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Article

Genotypic Diversity and Antimicrobial Resistance Profiles of Multidrug-Resistant *Escherichia coli* in Porcine Populations from Hubei, China

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Abstract

The indiscriminate and excessive use of antimicrobial agents in livestock production constitutes a significant contributor to antimicrobial resistance (AMR), posing substantial threats to global public health. Despite this critical concern, the genetic diversity and antibiotic resistance patterns of *Escherichia coli* (*E. coli*) in regional ecosystems remain insufficiently characterized. This study investigated the prevalence of antibiotic resistance, transmission mechanisms, and molecular epidemiology of *E. coli* strains isolated from swine farms in Hubei Province, China, while simultaneously analyzing their clonal and genetic diversity. A total of 148 *E. coli* isolates were collected from porcine sources in central China, revealing distinct regional variations in genetic diversity. Multilocus sequence typing (MLST) analysis identified 38 sequence types (STs) distributed across 7 clonal complexes (CCs) and several unassigned clones. ST46 emerged as the predominant sequence type (19.6% prevalence), followed by ST23 and ST10. Antimicrobial susceptibility testing demonstrated universal resistance to lincosamides and sulfonamides, with all isolates exhibiting multidrug resistance (MDR) to ≥ 9 antimicrobial classes. Genetic characterization detected 16 resistance determinants, with individual isolates carrying 5-7 resistance genes on average. The resistance profile included: Seven β -lactamase genes: blaTEM (61.5%), blaCTX-M-1G (57.4%), blaDHA (46.6%), blaSHV (39.2%), blaCTX-M-9G (24.3%), blaOXA (13.5%), and blaCMY-2 (1.4%). Eight aminoglycoside-modifying enzyme genes, polymyxin resistance gene mcr-1 (7.4%). Virulence factor screening through PCR detected nine associated genes, with EAST1, fyuA, STa, K88, STb, Irp2, and LT-1 present in 95.3% of isolates, while K99 and 987P were absent in all specimens. This investigation documents alarmingly high antimicrobial resistance rates in swine-derived *E. coli* populations while elucidating their genetic diversity. The findings suggest that intensive antibiotic use in porcine production systems has driven the evolution of extensively drug-resistant bacterial strains. These results emphasize the urgent need for implementing antimicrobial stewardship programs in livestock management to mitigate AMR proliferation.

Keywords: *E. coli*; antibiotic resistance; MLST; extended-spectrum β -lactamase; ESBLs; resistance genes

1. Introduction

Escherichia coli (*E. coli*) is a predominant commensal bacterium residing as part of the natural gut microbiota in humans and warm-blooded animals [1–4]. However, pathogenic strains of *E. coli* are a major cause of intestinal infections in neonatal piglets aged 1-10 days, primarily causing diarrhea and edema in this vulnerable population [5]. The lack of effective vaccines to prevent *E.*

coli outbreaks attributable to its diverse serotypes and region-specific epidemiological variations has led to heavy reliance on antimicrobial agents for disease management in most agricultural settings. The escalating use of antimicrobials in livestock production has precipitated the emergence of multidrug-resistant (MDR), extensively drug-resistant (XDR), and even pandrug-resistant (PDR) bacterial strains, raising significant global health concerns. Of particular urgency is the rapid dissemination of antimicrobial resistance (AMR), a critical public health crisis that now permeates multiple sectors of society and imposes substantial economic burdens worldwide [6].

Antimicrobial resistance (AMR) has evolved into one of the most critical global health threats of the past three decades [7]. Of particular concern is the rising prevalence of drug-resistant Gram-negative bacteria in livestock populations, which can transmit to humans through environmental pathways and food chain contamination [8]. The intensive use of antibiotics in swine production has exacerbated this trend, with resistance patterns in pig-derived pathogens showing accelerated development compared to other livestock sectors. Notably, surveillance data from Great Britain revealed higher AMR prevalence in porcine-derived *E. coli* isolates than those from cattle and sheep [9], underscoring the urgency to systematically characterize antibiotic resistance profiles of pathogenic *E. coli* in Chinese swine operations. Surveillance data from 2011-2012 revealed high resistance rates in swine *E. coli* isolates to tetracycline (79.57%), trimethoprim-sulfamethoxazole (73.12%), and kanamycin (55.91%) [10]. Subsequent monitoring in Guangdong Province (2013-2016) detected multidrug resistance (MDR) in 100% of 333 *E. coli* isolates from commercial pig operations [11]. These findings reflect global concerns where MDR Gram-negative pathogens contribute to 60-70% of antimicrobial treatment failures in veterinary practice, highlighting critical challenges for animal health management [12].

Extended-spectrum beta-lactamase (ESBL)-producing-*E. coli* is spreading worldwide and poses a public health issue[13]. *Escherichia coli* has evolved multiple β -lactamase-mediated resistance mechanisms, including: Extended-spectrum β -lactamases (ESBLs; SHV, TEM, OXA, and CTX-M types), Plasmid-mediated AmpC β -lactamases (CMY-2, a plasmid-mediated *AmpC*-like gene) and Carbapenemases (MBL, KPC, and class D oxacillinases) [14]. CTX-Ms was the largest group of ESBLs, and they have become globally disseminated. Currently, CTX-M-15 (a part of the CTX-M-1 group) is the most frequent CTX-M worldwide. This is closely followed by CTX-M-14 (a member of the CTX-M-9 group) [15]. Given the limited data on the epidemiology of MDR *E. coli* from pigs in Hubei province in China, it is interesting to investigate the spread of these resistant bacteria, particularly the ESBL types, which may be the cause of the spread of resistance genes from pork to human.

Several studies of epidemiological studies on *E. coli* have been initiated in parts of China. In Northeastern China, a survey showed that the separation rate of *E. coli* isolated from pig fecal samples reached 88% [16]. An investigation on pig farms in Henan province found that the positive rate of *E. coli* was 70.74%, of which the commonly sequenced types (STs) 10 and 101 were identified [17]. However, data on genetic diversity and antimicrobial resistance of *E. coli* is still restricted in the Hubei province of central China.

