotic resistance in various scale farms of swine, chickens and ducks in China (Cheng et al., 2013). Wang et al. (2019) investigated the distribution of *mcr-1* gene in poultry manure samples and found that the positive rate of *mcr-1* gene was not significantly different between east-west provinces or coastal-inland provinces. Therefore, the resistance profiles have similar trends in various provinces and different scales, which may be due to similar medication use background and frequent livestock inter-provincial transportation (Wang et al., 2019).

Furthermore, to explore the possible influencing factors for the differences in the distribution of ARGs, the physicochemical factors in 6 large-scale farms in Sichuan were measured. There was no correlation between ARGs and swine manure pH, which was agreed with the previous reports (Wang et al., 2016). Cu, Zn, and Fe had a significant positive correlation with multiple ARGs. Similar correlations of metals and ARGs were also found in soils and in livestock farms (Wang et al., 2016; Zhou et al., 2016). The co-occurrence of metals with ARGs could be ascribed to their rifeness and their cross-resistance and co-resistance mechanisms with ARGs (Zhang et al., 2020).

Due to the co-selection of traits such as physiological cross-resistance and genetic co-resistance, heavy metal pollution can serve as a selective pressure for ARGs (Imran et al., 2019; Zhou et al., 2021). Moreover, the widespread use of Cu and Zn in commercial feed and additives in swine farms may intensify this selection pressure (Hou et al., 2014; Qian et al., 2018). It was worth noting that Cu and Zn have higher correlations with *oprA* and *bla_{CTX-M}* respectively. The possible reason for this phenomenon is that metal ions (Cu and Zn) and antibiotics (chloramphenicol and β -lactams) have the same resistance mechanism such as rapid efflux (Levy, 2002; Nies, 2003) and reduction of membrane permeability (Ruiz et al., 2003; Silver and Phung, 1996) to metals and antibiotics.

Environmental nutrition also affected the distribution of ARGs, it has been reported that high nutrients are more conducive to the horizontal transfer of ARGs (Guo et al., 2015). TN, TP and TK showed positive correlations with the dominant ARGs in plasmid DNA. This result may be related to the potential role of environment nutrients in promoting the propagation of host bacteria and further affecting the ARGs carried by them, since environment nutrients (TN, TP, and TK) play a vital role in the growth and metabolism of microorganisms (Guo et al., 2020). Previous studies reported that high concentrations of nitrogen increased bacterial resistance to a variety of antibiotics (Wang et al., 2020a; Yu et al., 2009).

It is common to find positive correlations between antibiotics and ARGs in different types of environments (Luo et al.,

