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Prevalence and dissemination of antibiotic resistance genes and coselection of heavy metals in Chinese dairy farms

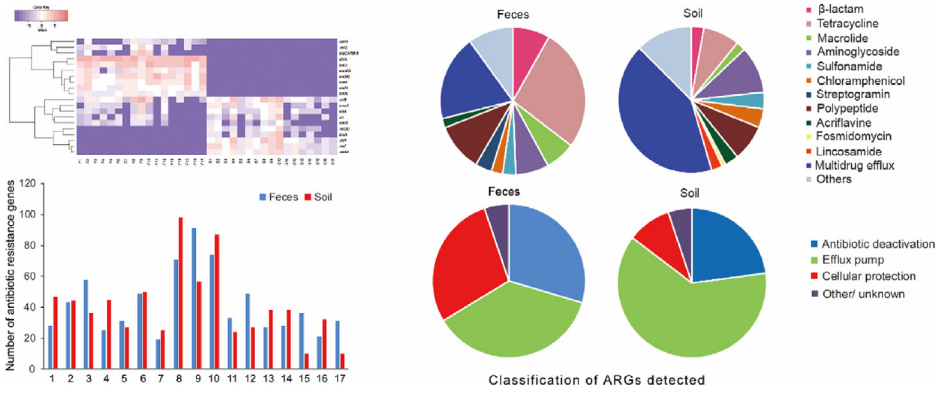
Bingrui Zhou [a], Chong Wang [a], Qin Zhao [b], Yang Wang [b], Meijun Huo [a], Jundong Wang [a,*], Shaolin Wang [b,*]

[a] Shanxi Key Laboratory of Ecological Animal Science and Environmental Medicine, College of Animal Science and Veterinary Medicine, Shanxi Agricultural University, Taigu, Shanxi 030801, People's Republic of China
[b] Beijing Advanced Innovation Center for Food Nutrition and Human Health, College of Veterinary Medicine, China Agriculture University, Beijing 100193, People's Republic of China

HIGHLIGHTS

- Abundant ARGs in dairy farms were detected using metagenomic sequencing.
- Significant correlation of ARG richness was observed between the feces and soil.
- Heavy metals participated in coselection processes for the ARGs in dairy farms.
- High level of transposases coexisting with ARGs increased ARG dissemination risk.
- Dairy feces is a huge pollution source of ARGs and heavy metals.

GRAPHICAL ABSTRACT



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ABSTRACT

This study aims to explore prevalence and dissemination of antibiotic resistance genes (ARGs) in dairy farms. A variety of ARGs conferring resistance to most classes of antibiotics were detected in feces and soil samples obtained from dairy farms, using a high-throughput metagenomic sequencing approach. The ARGs observed in the feces and the soil samples were significantly correlated ($p < 0.01$). The abundance of mobile genetics elements, such as transposase, was also examined to evaluate the potential risk of horizontal ARGs transfer. The positive correlation ($p < 0.001$) between the total abundance of transposase genes and ARGs in the soil samples suggested strong dissemination capacity of ARGs in soil. In addition, the ARGs and metal resistance genes (MRGs) were significantly correlated with heavy metals in the feces ($p < 0.01$), suggesting that the heavy metals promoted the emergence of metal resistance, and participated in the coselection processes for ARGs. The prevalence of ARGs with high levels of genetic mobile elements in the dairy farms suggests that cattle excrement is a major reservoir of ARGs with a high risk of dissemination, which increases the potential risk of environmental pollution and threatens public health.

Abbreviations: ARG, antibiotic resistance gene; LGT, lateral gene transfer; MRSA, methicillin-resistant *Staphylococcus aureus*; ORF, open reading frame; RND, resistance-nodulation-cell division; MRG, metal resistance gene.

* Corresponding authors.
E-mail addresses: wangjd53@outlook.com (J. Wang), shaolinwang@cau.edu.cn (S. Wang).

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1. Introduction

For over half a century, antibiotics have been widely used in the animal production industry. They are used not only for disease prevention and treatment, but also for promotion of growth. It is estimated that half of the total antibiotics produced are used for veterinary purposes [1]. Growing evidences suggest that the increase in number of antibiotic resistance genes (ARGs) in human pathogens is correlated with antibiotic use in farm animals [2,3]. Overuse of antibiotics in livestock industry could promote the antibiotic resistance worldwide, therefore, it has become a global concern. Moreover, the expansion and evolution of ARGs in multidrug-resistant pathogens pose a severe threat to the health of humans and animals [4]. Recently, Liu et al. first reported a plasmid mediated polymyxins resistance gene, mcr-1, which heralds the breach of the last group of antibiotics [5].

A recent study reported that approximately 54000 tons of antibiotics was excreted by animals and humans in China, 99.6% of which entered the environment [6]. Environmental antibiotic residues exert high selective pressure on bacteria, significantly increasing the frequency of ARGs acquired by bacteria and accelerating the spreading of ARGs via lateral gene transfer(LGT) [7]. Bacteria continue to evolve under the selective pressure of antibiotics, and the recent discovery of metallo-beta-lactamases suggests that the drug resistance is entering an era of pan-drug resistance [8]. Fournier et al. reported that multidrug resistant *Acinetobacter baumannii* strain AYE carries 45 resistance genes. The genome of this bacterium contains an ATPase open reading frame (ORF) that causes genomic instability, which contributes to ARG acquisitions from other bacteria and the environment [9]. Another study revealed that a remote human community in the Peruvian Amazonas, barely exposed to the antibiotics, was detected with high levels of acquired ARGs to various antibiotics in commensal *Escherichia coli* [10].