E. coli can induce porcine postweaning diarrhea (PWD) [18], and it may be associated with the expression of fimbriae to mediate adhesion and colonization of porcine enterocytes [19,20]. The most adhesins are related with fimbriae F18 and F4 (K88), F5 (K99), F6 (987P) and F41, while F18 and F4 (K88) are leading cause of PWD of piglets [21–23]. While the pathogenic *E. coli* predominant toxins including heat-labile (LT-I and LT-II) [24,25], heat-stable (STa and STb) [26], enteroaggregative *E. coli* heat-stable enterotoxin 1 (EAST1) [27] and produce other virulence factors, such as pathogenicity islands (PAI). In particular, the locus of enterocyte effacement (LEE) and high-pathogenicity island (HPI) are the significant PAIs. *Irp2* and *fyuA* genes, located in the high-pathogenicity island (HPI), encode a yesiniabactin-like iron scavenging system [28]. However, *E. coli* isolated from pigs with diarrhea carry different types of virulence genes, which can enhance the pathogenicity of *E. coli* through synergistic effects. However, data on characteristics of virulence genes of *E. coli* is still restricted in the Hubei province of central China.

Multilocus sequence typing (MLST) has been used to study the evolution and epidemiology of a number of bacterial pathogens. It has become the method of choice for typing epidemiologically important strains. MLST is a standard molecular subtyping technique that identifies the genetic relatedness of strains and determines the strains with high discriminatory power [29]. Investigating the trend characterization of epidemic strains will help us to better understand epidemiology.

This present study was carried out to investigate the prevalence and characteristics of *E. coli*, the MLST genotypes, antibiotic resistance and virulence genes of *E. coli* isolates were examined, collected from 2019 to 2022 in Hubei province of China. These findings provide information and implication for safeguarding and commanding the occurrence of diseases in future studies.

2. Methods

Isolation of *E. coli*

From 2019 to 2022, a total of 148 fecal swab samples were collected from pig farms in 4 cities, including Xiangyang, Yichang, Suizhou, Wuhan in Hubei province. The samples were taken from diseased piglets with diarrhea symptoms. These strains isolated by MacConkey agar incubated at 37°C for 24 h. Pink colonies were picked and purified again, then bacterial stains cultured in Luria-Bertani (LB) broth at 37°C for 24 h. And further confirmed by the PCR amplification of the 16S rRNA gene with primers (16S-F: 5'-AGAGTTTGATCATGGCTCAG-3'; 16S-R: 5'-TAGGGTTACCTTGTTACGACTT-3 ') as previously described[30] with some slight modifications.

Antibiotic Resistance Profiles

According to the guidelines of the Clinical and Laboratory Standards Institute[31], the confirmed *E. coli* was identified for antimicrobial susceptibility. *E. coli* isolates were examination for susceptibility to antimicrobial drugs utilizing a disk diffusion assay. All samples were analyzed for the presence of resistant bacteria. A total of 17 antimicrobials were tested, comprising cefuroxime (CXM), ceftriaxone (CRO), cephalothin (CEP), cefotaxime (CTX), ampicillin (AMP), amoxicillin (AMX), lincomycin (MY), doxycycline (DOC), tetracycline (TET), kanamycin (KMC), gentamicin (GEN), amikacin (AMK), ciprofloxacin (CIP), enoxacin (ENO), lomefloxacin (LOM), azithromycin (AZM), sulfafurazole (SFN). Inoculated plates were incubated at 37°C for 24 h, subsequently the diameters (in mm) of the inhibition zone were measured. Based on the minimal inhibitory concentration determined for each drug, the isolates were classified as “susceptible”, “intermediate”, or “resistant”. The *E. coli* strain ATCC 25922 was utilized for quality control. Multidrug-resistant (MDR) of an isolate was designated as clinical resistance to at least one agent in three or more antimicrobial categories[32].

Detection of Antibiotic Resistance Genes

The boiling the lysis of isolated colonies method was used to extract total DNA of each *E. coli* strain. PCR was used to identify genes responsible for resistance to β-lactamase genes (*bla_{DHA}*, *bla_{CMY-2}*, *bla_{TEM}*, *bla_{SHV}*, *bla_{CTX-M-1G}*, *bla_{CTX-M-9G}*, *bla_{OXA}*), aminoglycoside modifying enzyme genes (*aac(3')-Ia*, *aac(3')-IIc*, *aac(3')-IV*, *aac(6')-Ib*, *aadA1*, *aadA2*, *rmtA*, *rmtB*), and polypeptide resistance gene *mcr-1*. The primers for PCR are listed in Table 1 and were synthesised at Sangon Biotech, Shanghai.

Table 1. Primers used in this study to amplify *E. coli* drug resistance genes.

Genes	Primer sequence (5 ' -3 ')	Size of product (base pairs)	Reference
<i>bla_{DHA}</i>	F: AACTTTCACAGGTGTGCTGT	387	Pai, Seo & Choi, 2007[60]
	R: CCGTACGCATACTGGCTTTC		
<i>bla_{CMY-2}</i>	F: ATGATGAAAAAATCGTTATGC	1143	Yan et al. 2004[61]

	R: TTGCAGCTTTTCAAGAATGCG		
<i>bla</i> _{TEM}	F: ATAAAATTCTTGAAGACGAAA R: GACAGTTACCAATGCTTAATC	1080	Weill et al. 2004[62]
<i>bla</i> _{SHV}	F: CACTCAAGGATGTATTGTG R: TTAGCGTTGCCAGTGCTCG	885	Brinas et al. 2005[63]
<i>bla</i> _{CTX-M-1G}	F: CTTCCAGAATAAGGAATCCC R: CGTCTAAGGCGATAAACAAA	949	Liu et al. 2007[64]
<i>bla</i> _{CTX-M-9G}	F: TGACCGTATTGGGAGTTTG R: ACCAGTTACAGCCCTTCG	902	Liu et al. 2007[64]
<i>bla</i> _{oxA}	F: ATATCTCTACTGTTGCATCTCC R: AAACCCTTCAAACCATCC	619	Colom et al. 2003[65]
<i>aac</i> (3')-Ia	F: TTACGCAGCAGCAACGATGT R: GTTGGCCTCATGCTTGAGGA	402	Sun et al. 2012[66]
<i>aac</i> (3')-IIc	F: AACCGGTGACCTATTGATGG R: TGTGCTGGCACGATCGGAGT	774	Sun et al. 2012[66]
<i>aac</i> (3')-IV	F: GGCCACTTGGACTGATCGAG R: GCGGATGCAGGAAGATCAAC	609	Sun et al. 2012[66]
<i>aac</i> (6')-Ib	F: TTGCGATGCTCTATGAGTGGCTA R: CTCGAATGCCTGGCGTGTTT	482	Park et al. 2006[67]
<i>aadA1</i>	F: AGGTAGTTGGCGTCATCGAG R: CAGTCGGCAGCGACATCCTT	589	Sun et al. 2012[66]
<i>aadA2</i>	F: GGTGCTAAGCGTCATTGAGC R: GCTTCAAGGTTTCCCTCAGC	470	Sun et al. 2012[66]
<i>rmtA</i>	F: CTAGCGTCCATCCTTTCCTC R: TTGCTTCCATGCCCTTGCC	635	Chen et al. 2004[68]
<i>rmtB</i>	F: ACATCAACGATGCCCTCAC R: AAGTTCTGTTCCGATGGTC	724	Chen et al. 2004[68]
<i>mcr-1</i>	F: CGGTCAGTCCGTTTGTTTC R: CTTGGTCGGTCTGTAGGG	309	Liu et al. 2016[53]