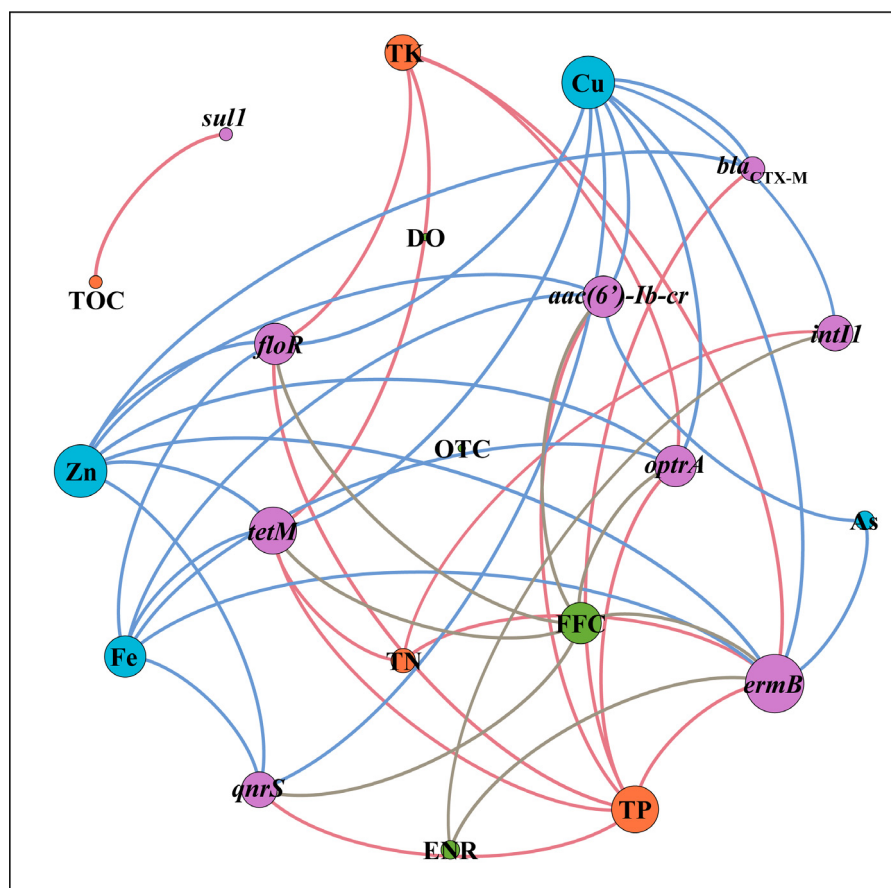


Fig. 4 – Network analysis revealing the co-occurrence patterns between ARGs and physicochemical factors in swine manure. The nodes were colored according to physicochemical factors types. A connection represents strong (Pearson correlation coefficient $r \geq 0.5$) and significant ($P < 0.05$) relationships between ARGs and physicochemical factors. The size of each node is proportional to the number of connections. FFC: florfenicol; DO: doxycycline; OTC: oxytetracycline; ENR: enrofloxacin; Cu: copper; Zn: zinc; Fe: iron; As: arsenic; TP: total phosphorus; TK: total potassium; TOC: total organic carbon.

2017; Zhu et al., 2013). FFC was positively correlated not only with *optrA* and *floR*, but also with other ARG such as *aac(6')-Ib-cr*, *ermB*, *tetM*, etc, indicating that they not only have an impact on its resistance genes but also screen other types of antibiotic resistance genes. The co-transmission of ARGs may be the reason why antibiotics affect different types of ARGs (Zhao et al., 2017).

In summary, this is the first large-scale investigation of ARGs carried by plasmids from farms of different scales in Sichuan, Hubei and Hebei. ARGs in manure plasmidome may be used as monitoring factors for the farm's antibiotic resistance gene contamination. Importantly, we found that *optrA* and *bla*_{CTX-M} are positively correlated with Cu and Zn respectively. Our results demonstrated that physicochemical factors (Cu, Zn, Fe, TN, TK and FFC) had a significant positive correlation with the abundance of multiple ARGs, which was consistent with the results of previous studies (Deng et al., 2020; Wang et al., 2020a; Zhang et al., 2020). Heavy metals, environmental nutrients, and antibiotic residues in the fecal environment of swine farms can affect the distribution of ARGs, and the irregular treatment of animal feces or reuse of manure

might be an important way to increase the expansion of antibiotic resistance.

4. Conclusions

The prevalence of several ARGs in plasmid DNA in swine manure from farms at different scale across various geographical locations were investigated in this study. Ten ARGs and the class 1 integron gene *int11* were found in plasmid DNA of fecal samples, offering additional evidence that plasmids are important reservoirs of ARGs in environment. Of particular note, high gene copies of *optrA* and high detection rate of *mcr-1* were found in plasmid DNA of swine manure. Furthermore, the concentrations of FFC, Cu, Zn, Fe, TP and TK had significant correlations with assessed ARGs, suggesting that antibiotics, heavy metals and environmental nutrients are likely to jointly contribute to the long-term persistence of ARGs in swine production. Consequently, further studies are needed to explore the role of physicochemical factors in the transmission of ARGs in animal feces and farm environments.

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Appendix A Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jes.2021.11.030.

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