China produces and consumes the most antibiotics in the world. It has been estimated that 200,000 tons of antibiotics are annually produced in China, over half of which is used in the livestock industry (about 10 times greater than the consumption in the United States) [11]. The use of antibiotics for treating disease and promoting growth of animals in China is not well regulated and frequently leads to their overuse. Most antibiotics used in livestock are expelled in animal excrement, which is usually applied to farm lands as fertilizer. Consequently, a high level of antibiotic residues is often detected not only in the animal manure, but also in the environment. Swine manure is considered as a major source of antibiotics and ARGs, and promotes the accumulation of antibiotic residue in the environment [12,13]. A recent study showed that three veterinary antibiotics, tylosin, chlortetracycline, and enrofloxacin, were found in the urine samples of Chinese school children, likely because they were exposed to polluted food and environment containing these antibiotics [14].

Dairy farms produce vast amounts of excrement which is a potential ARG pollution source. Most published studies focus on single bacterial strains with specific antibiotic resistance in feces of dairy cattle [15,16]. A previous study detected the abundance of five ARGs in Chinese dairy farms [17]. However, comprehensive antibiotics resistance profiles and dissemination risks in Chinese dairy farms are obscure.

Although the ARGs of specific microbes isolated from animal feces and farm soil have been investigated, these were detected using conventional culture methods [16,18]. Unculturable resistant populations or unexpressed ARGs cannot be examined by these methods. Nevertheless, these undetected ARGs may potentially serve as a large ARG reservoir, which can be transferred to human and animal pathogens through LGT under selection pressure in the future. ARGs in beef cattle fecal microbiota have been studied using metagenomic approach [19]. In this study, we performed high-throughput metagenomic sequencing for in-depth analysis of antibiotic resistance profiles in dairy farms in China.

2. Material and methods

2.1. Feces and soil sampling

Samples were obtained from a total of 17 dairy farms in the Shanxi province of China in 2013. Fresh fecal samples were collected immediately after a cow defecated in the cattle pen. Soil samples were obtained from the top 0–10 cm of the ground in the dairy farms. At least 5 fecal and 5 soil samples were collected randomly from the same farm and were then mixed into a single fecal or soil sample to represent the farm. All samples were kept on ice during transportation and stored at −80 °C before DNA isolation.

2.2. DNA isolation, library construction, and metagenome sequencing

The metagenomic DNA from fecal and soil samples was extracted using the Powersoil DNA isolation kit (MOBIO, Carlsbad, CA, USA). The quantity and quality of extracted DNA were evaluated using NanoDrop spectrophotometer 2000 (Thermo Fisher Scientific, Woburn, MA, USA) and agarose gel electrophoresis. The extracted DNA (1 μg at a time) was fragmented to 200 bp using a Covaris E210 sonicator (Covaris Inc., Woburn, MA, USA). Fragmented DNA was end-repaired, A-tailed, ligated to standard Illumina multiplexing adapters, and amplified with 0.5 μM Illumina multiplexing PCR primers 1.0 and 2.0 using TruSeq DNA Sample Prep kits (Illumina, San Diego, CA, USA). Subsequently, the PCR products were purified using the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) and subjected to a final size-selection step on a 2% agarose gel. Ethidium bromide-stained gel slices containing fragments within the range of 300–400 bp were excised and purified using the QIAquick kit. DNA was quantified using the Quant-iT PicoGreen dsDNA kit (Life Technologies, Carlsbad, CA, USA) before sequencing on Illumina HiSeq 2000 with TruSeq SBS Kit v3-HS (200-cycles).

2.3. Quantitative PCR

The relative abundance of transposase genes in the fecal and soil samples were detected by quantitative PCR using the nine primers (Table S1). All PCR experiments were conducted in 96-well plates on a Quantitative 7 PCR instrument (Life, Technologies, Singapore), using the following protocol: 95 °C for 2 min, followed by 40 cycles of 95 °C for 30 s, 57 °C for 30 s, and 72 °C for 34 s. The reaction volume was 20 μL, and consisted of 10 μL SYBR Premix Ex Taq™ (Takara, Dalian, China), 0.4 μM each primer, 6 μL sterile water, 0.4 μL ROX reference dye, and 2 μL template DNA. Negative controls without DNA templates were also run. The copy number of each target gene was calculated according to Liang et al. [20]. Calibration curves (Ct values versus log10 values of target gene copy number) with six points were generated using a tenfold serial dilution of the plasmid carrying the target transposase genes. PCR amplification efficiencies ranged from 96 to 113% and all R^2 values were greater than 0.993 (Table S2). The target gene copy numbers were calculated using calibration curves and normalized to 16S rRNA gene copies.

2.4. Heavy metal analysis

Air dried and sieved samples (0.100 g) were digested using HNO_3 (2 mL), HCl (3 mL), and $HClO_4$ (1 mL) in 50 mL digestion tube, incubated overnight. The samples were digested in 120 °C