Detection of Virulence-Associated Genes

Considering the contribution of virulence genes to the invasiveness and pathogenicity of *E. coli*, DNA was isolated and identified nine virulence-associated genes of each strain, including *LT-1*, *STa*, *STb*, *EAST1*, *irp2*, *fyuA*, *K88*, *K99*, *987P* were amplified by PCR, as previously described[33–36]. The primer sequences of virulence genes are shown in Table 2. PCR reactions were carried out in a final volume of 25 µl containing 12.5 µl mix (Vazyme Biotech, Nanjing, China), 1 µl each of primers, and 2 µl bacterial DNA. PCR was performed in a GeneAmp PCR System 9700 (Applied Biosystems, Darmstadt, Germany), under the following conditions: initial denaturation at 95°Cfor 5 min, cycling consisted of 35 cycles of 30 s at 94 °C, 30 s at 58°Cand 1 min at 72°Cand a final extension at 72°Cfor 10 min. The resulting amplification products were separated by electrophoresis in 1% agarose gel, stained with ethidium bromide and visualized using a GelDoc XR System (Bio-Rad, Shanghai, China).

Table 2. Primers used in this study to amplify *E. coli* virulence-associated genes.

Virulence factors	Primer sequence (5 ' -3 ')	Size of product (base pairs)
LT-1	F: TAGAGACCGGTATTACAGAAATCTGA R: TCATCCCGAATTCTGTTATATATGTC	282
STa	F: GGGTTGGCAATTTTATTTCTGTA R: ATTACAACAAAGTTCACAGCAGTA	183
STb	F: ATGTAAATACCTACAACGGGTGAT R: TATTTGGGCGCCAAAGCATGCTCC	300
EAST1	F: ATGCCATCAACACAGTATATC R: TCAGGTCGCGAGTGACGG	117
<i>irp2</i>	F: AAGGATTCGCTGTTACCGGAC R: TCGTCGGGCA GCGTTTCTTCT	301
<i>fyuA</i>	F: TGATTAACCCCGCGACGGGAA R: CGCAGTAGGCACGATGTTGTA	787
K88	F: GATGAAAAAGACTCTGATTGCA R: GATTGCTACG TTCAGCGGAGCG	841
K99	F: CTGAAAAAAACACTGCTAGCTATT R: CATATAAGTGACTAAGAAGGATGC	543
987P	F: GTTACTGCCAGTCTATGCCAAGTG R: TCGGTGTACCTGCTGAACGAATAG	463

MLST and Phylogenetic Tree

The multilocus sequence typing (MLST) was executed on *E. coli* isolates according to the *E. coli* MLST database guidelines ([http://enterobase.warwick.ac.uk/species/ecoli/allele st search](http://enterobase.warwick.ac.uk/species/ecoli/allele%20st%20search)), accreting to the protocols published on the web site. Briefly, the seven house-keeping genes *adk*, *fumC*, *gyrB*, *icd*, *mdh*, *purA* and *recA* were amplified employing a PCR protocol, and the amplicons sequenced utilizing the amplification primers. Investigated individual gene sequences and allocated an allelic profile number in line with the MLST database. Sequence type (STs) and clone complexes (CCs) designations of each strain were comprised of seven alleles. The calculated tree of the *E. coli* resistant isolates was constructed by applying the MEGA cluster analysis based on seven housekeeping gene sequences.

Statistics Analysis

The student’s t-test was employed to analyze the data. When *P* < 0.05, difference was considered statistically significant. The analysis was performed using GraphPad Prism version 8 (GraphPad Software, Inc., USA).

3. Result

Isolation of E. coli Strains and Antimicrobial Susceptibility Profile

E. coli isolates were obtained from fecal of weaned piglets from Hubei province. A total of 148 strains of *E. coli* were separated from samples and further identified by PCR tests as *E. coli*. There are 15 strains of *E. coli* isolated from Suizhou city, 37 strains of *E. coli* isolated from Xiangyang city, 82 strains of *E. coli* isolated from Wuhan city and 14 strains of *E. coli* isolated from Yichang city (Figure 1).



Figure 1. Geographical distribution of the farms where the experimental samples were collected. Farms represent the different farms where samples were collected and the number of samples; Wuhan (82), Xiangyang (37), Yichang (14), Suizhou (15).

Antimicrobial Susceptibility Profile of E. coli Isolates

Antimicrobial susceptibility testing (AST) results showed that all of the *E. coli* isolates showed resistance to lincosamides and sulfonamide (Figure 2A). A high rate of resistance to sulfaisoxazole, tetracycline, lomefloxacin, enoxacin, ampicillin, amoxicillin, azithromycin, gentamicin, has also been observed for the isolates, among which 100% of the *E. coli* was resistant to sulfaisoxazole; 97.97% of the *E. coli* was resistant to tetracycline; 95.95% of the *E. coli* was resistant to lomefloxacin; 93.24% of the *E. coli* was resistant to enoxacin; 91.89% of the *E. coli* was resistant to ampicillin; 91.89% of the *E. coli* was resistant to amoxicillin; 89.19% of the *E. coli* was resistant to azithromycin; 82.43% of the *E. coli* was resistant to gentamicin. The isolates demonstrated a relatively low rate of resistance to cephalothin (77.7%) cefuroxime (68.92%), doxycycline (68.92%), cefotaxime (68.24%), ceftriaxone (62.84%), ciprofloxacin (61.49%), amikacin (52.7%) and kanamycin (51.35%) (Figure 2A, Figure 3).

