

# Research Note: Molecular characterization of antimicrobial resistance and virulence gene analysis of *Enterococcus faecalis* in poultry in Tai'an, China

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**ABSTRACT** *Enterococcus faecalis* (*E. faecalis*) is a zoonotic pathogen that causes severe economic losses in the poultry-breeding industry. In our study, cecal samples from broilers with cecal enlargement at slaughterhouses in Tai'an, China, were analyzed. The results revealed that the 61 *E. faecalis* strains had drug resistance rates ranging from 96.72 to 8.20% against 11 antibiotics in 5 classes, of which erythromycin (96.72%) and tetracycline (96.72%) had the highest rates and vancomycin (8.20%) the lowest. The highest detection rate of multiple drug-resistant strains in 61 isolates was 72.13%. The results of polymerase chain reaction showed that, of the 12 virulence genes, *ccf* had the highest detection rate (80.33%), followed by *asal* and *cob* (both

78.69%), whereas *hyl* had the lowest (6.56%). Among 15 drug resistance genes, *ermB* had the highest detection rate (95.08%), followed by *tetM* (91.80%) and *tetL* (90.16%), whereas *tetK* (0.00%) and *vanB* (0.00%) remained undetected. Of the 34 sequence types found with multilocus sequence typing, the most predominant were ST631 (13.11%, 8/61) and ST634 (8.2%, 5/61). Our results provide a theoretical basis for guiding the rational use of antibiotics and preventing the spread of drug-resistant bacteria, along with epidemiological data for the risk analysis of food-borne bacteria and antimicrobial resistance in poultry farms in Shandong Province.

**Key words:** *Enterococcus faecalis*, multiple drug-resistant, drug resistance gene, multilocus sequence typing

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## INTRODUCTION

*Enterococcus faecalis* is a common intestinal bacterium found in humans and animals, including mammals and birds, that is associated with various clinical zoonotic bacterial diseases. In poultry, *E. faecalis* is commonly associated with septicemia, cecal enlargement, endocarditis, balloon inflammation, salpingitis, bacteremia, pulmonary hypertension, amyloid arthropathy, encephalomalacia, and neurological disorders (Gregersen et al., 2010). The colonization of *E. faecalis* in newly hatched chicks, including from environmental sources, has shown both vertical and horizontal transmission (Olsen et al., 2012).

Transmitted between bacteria, animals, and humans, bacterial resistance is a public health problem that has attracted significant attention from scholars all over the world. With the continuous expansion of livestock and poultry breeding in China, massive amounts of antibiotics have been used in husbandry in the country. Worldwide, antibiotic use in livestock is predicted to increase by 67% by 2030 according to a report by the UN Environment Programme (<https://www.unep.org/>). However, the use of antimicrobial agents can affect the proportion of related drug-resistant bacteria in the environment, while the prophylactic use of antimicrobial agents in livestock and poultry breeding can increase the number of resistant bacteria. For those reasons, the World Health Organization has identified antibiotic tolerance as a global public health crisis that urgently needs to be solved.

Because *E. faecalis* spreads horizontally between humans and animals, the threat of the drug resistance of *Enterococcus* species to public health has attracted considerable attention from the scientific community. To help to counter that threat, we investigated the resistance of clinical *E. faecalis* isolates in the Tai'an area of

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China's Shandong Province in the past 2 yr, its carrying status of resistance genes, and the characteristics of its genetic evolution. Our aims were to understand the current situation and mechanism of *E. faecalis*'s resistance in the area, to furnish guidance for the prevention and treatment of *E. faecalis*-based diseases in Tai'an, Shandong Province, and to provide a reference for theoretical research on bacterial resistance.

## MATERIALS AND METHODS

### Sample Collection

The cecal tissue of broiler chickens with swollen cecal lesions was collected from a slaughterhouse in Tai'an, Shandong Province, from August 2019 to February 2020. The broilers slaughtered in the slaughterhouse generally come from various large-scale chicken farms in the Tai'an area. All 90 tissue samples with suspected lesions were stored at 4°C before being processed within 12 h of collection.

