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Brief Original Article

Prevalence and antimicrobial resistance profiles of *Escherichia coli* isolated from free-range pigs

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Abstract

Introduction: Numerous studies about antimicrobial resistant *Escherichia coli* (*E. coli*) of animal origins have been conducted around the world, most of them focus on bacteria from animals raised in intensive breeding farms, but systematic studies on antimicrobial resistance in *E. coli* of free range animals are still lacking.

Methodology: This study aimed to investigate the prevalence and antimicrobial resistance profiles of *E. coli* from free-range pigs in Laiwu mountainous areas, eastern China.

Results: Among 123 fecal samples, 123 non-duplicate *E. coli* were obtained with an isolation rate of 100.0% (123/123). These *E. coli* showed the highest resistance rate to tetracycline (77/123, 62.6%), but all were sensitive to amoxicillin/clavulanic acid. Thirty-eight *E. coli* (38/123, 30.9%) showed multidrug resistance (MDR). Among 123 *E. coli* isolates, only 39 carried antimicrobial resistant genes detected in this study. Of these 39 isolates, $bla_{\text{TEM-1}}$, $bla_{\text{CTX-M-14}}$, $bla_{\text{CTX-M-15}}$, *qnrB*, *qnrD*, *qnrS1*, *floR* and *cfr* genes were detected in 13, 9, 4, 7, 10, 7, 20, and 7 isolates, respectively. $bla_{\text{TEM-1}}$ and $bla_{\text{CTX-M-14}}$ genes were concomitantly detected in 6 isolates, and bla_{TEM}, *qnrB, qnrS* and *qnrD* genes were concomitantly detected in 7 isolates.

Conclusions: Free-ranging pigs may be regarded as a potential reservoir for antibiotic resistant genes.

Key words: Free-ranging pig; *E. coli*; antimicrobial sensitivity; resistance genes

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Introduction

Escherichia coli (*E. coli*) is a Gram-negative opportunistic pathogen, which can be found in the digestive tract of humans and animals [1]. It can be isolated from various natural environments such as: air, soil, water, and human and animal feces, and it is an important indicator bacterium for epidemiological survey of drug-resistant bacteria [2-5]. At present, studies on drug-resistant bacteria of animal-origins have attracted great concern [6]. Numerous studies on drug-resistant bacteria of animal-origins have been conducted around the world, most of them focusing on bacteria from animals raised in intensive breeding farms [7-10], but systematic studies on drug resistance in bacteria of free range animals are still lacking.

A large amount of woodland in mountainous areas of Laiwu, China, provides free-ranging conditions for Laiwu black pigs, and local farmers like to raise Laiwu black pigs by grazing. To note, antibiotics are not used except when pigs contract disease, and owners occasionally add pelleted feed without containing any antibiotics. This unique way of rearing is very different from modern intensive breeding farms. Our study was therefore focused on investigating prevalence and drug-resistant characteristics of *E. coli* from Laiwu black pigs, providing insights into the development of free range livestock husbandry in mountainous areas, as well as human health and ecological security.

Methodology

Sample collection

From May to October 2015, 123 samples of fresh manure were randomly collected from different pigs from 10 herds of Laiwu black pigs raised in the woodlands in the Laiwu region (12-16 months of age), eastern China (36.02N, 117.19E). Pig fecal samples obtained by swabbing fresh manure using disposable sterile cotton swabs were placed in sterile centrifuge tubes, transported to our lab with an icebox, and processed for bacterial isolation within 6 h. One herd of pigs was sampled only once.

Isolation and identification of E. coli

Fecal swabs were plated onto MacConkey plates (Haibo, Qingdao, China) and cultured at 37 ℃ for 24 h. A pure colony per sample with typical *E. coli* morphology was picked up for further identification by Vitek MS system (BioMérieux, Marcy l'Étoile, France) and by polymerase chain reaction (PCR) for the *uidA* gene [11].

Antibiotics susceptibility testing

Antimicrobial susceptibility of *E. coli* isolates was tested using the disk diffusion and broth microdilution methods based on the standards and interpretive criteria described by the Clinical and Laboratory Standards Institute (CLSI, 2013) [12]. The disk diffusion method was conducted on Mueller-Hinton agar (Haibo, Qingdao, China), using the following antibiotics (Oxoid, Beijing, China): ampicillin (10μg), amoxicillin/clavulanic acid (25 μg), gentamicin (5 μg), ciprofloxacin(10 μg), co-trimoxazole (1.25/23.75 μg) and tetracycline (30 μg). Commercial disks for florfenicol and ceftiofur are not available, minimum inhibitory concentrations of them were determined using the broth microdilution method. The breakpoint for antimicrobial drugs was based on the guidelines provided by the CLSI. In addition, ≥32 mg/mL was the tentative breakpoint for florfenicol, and ≥2 mg/mL was used for ceftiofur, according to EUCAST (http://www.eucast.org/mic_distributions/).

Eight antibiotics used in this study are clinical drugs commonly used by local veterinarians. Isolates that showed resistance to three or more antibiotics were considered to be multidrug resistance (MDR). *E. coli* ATCC 25922 was used as the quality control strain.

Resistance genes

The presence of β-lactamase genes (*bla*$_{TEM}$, *bla*$_{CTX-M}$ and *bla*$_{SHV}$) was detected by PCR amplification as described previously [13]. Plasmid-mediated quinolone resistance (PMQR) genes (*qnrA, qnrB, qnrC, qnrD, qnrS, aac(6')-Ib, qepA, oqxA* and *oqxB*) were detected as described previously [14]. The presence of florfenicol-resistant genes (*florR* and *cfr*) was determined by PCR amplification as described previously [15,16]. After the PCR products were sequenced, BLAST sequence alignment was performed at www.NCBl.nlm.nih.gov.