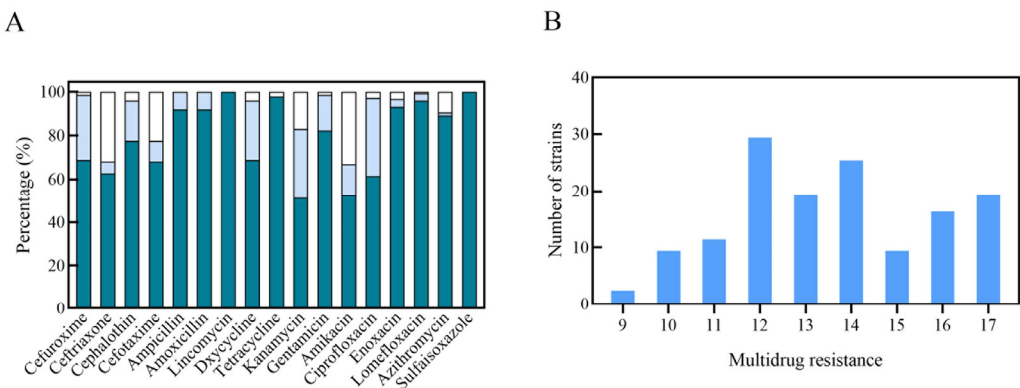


Figure 2. Antimicrobial susceptibility results of *E. coli* ($n=148$) isolates. **(A)** Antimicrobial resistance profiles of *E. coli* isolates to 17 agents. **(B)** Analysis of multidrug resistance. X-axis indicates multidrug resistance of the isolates to 17 agents. (■ drug resistance rate; ■ drug intermediation rate; □ drug sensitivity rate).

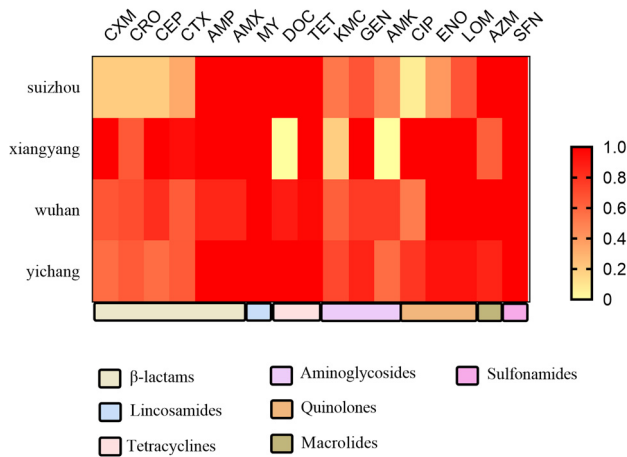


Figure 3. Heatmap showing the percentage of pig farm *E. coli* isolates resistant to each of the antibiotics tested. The resistance rate of *E. coli* isolates to 17 agents. CXM, cefuroxime; CRO, ceftriaxone; CEP, cephalothin; CTX, cefotaxime; AMP, ampicillin; AMX, amoxicillin; MY, lincomycin; DOC, doxycycline; TET, tetracycline; KMC, kanamycin; CEN, gentamicin; AMK, amikacin; CIP, ciprofloxacin; ENO, enoxacin; LOM, lomefloxacin; AZM, azithromycin; SFN, sulfafurazole.

As shown in the Figure 2B, all of the isolates were multidrug-resistant (MDR) strains, and most of the isolates were resistant to more than nine drug classes. AST revealed that the 20 isolates showed severe resistance profiles that was resistant to the seventeen antimicrobial agents tested. Overall, most of the isolates were conferring resistance to 12-14 antimicrobial agents tested. Among antibiotics tested here, resistance to sulfaioxazole, azithromycin, lomefloxacin, enoxacin, amikacin, tetracycline, doxycycline, lincomycin, cefuroxime, ceftriaxone, cephalothin, cefotaxime.

E. coli in different regions has separate antibiotic resistance. The AST results revealed that resistance to lincomycin, and sulfaioxazole was a common phenotype of the isolates from the pig farms in 4 cities, including Xiangyang, Yichang, Suizhou, Wuhan in Hubei province (Figure 3). *E. coli* strains resistant to doxycycline and amikacin were isolated including Yichang, Suizhou, Wuhan in Hubei province, respectively; Xiangyang was the only region where no strains from pig farms with the abovementioned resistant phenotypes were detected (Figure 3). Notably, MDR isolates were identified on pig farms in Hubei province, but a relatively high proportion of the MDR isolates were identified on farms in wuhan and yichang relative to those from the other city.

Detection of Antimicrobial Resistance Genes

16 ARGs (Antibiotic resistance genes, ARGs) in 148 strains of *E. coli* were detected (Figure 4A) using PCR-based assays. Of which, *aac(6')-Ib* (71.6%, 106/148), *bla_{TEM}* (61.5%, 91/148), *aadA1* (60.1%, 89/148), *bla_{CTX-M-1G}* (57.4%, 85/148), *aac(3')-Iv* (55.4%, 82/148), *aac(3')-IIc* (54.1%, 80/148), *aadA2* (52.7%, 78/148), *bla_{DHA}* (46.9%, 69/148) were prevalent with higher detection rates (Figure 4B). Among the 148 *E. coli* strains, 147 strains carried β -lactamase genes with a detection rate of 99.3%, of which, the detection rates of *bla_{SHV}*, *bla_{TEM}*, *bla_{OXA}*, *bla_{CMY-2}* and *bla_{DHA}* were 39.2% (58/148), 61.5% (91/148), 13.5% (20/148), 1.4% (2/148) and 46.6% (69/148), respectively. The detection rate of *bla_{CTX-M-1G}* in CTX-M was 57.4% (highest) and the detection rate of *bla_{CTX-M-9G}* was 24.3%.