### Isolation and Identification of *E. faecalis*

The contents of the cecal tissue were extracted aseptically by burning the inoculation ring with a flame, and 3 zones were made on *Enterococcus* or LB medium. After overnight culture at 37°C, typical single colonies were selected; in general, *E. faecalis* forms large black round colonies with needle-shaped tips on *Enterococcus* medium and shows *E. faecalis* forms the size of rice grains on LB medium. Next, for purification, *E. faecalis* was inoculated again on *Enterococcus* or LB medium, putative *E. faecalis* isolates were selected based on *E. faecalis*-specific polymerase chain reaction (Kariyama et al., 2000). The colonies suspected of *E. faecalis* were further purified 3 to 5 times according to their characteristics as colonies. A microbial mass spectrometer (IVD MALDI Biotyper, Bruker, Bremen, Germany) was used to quickly identify *E. faecalis* and confirmed. In that process, a toothpick was first used to select a single colony to coat the metal identification plate, after which 1 µL of 70% formic acid was added. Once the plate was dry, 1 µL of matrix solution was added, and the metal plate was placed in the instrument for subsequent comparison with the standard fingerprint in the database.

### Drug Sensitivity Test

The *E. faecalis* isolates were screened for their susceptibility to various antibiotics using the MicroScan Walk-Away-96 automatic drug sensitivity analyzer (Siemens, Germany). The standard drug sensitivity plate includes 11 antibiotics in 5 classes: tetracycline (TE), erythromycin (E), streptomycin (S), gentamicin (CN), ciprofloxacin (CIP), levofloxacin (LEV), penicillin (P), linezolid (LZD), daptomycin (DAP), ampicillin (AMP), and vancomycin (VA).

## Detection of Virulence and Drug-Resistance Genes in *E. faecalis*

Drug resistance detection revealed that *Enterococcus* species in the Tai'an area were primarily resistant to TE and E. To further clarify the results of testing and better explain the mechanism of drug resistance, we examined 12 virulence genes (i.e., *ace*, *asal*, *agg*, *cob*, *ccf*, *cpd*, *cylA*, *cylB*, *cylM*, *esp*, *hyl*, and *mefA*) and 15 drug resistance related genes (i.e., *aac(6')*/*aph(2')*, *ant(6)-I*, *aph(3')*-III, *cat*, *ermB*, *gelE*, *gyrA*, *parC*, *tetK*, *tetL*, *tetM*, *tetS*, *vanA*, *vanB*, and *Int11*) by PCR.

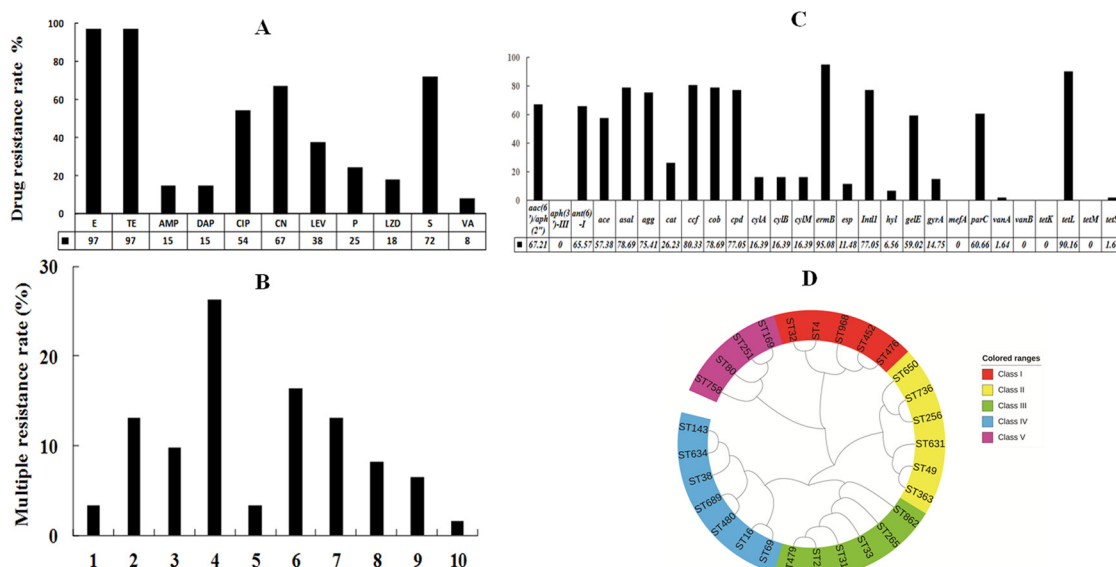
### Multilocus Sequence Typing

The primers were acquired from a housekeeping gene design website (<http://efaecalis.MLST.net/misc/info.asp>). The 6 or 7 alleles of the housekeeping genes of *E. faecalis* were amplified and sequenced and subsequently spliced and intercepted with Mega-X software. The corrected sequences were submitted to *E. faecalis*-relevant MLST software for comparison with specific standard sequences to obtain the sequence number of alleles of each housekeeping gene. After *E. faecalis* was aligned with Mega-X software, a genetic evolutionary tree was generated and subsequently decorated and beautified using EvoView while a minimum spanned tree was generated in PHYLOViZ.