Results

E. coli isolates from pigs

One hundred and twenty-three fresh fecal samples of Laiwu black pigs were collected in this study, and 123 *E. coli* isolates were obtained with an isolation rate of 100.0%.

Table 1. Multidrug resistance patterns of *E. coli* isolates in this study.

No. of antibiotics	Resistance spectrum	No. of *E. coli*
7	T, A, C, P, G, F, R	2
5	T, A, C, F, P	3
5	T, A, C, F, R	6
4	T, A, C, F	7
4	T, A, P, G	9
3	T, P, R	11

Notes: T: tetracycline, A: ampicillin, C: ceftiofur, P: ciprofloxacin, G: gentamicin, F: florfenicol, R: co-trimoxazole.

Antimicrobial susceptibility

E. coli isolates showed a high resistance rate to tetracycline (77/123, 62.6%), intermediate resistance rates to florfenicol (39/123, 31.7 %), ampicillin (37/123, 30.1%), co-trimoxazole (33/123, 26.8%) and ceftiofur (31/123, 25.2 %), low resistance rates to ciprofloxacin (23/123, 18.7%) and gentamicin (21/123, 17.1 %), and no resistance to amoxicillin/clavulanic acid. Additionally, 38 E. coli (38/123, 30.9%) showed MDR (Table 1).

Characterization of resistance genes

All the 123 *E. coli* isolates were all negative for *bla*$_{SHV}$-, *qnrA-, qnrC-, qepA-*, *aac(6')-Ib-, oqxA-* and *oqxB*-resistant genes, and only 39 carried antimicrobial resistant genes detected in this study. Among these 39 isolates, *bla*$_{TEM-1}$, *bla*$_{CTX-M-14}$, *bla*$_{CTX-M-15}$, *qnrB*, *qnrD*, *qnrS1*, *floR* and *cfr* genes were detected in 13, 9, 4, 7, 10, 7, 20, and 7 isolates, respectively. *bla*$_{TEM-1}$ and *bla*$_{CTX-M-14}$ genes were concomitantly detected in 6 isolates, and *bla*$_{TEM,}$ *qnrB, qnrS* and *qnrD* genes were concomitantly detected in 7 isolates (Table 2).

Table 2. Antibiotic resistant genes from *E. coli* isolates in this study.

Patterns of resistance genes	No. of *E. coli*
***bla*$_{TEM-1}$+*bla*$_{CTX-M-14}$**	6
bla*$_{TEM-1}$+*qnrB*+*qnrS1*+*qnrD	7
bla*$_{CTX-M-14}$+ *qnrD	2
bla*$_{CTX-M-15}$+ *qnrD	1
bla*$_{CTX-M-14}$+*floR	1
***bla*$_{CTX-M-15}$**	3
floR*+*cfr	7
floR	12

Discussion

In the present study, except that most of *E. coli* isolates showed resistant to tetracycline, the resistance rates of *E. coli* to the antibiotics tested in this study were lower than those reported in pigs raised in intensive breeding farms in China [2,17-19], and the difference may be related with the long term use of antibiotics for disease treatments and growth promoters in intensive breeding farms. However, as compared with *E. coli* isolated from free-range Tibetan pigs, *E. coli* isolates showed relatively higher resistance to antibiotics used in this study [20], and the difference may be related to the feeding environment.

The most prevalent resistance genes encoding ESBL in *E. coli* isolates were $bla_{\text{CTX-M-14}}$ and $bla_{\text{CTX-M-15}}$, which was consistent with the results of other studies conducted in China, such as the finding of $bla_{\text{CTX-M-14}}$ as the major genotype in genotypic study of $bla_{\text{CTX-M}}$ in *E. coli* originating from animals of Guangdong province, China [21]. Similarly, $bla_{\text{CTX-M-14}}$ was the major $bla_{\text{CTX-M}}$ genotype of *E. coli* isolated from cows with mastitis in China [22]. In addition, $bla_{\text{CTX-M-14}}$ and $bla_{\text{CTX-M-15}}$ are frequently detected in patients, food animals and water environment, China [23-25], indicating the clonal spread of *E. coli* between humans and animals via food chains. To note, among 31 isolates phenotypically resistantt to ceftiofur, $bla_{\text{CTX-M}}$ genes were only detected in 13 isolates, which may be associated with the gene mutation or expression status. And these disproportionate results were also found in other resistance genes detected in this study, which needs to be studied further.

Thirty-nine *E. coli* isolates were positive for *floR* with a detection rate of 31.7%, which was lower than that found in *E. coli* isolated from chickens and pigs raised in intensive breeding farms in China [26,27]. Nevertheless, this result indicated that *floR* gene was relatively prevalent in Laiwu black pig-derived *E. coli*. The prevalence of *floR* genes may, to a great extent, be due to the fact that the resistance gene is generally located on mobile elements such as plasmids or transposons [28-30]. What is more, *qnr* genes were detected in 23 isolates phenotypically resistant to ciprofloxacin, but 2 containing *qnr* resistance genes showed phenotypically sensitive, which may be related with the expression status of *qnr* genes.

Conclusions

In summary, although antimicrobial resistance rate and resistance genes detection rate in *E. coli* from free range Laiwu black pigs were relatively lower compared to those from intensive breeding farms, these pigs still carry many antimicrobial resistance genes and may threaten human health and ecological environments. Therefore, free-range pigs are still a potential reservoir for antimicrobial resistant bacteria, and the results warrant the long-term surveillance of antibiotic-resistant bacteria from free-range animals.

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