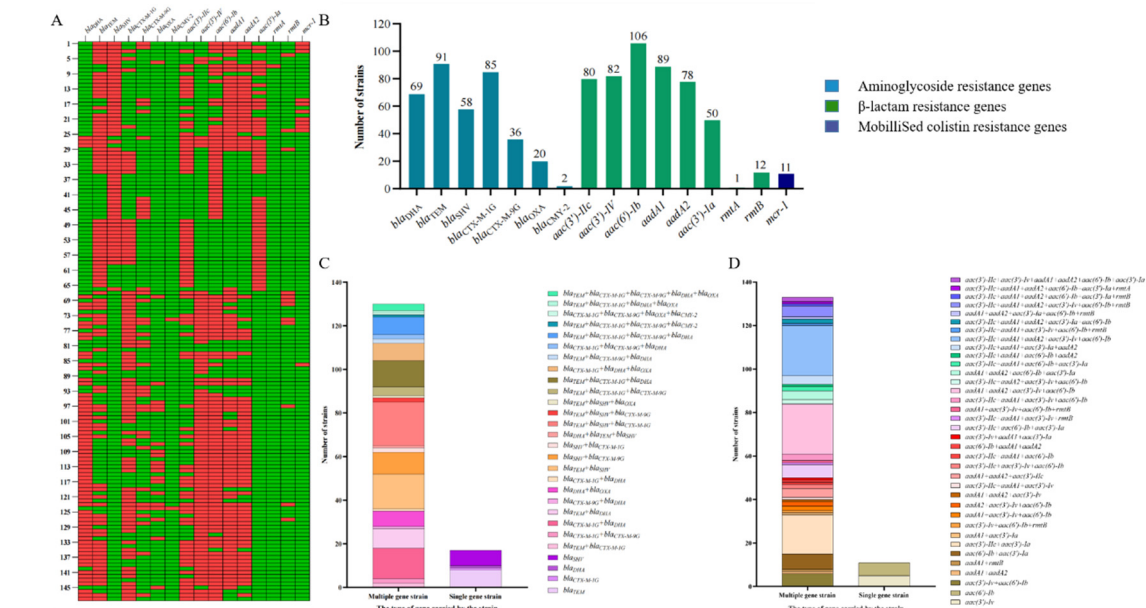


Figure 4. Detection rate of antibiotic resistance genes in *E. coli* isolates. **(A)** Heat map showing detected antimicrobial resistance genes carried by the isolates. Small rectangles marked in red and green represent positive and negative. **(B)** The bar graph showing numbers of positive strains for different detected antibiotic resistance genes. Antimicrobial resistance genes accounting for resistance to the same class of antibiotics are marked with bars in the same color. **(C)** Genetic compositions of extended-spectrum β-lactamase strains. Left column: multiple gene strains, Right column: single gene strains. **(D)** Genetic compositions of aminoglycoside-modifying enzyme strains. Left column: multiple gene strains, Right column: single gene strains.

Among the 148 *E. coli* strains, 144 strains carried aminoglycoside-modifying enzyme genes with a detection rate of 97.3%, of which, the detection rates of *rmtB* (8.1%) and *aac(3')-Ia* (33.8%) were prevalent with lower detection rates. Amongst the strains positive for aminoglycoside-modifying enzyme genes, only one strain was positive for *rmtA*. In addition, the detection rate of the mobilised colistin resistance gene *mcr-1* was 7.4%.

Antibiotic resistance genes were detected in all 147 strains, only one strain was not detected anyone antibiotic gene. Of which, 91 (61.5%) strains had more than six ARGs, four (2.7%) strains had more than 10 ARGs, and two strains had 11 ARGs (Supplementary Table 1).

Various Extended-Spectrum β-Lactamase Genes Were Present in the Isolates

Amongst the 148 strains tested, 147 (99.3%) strains carried ESBLs and 130 of them harbored various number of ESBLs. The most frequent was *blaTEM*+*blaSHV*+*blaCTX-M-1G* (13.5%, 20/148); 64 strains were found to have two different ESBLs genes; 52 strains were found to have three different ESBLs genes; 11 strains were found to have four different ESBLs genes; 3 strains were found to have five ESBLs genes *blaTEM*, *blaCTX-M-1G*, *blaCTX-M-9G*, *blaDHA* and *blaOXA* (Figure 4C, Supplementary Table 1).

Various Extended-Spectrum Aminoglycoside-Modifying Enzyme Genes Were Present in the Isolates

Amongst the 148 strains tested, 144 (97.3%) strains carried aminoglycoside-modifying enzyme genes and 133 of them harbored various number of aminoglycoside-modifying enzyme genes. The most frequent were *aadA1*+*aadA2*+*aac(3')-Iv*+*aac(6')-Ib* and *aac(3')-Iic*+*aadA1*+*aadA2*+*aac(3')-Iv*+*aac(6')-Ib* (15.5%, 23/148); 34 strains were found to have two different ESBLs genes; 22 strains were found to have three different ESBLs genes; 41 strains were found to have four different ESBLs genes; 27 strains were found to have five different ESBLs genes; 9 strains were found to have six different ESBLs genes (Figure 4D, Supplementary Table 1).

Prevalence of Virulence Genes in *E. coli* Isolates from Pigs

The distribution of 8 virulence genes in 148 strains of *E. coli* has been examined in this study. As shown in Figure 5A/B, the virulence gene *EASTI* (80.4%, 119/148) were found in most of the *E. coli* isolates, followed by *fyuA* (55.4%, 82/148), *STa* (52%, 77/148), *K88* (38.5%, 57/148) and *irp2* (3.1%, 49/148). While *K99* and *987P* were not detected in all isolates.