## RESULTS AND DISCUSSION

In China, a country with massive livestock and poultry-breeding industries, Shandong has emerged as a major, rapidly developing agricultural province where poultry production reached 3.337 million tons in 2019 (Liu et al., 2021). There, large-scale broiler breeding has involved high breeding density and, as a consequence, a variety of viral and bacterial diseases. Because the prevention and control of bacterial diseases has long relied on antibiotics, the extensive, long-term use of various antibiotics, especially for overdose, has increased drug-resistant bacteria and plagued China with some of the world's most intense bacterial resistance in livestock and poultry (Li et al., 2022; Song et al., 2020; Wang et al., 2021). In turn, the ineffectiveness of antibiotics has increased the difficulty of preventing and controlling bacterial disease, raised the rate of frail chicks and mortality, and decreased both the feed conversion rate and production incomes.

In our study, the cecal tissue of broiler chickens with swollen cecal lesions was collected from a slaughterhouse in Tai'an, China. Sixty-one strains of *E. faecalis* were initially identified in the laboratory, and the overall isolation rate of *E. faecalis* was 67.78% (61/90). The total drug resistance rates of isolated *E. faecalis*, also shown in Figure 1A, were as follows: E (96.72%), TE (96.72%), S (72.13%), CN (67.21%), CIP (54.10%), LEV (37.70%), P (24.59%), LZD (18.03%), DAP (14.75%), AMP (14.75%), and VA (8.20%).



**Figure 1.** (A) The drug resistance rate of 61 *E. faecalis* strains. (B) The multidrug drug resistance rate of 61 *E. faecalis* strains. (C). Detection rate of virulence gene and drug resistance gene of 61 *E. faecalis* strains. (D) Phylogenetic tree based on MLST data.

In past work on the subject, Ci et al. (2020) determined that the resistance rate of *E. faecalis* to E on fur-bearing animals was 80% and to TE was 100%. Meanwhile, Yang et al. (2019) studied the drug resistance of *E. faecalis* isolated from cows in multiple areas of China and observed that the resistance rates to E and TE were the highest. Added to that, in their study on *E. faecalis* among pigs in Hunan Province, Jiang et al. (2020) revealed that the drug resistance rate of *E. faecalis* to E and TE has exceeded 90% since 2015. The results of our study, demonstrating general drug resistance to E and TE, were similar to those findings. Taken together, those results urge close attention to the TE resistance of livestock and poultry pathogens as well as the minimal use of TE, macrolides, and aminoglycosides as antibiotics in treating enterococcal infections in poultry in the Tai'an area. The 61 strains of *E. faecalis* were resistant to at least one but up to 10 antibiotics. Totaling 16, the strains resistant to 4 antibiotics were the most common (26.13%), as detailed in Figure 1B. Beyond that, the average multiple drug resistance rate of 72.13% in 44 of 61 isolates indicates severe multiple drug resistance in the region.

Statistical analysis revealed the detection rates of 15 drug related resistance genes in 61 *E. faecalis* strains: *aac(6')/aph(2'')* (67.21%), *ant(6)-I* (0.00%), *aph(3')-III* (65.57%), *cat* (26.23%), *ermB* (95.08%), *hyl* (6.56%), *gelE* (59.02%), *gyrA* (14.75%), *parC* (60.66%), *tetK* (0.00%), *tetL* (90.16%), *tetM* (91.80%), *tetS* (1.64%), *vanA* (1.64%), *vanB* (0.00%), and *Int1* (77.05%). The detection rates of 12 virulence genes were also obtained: *ace* (57.38%), *asal* (78.69%), *agg* (75.41%), *cob* (78.69%), *ccf* (80.33%), *cpd* (77.05%), *cylA* (16.39%), *cylB* (16.39%), *cylM* (16.39%), *esp* (11.48%), and *mefA* (8.20%). Among them, the most prominent drug-resistant genes were *ermB* (95.08%) and *tetM* (91.80%), while the most prominent virulence genes were *ccf* (80.33%), *asal* (78.69%), and *cob* (78.69%), as shown in