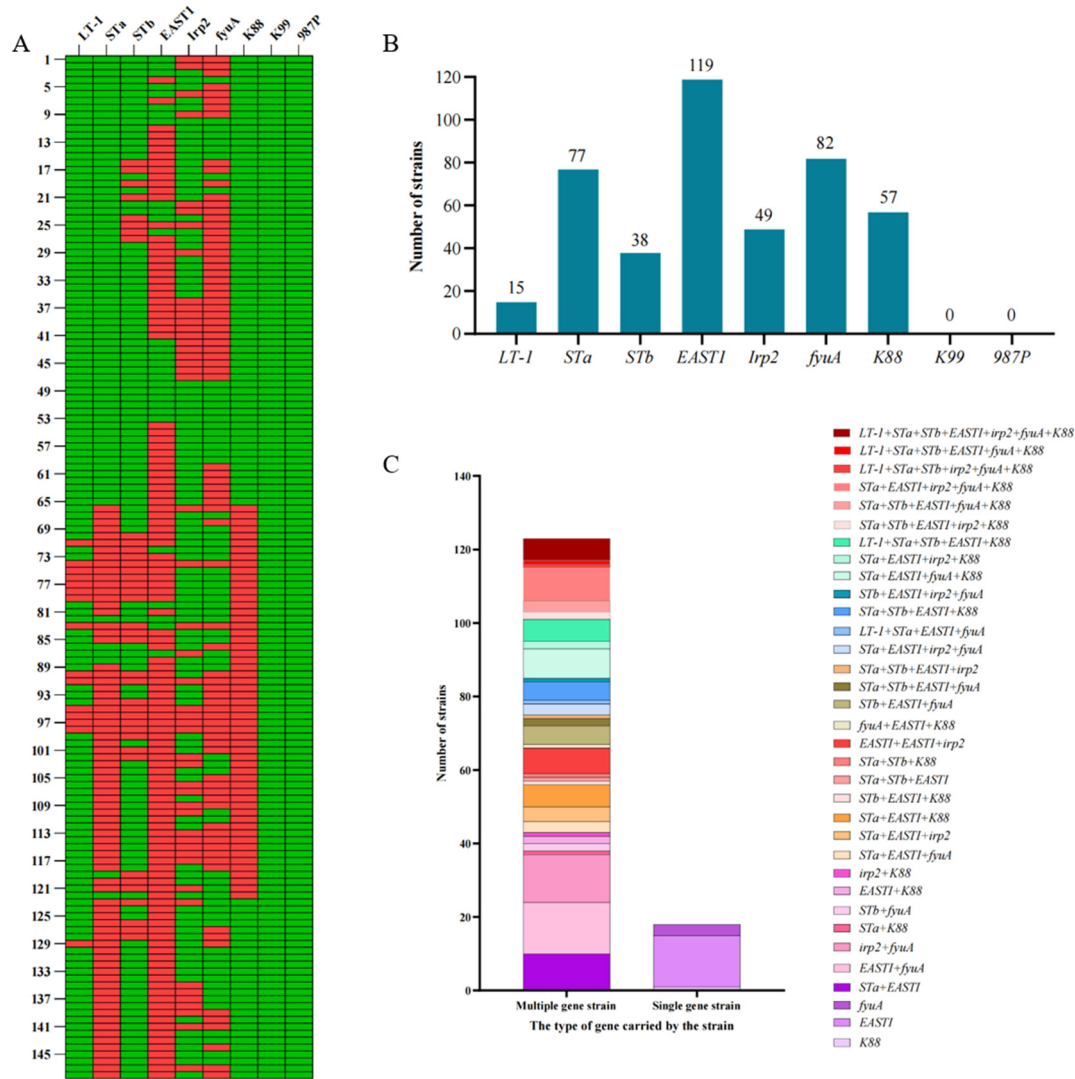


Figure 5. Distribution and category of *E. coli* isolates positive for virulence-associated genes. **(A)** Heat map showing detected virulence genes carried by the isolates. Small rectangles marked in red and green represent positive and negative. **(B)** The bar graph showing numbers of positive strains for different detected virulence genes. **(C)** Genetic compositions of virulence-associated strains. Left column: multiple gene strains, Right column: single gene strains.

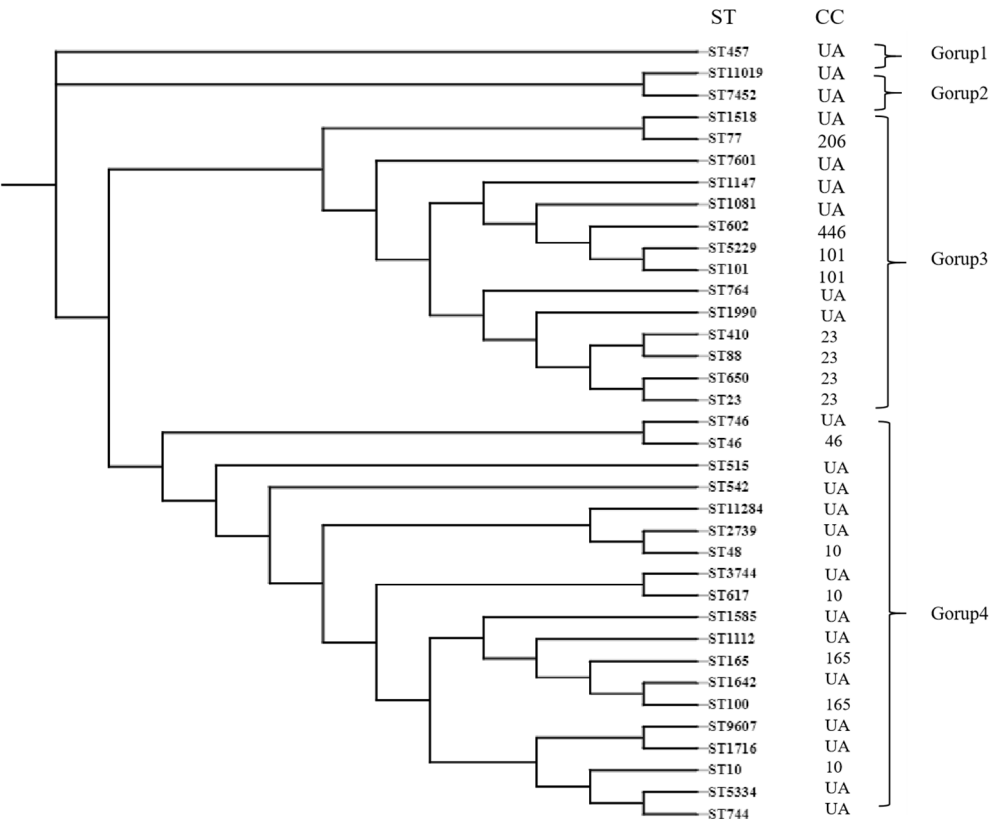
Amongst the 148 strains tested, 141 (95.3%) strains carried virulence genes and 123 of them harbored various number of virulence genes. The most frequent were *EASTI*+ and *EASTI*+*fyuA*+ (9.9%, 14/141), followed by *irp2*+*fyuA*+ (9.2%, 13/141), *STa*+*EASTI* (7.1%, 10/141), *STa*+*EASTI*+*irp2*+*fyuA*+*K88*+ (6.4%, 9/141), *STa*+*EASTI*+*fyuA*+*K88*+ (5.7%, 8/141), *EASTI*+*EASTI*+*irp2*+ (5.0%, 7/141), *LT-1*+*STa*+*STb*+*EASTI*+*irp2*+*fyuA*+*K88*+ (4.3%, 6/141), *LT-1*+*STa*+*STb*+*EASTI*+*K88*+ (4.3%, 6/141), *STa*+*EASTI*+*K88*+ (4.3%, 6/141) (Figure 5C, Supplementary Table 1).

Multi-Locus Sequence Typing (MLST)

The genetic diversity of these *E. coli* isolates was analyzed with MLST. A total of 148 isolates were analyzed utilizing MLST, the identification of 38 sequence types (STs). Among these isolates, 101 out of the 148 isolates possessed 14 different STs which belonged to 7 CCs. The remaining 46 isolates belonged to 23 different unassigned STs. A new STs were due to new combinations of previously known alleles in *adk* (allele 7), *fumC* (allele 7), *gyrB* (allele 193), *icd* (allele 1), *mdh* (allele 986), *purA* (allele 159) and *recA* (allele 139). The predominant STs were ST46, ST23 and ST10 containing 29 (19.59%), 20 (13.51%),12 (8.11%) isolates respectively. Ten STs contained 3 or more isolates with ST602, ST165, ST100, ST744, ST7452, ST1642, ST1081, ST515, ST48 and ST101 comprising 9 (6.08%), 8 (5.41%), 8 (5.41%), 7 (4.73%), 5 (3.38%), 4 (2.70%), 4 (2.70%), 4 (2.70%), 3 (2.03%) and 3 (2.03%) isolates respectively. Seven STs (ST410, ST650, ST764, ST1147, ST1518, ST2739 and ST5229) contained 2 isolates each. Eight STs (ST77, ST88, ST457, ST542, ST617, ST710, ST746, ST1112, ST1585, ST1716, ST1990, ST3744, ST5334, ST7601, ST9607, ST11019 and ST11284) had only 1 isolate each (Figure 6).

Minimum-spanning trees showed that the tested *E. coli* mainly classed into seven clonal complexes and other unassigned clone complexes. CC-46 was the most frequently isolated clonal complex which contained 29 isolates belonging to one STs, and accounted for 19.59% (29/148) of all the isolates. The major clonal complexes also included CC-23 (25/148, 16.89%), CC-10 (16/148, 10.81%) and CC-165 (16/148, 10.81%). The other isolates covered 28 STs belonging to 3 CCs and unassigned.

To further analyzing STs utilized the UPGMA cluster analysis (Figure 6). 37 identified STs were classified into four major groups. Group 1 included 1 isolate that only belonged to one unassigned clone complexes. There are 6 isolates included two different unassigned clone complexes constitute Group 2. Group 3 and group 4 covered a great majority of STs, containing 140 isolates, belonging to the CC-10, CC-23, CC-46, CC-101, CC-165, CC-206, CC-446 and unassigned clone complexes. CC-10 contained 3 of our STs (ST10, ST48 and ST617). CC-23 contained 4 of our STs (ST23, ST88, ST410 and ST650). CC-101 contained 2 of our STs (ST101 and ST5229). CC-165 contained 2 of our STs (ST100 and ST165) (Figure 6).



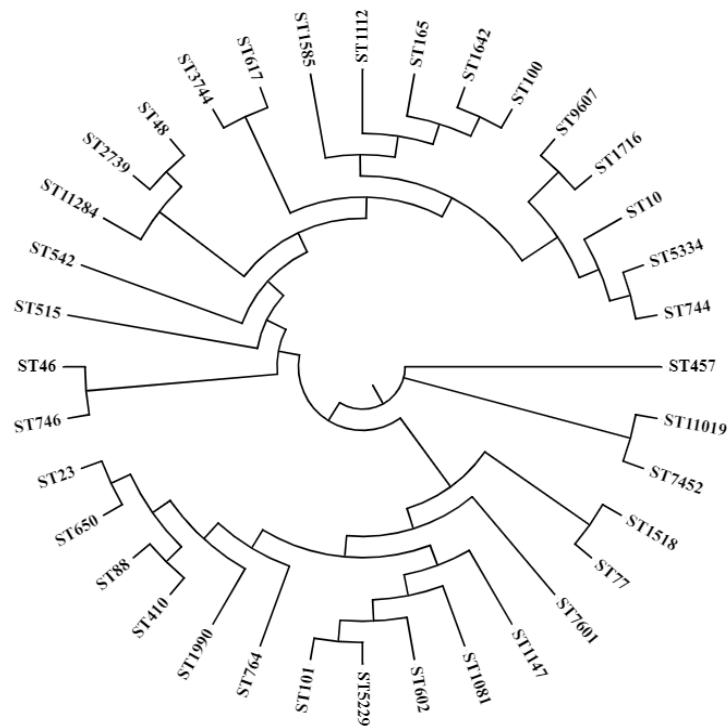


Figure 6. Dendrogram of multilocus sequence typing (MLST) profiles among the *E. coli* isolates.

4. Discussion

E. coli is one of the main pathogenic bacteria that impact the production and growth of pigs in pig farms. It is associated with gastrointestinal diseases such as diarrhea, edema disease, and systemic infections such as septicemia and polyserositis [2]. These diseases caused by *E. coli* can increase mortality, morbidity and growth delays of piglets, which are responsible for economic losses. This study analyzed the prevalence, genetic diversity and antibiotic resistance of disease, which may help us to improve methods of prevention and treatment.

The distribution of swine-origin *E. coli* in pigs differs between countries and regions. From 2002 to 2008, the prevalence of *E. coli* isolated from pork chop samples was 44% in the United States [37]. However, various incidence rates have also been reported in different regions of China. From 2003 to 2009, the prevalence of *E. coli* isolates from pig farms was 77.78% in China [38]. From 2013 to 2016, the positive rates of *E. coli* between farm 1 and farm 2 were 40.25% and 59.75% in Guangdong province [11]. Between 2016 and 2017, a survey indicated that the separation rate of *E. coli* isolated from pig fecal swabs reached 88% in northeastern China, including Heilongjiang, Jilin and Liaoning [16]. In this study, we collected samples from the fecal of weaned piglets, and a total of 148 strains of *E. coli* were isolated from 4 cities in Hubei province.

Diarrhea in weaned piglets driven by *E. coli* remains a principal cause of economic losses for the pig industry. This commonly seeks antimicrobial drug treatment, which is considerable to cure pathogen animals. In 2011, Danish scholar Agerse et al. study found that 32% of isolates have multi-drug resistance, mainly concentrated on ampicillin (27%) and tetracycline (29%) [39]. In 2012, Tadesse et al. tested 1729 isolates of *E. coli* antibiotic susceptibility varied from different sources, the resistance rate of *E. coli* increased from 7.2% to 63.6% but the most common resistance to tetracycline, streptomycin and sulfonamides [40]. Total of 131 *E. coli* isolates were obtained from the pigs presenting from diarrhea in Switzerland from 2014 to 2015, isolates exhibited resistance to tetracycline (50%), sulfamethoxazole (49%), ampicillin (26%), gentamicin (17%), ciprofloxacin (8%)

[41]. However, this caused a rise in the employment of various antimicrobial agents, such as lincosamides, tetracyclines and sulfonamides, which may expand antimicrobial resistance.