Figure 1C. The screening results of the experiment are consistent with results from other countries (Nowakiewicz et al., 2017; Stępień-Pyśniak et al., 2021). The highest percentage of *E. faecalis* strains was resistant to TE (60.5%) and E (54.4%) in Poland, where phenotypic resistance to erythromycin was encoded in 98.4% strains by the *ermB* gene (Nowakiewicz et al., 2017). Among the aminoglycosides genes, *aac(6')/aph(2'')* and *aph(3')-III* were also the dominant carried genes, and the virulence gene test results were very similar to other published data (Stępień-Pyśniak et al., 2021). The only difference that we detected was a small number of strains carrying the *hyl* (4/61) gene that were not detected in Poland or in the Netherlands. The presence of resistant integrons in pathogens may prompt an increase in the number and cost of clinical failures due to bacterial diseases. In our study, the integrase gene, *Int1*, was found to have a multiple drug-resistant (MDR) phenotype in more than three-fourths of the strains investigated (77.05%), which indicates that the gene level is more significant to the study of drug resistance and that more studies on drug resistance should be conducted at the gene level in the future.

MLST technology has increasingly been applied in bacterial typing to study the relationship between bacterial virulence and genotype. The 61 *E. faecalis* strains in our study had 34 ST types, and the proportion of each ST type was ST631 (11.48%, in 7 strains); ST634 (8.20%, in 5 strains); ST4, ST480, and ST758 (6.56%, in 4 strains each); ST32 and ST195 (4.92%, in 3 strains each); ST10, ST257, ST314, ST363, and ST968 (3.28%, in 2 strains each); and ST16, ST33, ST38, ST49, ST69, ST80, ST143, ST169, ST198, ST251, ST256, ST262, ST265, ST452, ST476, ST479, ST650, ST689, ST736, ST862, and ST991 (1.64%, in 1 strain each). The dominant ST types were ST631 and ST634. It has been reported that ST631 derived from *E. faecalis* in China, whereas related strains were primarily isolated from the

blood of the gastrointestinal system, surgical urine, and ascites in intensive care units in hospitals (Jiang et al., 2020). Relevant studies in China have shown that the pathogenicity of human-origin ST631 primarily manifests in diseases of the abdominal cavity, gastrointestinal tract, and other related sites.

According to genetic tree analysis, the 61 strains of *E. faecalis* formed 5 gene clusters (Figure 1D). The two dominant strains, ST631 and ST634, were distributed in gene clusters II and IV, respectively, in the genetic tree. Those results indicate that the ST types of *E. faecalis* isolated from the samples were closely related. According to the results of virulence and drug resistance gene detection, all strains of ST631 had *asal*, *ccf*, *cpd*, and *gelE*, which were related to *E. faecalis* adhesion, colonization, and gelatinase production. All strains of ST634, meanwhile, had *ace*, *asal*, *ccf*, *cpd*, *Int11*, *ermB*, and *tetM*, which indicates that along with adhesion- and colonization-related genes, it also had *Int11*-mediated multiple drug resistance genes. *E. faecalis* has been identified as an opportunistic pathogen that may cause clinical infections in humans and animals, which also underscores the potential threat of *E. faecalis* in zoonosis.

In sum, we systematically investigated *E. faecalis* carriers and their drug resistance, the characteristics of their drug resistance genes, and the characteristics of their genetic evolution in infected chickens in the Tai'an area in the past 2 yr. The 61 *E. faecalis* strains isolated and identified highlight that *E. faecalis* has generally been resistant in broilers in the area. The highest resistance was with TE (96.72%) and E (96.72%), and the MDR rate peaked at 72.13%. The most prominent virulence genes carried by *E. faecalis* in the Tai'an area were *ccf* (80.33%), *asal* (78.69%), and *cob* (78.69%), whereas the most prominent drug resistance genes were *tetM* (91.80%) and *ermB* (95.08%). Of the many types of *E. faecalis* in the Tai'an area, the dominant ST types were ST631 and ST634.

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Ethics statement: The study protocol and the poultry studies were approved by the Animal Care and Use

Committee of Shandong Agricultural University, Tai'an, China.

## DISCLOSURES

No conflict of interest exists in the submission of the manuscript, and the manuscript has been approved for publication by all authors.

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