In this study, *E. coli* isolates results of the antimicrobial susceptibility tests presented that the most prevalent antibiotic resistance was to lincosamides, tetracyclines, sulfonamides. This may be related to the long-term irrational use of these three antibiotics to control bacterial diarrhea in piglets in Hubei province. More than 80% of *E. coli* isolates presented high resistance rates to tetracycline, lomefloxacin, enoxacin, ampicillin, amoxicillin, azithromycin and gentamicin. The isolates are also resistant to beta-lactams drugs, the resistance to first-generation cephalosporin drug cephalothin 77.7%, higher than the second-generation cephalosporins cefuroxime (68.9%) and the third-generation drugs cefotaxime (68.2%) and ceftriaxone (62.8%). All of the isolates were resistant to test antibiotics to vary degrees, and Most of *E. coli* isolates advent high prevalent multi-drug resistance. There were twenty *E. coli* strains that were resistant to the seventeen antimicrobial agents tested. Overall, more than half of the isolates were conferring resistance to 9-17 antimicrobial agents tested. The most frequent multidrug resistance pattern was resistance to cefuroxime, amikactin, ampicillin, amoxicillin, lincomycin, doxycycline, tetracycline, gentamicin, lenoxacin, lomefloxacin, azithromycin and sulfaisoxazole, which covered 30 isolates. In some other studies in China, Jiang et al. revealed that *E. coli* isolates had high rates of resistance to ampicillin (99.5%), tetracycline (93.4%) and amoxicillin (65.1%). Resistance to cephalosporins, quinolones, and aminoglycosides was also quite prevalent[42]. Meng et al. study results showed that the great majority of *E. coli* isolates resistance to tetracycline (79.57%), trimethoprim-sulfamethoxazole (73.12%) and kanamycin in China (55.91%) [10]. However, *E. coli* isolates showed the highest resistance to sulfamethoxazole (61.6%), followed by tetracycline (61.2%), ampicillin (48.2%) and kanamycin (22.4%) in Sichuan province between 2012 and 2013 [43]. *E. coli* were isolated from pig farms from seven provinces that the resistance rate to ampicillin was 81.44%, 94.37% to tetracycline and 88.36% to sulfaisoxazole [44]. These findings provide important information and implications for the application of antibiotics in future studies.

It has been documented that *E. coli* has a great ability to accumulate ARGs, mainly through horizontal gene transfer [45]. In this study, a large number of ARGs are detected in various strains and it was found that a higher proportion contained six or more resistance genes. One of the most common antibiotic resistance mechanisms in *E. coli* is mediated by the production of β -lactamase, ESBLs are an increasing cause of resistance to third-generation cephalosporins, including the fourth-generation cephalosporins in Enterobacteriaceae [46,47]. The *bla*_{TEM}, *bla*_{CTX-M} and *bla*_{SHV} types have been recognized as the most prevalent ESBL genes that confer antibiotic resistance among pathogens [48,49]. In this study, five ESBL genes and two AmpC enzyme gene (*bla*_{DHA}, *bla*_{CMY-2}) were detected. Among the 5 ESBL genes, *bla*_{TEM} (61.5%) was the most prevalent gene, similar to the ESBLs epidemic in Guizhou in 2021 [50]; followed by *bla*_{CTX-M-1} at 57.4%, *bla*_{SHV} at 39.2%, *bla*_{CTX-M-9G} at 24.3% and *bla*_{OXA} at 13.5%. ESBLs are paradigmatic of resistance, usually encoded on mobile genetic elements that accelerate their dissemination, has become another challenge for drug resistance control in pig farms [51].

It is inevitable for bacteria to develop resistance to aminoglycosides due to their widespread use. There have been reports of aminoglycoside resistance in both Gram-negative and Gram-positive bacteria [52]. In this study, the detection rate of five aminoglycoside-modifying enzyme genes (*aac*(6')-Ib, *aac*(3')-IV, *aac*(3')-IIc, *aadA1*, *aadA2*) exceeds 50%, and it is possible to carry multiple aminoglycoside modifying enzyme genes in a single strain, leading to high levels of resistance to aminoglycosides. In 2015, the plasmid-mediated colistin resistance gene, *mcr-1*, was reported for the first time in *E. coli* isolate from the animals and their food in China [53], it has quickly spread to human pathogens [54]. The transfer of colistin resistance by plasmid has been ascribed to the *mcr-1* gene, which is the most predominant type of *mcr* genes [55]. The low detection rate (7.4%) of *mcr-1* in this study likely results from low use of polymyxins in pig feed in Hubei. It is important to monitor such strains closely to prevent their spread. The concern of antibiotics resistance in these categories is serious and need urgent attention. It demands urgent attention for regularization

monitoring of antimicrobial susceptibilities and efficient administration of bacterial infections to restrict the further spread of multidrug resistance in Hubei province.

In the past decades, the occurrence and spread of PWD in piglets have caused massive economic losses to the development of the pig farming industry in China [36]. The highest detection rate of virulence factor *EAST1* (80.4%), followed by *fyuA* (55.4%). Fimbriae adhesins are necessary in the pathogenetic mechanism, the most common adhesins on *E. coli* from PWD in pigs are fimbriae *F4* and *F18* [56]. *F4* (*K88*) (38.5%) was identified in this study, which indicated that it was closely related with pathogenic *E. coli*. For enterotoxins detected in this study, the gene encoding the heat-stable enterotoxin *STa* was frequently detected (52.0%), which was similar with the finding reported in Korea and other countries [57,58], followed by the heat-stable enterotoxin *STb* (25.7%). The detection of heat-labile enterotoxin (*LT-1*) (10.1%) was less than the *STa* and *STb* in this study, it is possible that the comparative identified of enterotoxins seem to vary from one geographic area to another. This study finding the distribution and characteristics of virulence factors in *E. coli* in Hubei province of, and the data may be useful for establishing preventive measures for PWD.

The application of MLST in *E. coli* isolates better comprehending the genetic diversity of these *E. coli* isolates. In this study, a total of 148 isolates were analyzed utilizing MLST, the identification of 38 sequence types (STs), the most frequent ST was ST46, followed by ST23, ST10, ST602, ST165, ST100, ST744, ST7452, ST1642, ST1081, ST515, ST48, ST101, ST410, ST650, ST764, ST1147, ST1518, ST2739 and ST5229, and then the other strains were individually classified into 8 different STs. These isolates belonged to 7 CCs and other unassigned clone complexes, including CC46, CC23, CC10 and so on. It reveals that there is diversity in ST types in Hubei province. In Yang's study, the common ST was ST10 (22/171, 12.9%), followed by , ST48 (16/171, 9.4%), ST744 (8/171, 4.7%), ST101 (7/171, 4.1%), ST617 (6/171, 3.5%), ST165 (5/171, 2.9%)[30]. Recently, *E. coli* ST10 has not only been presented by animals in China but also raised the ST10 from human infections in China [59]. Zhang et al. showed that the most prevalent ST was ST10 (16/32, 50%) [11]. However, ST10 (12/148, 8.11%) of the result in this study seem to conflict with those from most previous studies. The potential reason for these results is the limitation of the data and region.

In this study, a high antimicrobial resistance and the genotypic diversity of *E. coli* were observed isolated from swine-origin in Hubei province. From the results obtained it can be concluded that these isolates present high prevalent multi-drug resistance. These data provide a greater understanding of the genetic diversity and antimicrobial resistance of *E. coli*.

Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org.

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