RESEARCH ARTICLE Indian Journal of Animal Research, Volume 55 Issue 6: 697-703 (June 2021)

Effect of Biofilm Formation on the *Escherichia coli* Drug Resistance of Isolates from Pigs in Central China

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ABSTRACT

Background: Multi-drug resistant *Escherichia coli* (*E. coli*) can cause a variety of diseases that lead to considerable economic losses in the swine industry. In the past, the mainstream view believed that most bacterial resistance was caused by planktonic bacteria, but the ability of bacteria to form biofilms was ignored. Here, we isolated and identified 185 strains of *E. coli* from pigs in central China and analyzed the relationship between their genetics, antibiotic sensitivity and biofilm formation ability.

Methods: First, the isolates were classified according to biofilm formation ability by semi-quantitative staining of crystal violet. Then, Phylogenetic group analysis of isolates by polymerase chain reaction. In addition, *E. coli* with different biofilm-forming abilities were evaluated for antimicrobial susceptibility in its planktonic and biofilm state. Finally, the drug resistance pattern of the isolates with different biofilm formation capabilities were compared.

Result: most of the collected strains showed biofilm formation ability (87.57%, 162/185). The isolated *E. coli* with biofilm formation ability were classified into the following groups: A (16.05%, 26/162), B1 (10.49%, 17/162), B2 (33.33%, 54/162) and D (40.12%, 65/162). Simultaneously, the isolated *E. coli* were classified into the following groups according to the biofilm formation ability: Strong (34.57%, 56/162), Moderate (33.33%, 54/162), Weak (32.10%, 52/162) and Absent (12.43%, 23/162). Compared with the planktonic cells, the isolates showed a significant increase in the resistance rate in the biofilm form. And the isolates of the strong biofilm-forming ability group had a high drug resistance pattern. This study provides data of the drug resistance of pig-derived *E. coli* with different biofilm-forming abilities and provides a scientific basis for guiding veterinary clinical treatment and disease prevention.

Key words: Antibiotics resistance, Biofilm, *Escherichia coli*, Phylogenetic group, Pig.

INTRODUCTION

Escherichia coli (*E. coli*) *is a common porcine enteric bacterium that causes* colibacillosis, including a variety of diseases that lead to considerable economic losses in the swine industry (Marchant and Moreno, 2013). Antimicrobial therapy is a widely used strategy to prevent and control clinical infections (Bassetti and Righi, 2015). However, it is generally accepted that the use of antimicrobial agents in livestock production contributes to the increased incidence of antibiotic resistance in pathogens (de la Torre *et al.*, 2015). Infect, several studies have demonstrated that antimicrobial treatment regimes commonly used in the industry are responsible for increasing the number of antibiotic-resistant bacteria in pigs (Lalak *et al*., 2016; Yim *et al*., 2017).

E. coli can exchange antibiotic-resistance genes between strains of *E. coli*, as well as other bacteria. Given *E. coli* tendencies to become resistant against antimicrobial drugs, It is commonly used as an indicator of antimicrobial resistance in animals, humans and food products (Lalak *et al*., 2016). Therefore, limiting the use of antimicrobials can reduce the prevalence of resistance among bacteria (Yim *et al*., 2017). However, it will be difficult to entirely eliminate the use of antimicrobials for livestock for animal welfare and economic purposes. It is therefore important to find ways to reduce the occurrence of antibiotic resistance, while maintaining the capacity to treat diseased animals.

In the past, the mainstream view was that most infectious diseases inflicted mostly by planktonic bacteria. However, approximately 99% of bacteria have the ability to form biofilms (Frank *et al*., 2007). Biofilm-associated infections are now a concern because the concentration required to kill the identical strains that form biofilms can be increased a thousand times compared to the concentration of antibiotics that kill plankton (Mandell *et al*., 2019). This shows that biofilm formation is one of the important reasons for the improvement of bacterial antibiotic resistance. Several features in the biofilm create conditions for their antimicrobial resistance. The biofilm is a membrane-like structure formed by bacteria adhering to the surface of a biotic or abiotic body, secreting polysaccharide protein

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How to cite this article: Li, J., Fan, Q., Mao, C., Jin, M., Yi, L. and Wang, Y. (2021). Effect of Biofilm Formation on the *Escherichia coli* Drug Resistance of Isolates from Pigs in Central China. Indian Journal of Animal Research. 55(6): 697-703. DOI: 10.18805/IJAR.B-1304.

Submitted: 12-07-2020 **Accepted:** 16-10-2020 **Online:** 19-01-2021

complexes and wrapping it self. The extracellular matrix of the biofilm forms a dense architecture (Wang *et al*., 2014) and the molecular barrier and charge barrier formed outside the cell can prevent or delay the infiltration of certain antibiotics, in which the diffusion rate of the antibiotic is slowed down and cannot be effectively penetrated into the biofilm (Arciola *et al*., 2018). Even if antibiotics may penetrate into the biofilm, the concentration is greatly reduced and the purpose of cure is not achieved, but it is the cause of bacterial resistance (Harmsen *et al*., 2010; Wu *et al*., 2019). Moreover, the proximity of cells within the biofilm provides a brilliant chance for the exchange of genetic material and accelerate the horizontal spread of antibiotic resistance genes between cells (Yi *et al*., 2019).

The aim of this study was to isolate, classify and analyze antimicrobial resistance and biofilm formation ability of *E. coli* isolates from pigs in central China. The findings provide a better understanding of the epidemiological background of regional drug resistance in *E. coli*, facilitate future research on the mechanisms behind horizontal transmission of drug resistance and provide an experimental basis for preventing and treating swine bacterial diseases.

MATERIALS AND METHODS

Bacterial strains and growth conditions

E. coli strains were isolated from 20 swine farms in the cities of Luoyang, Xinyang, Shangqiu, Xian, Wuhan and Zhengzhou in central China between 2014 and 2017. All field isolates were derived from rectum of pigs and grown in Luria-Bertani (LB) broth and *E. coli* chromogenic media. The project was approved as ethical from the College of Animal Science and Technology, Henan University of Science and Technology (Protocol No.2014-118). All cultures were stored at -80°C in LB broth containing 15% glycerol and were freshly streaked on LB agar before each assay. In addition to the experimental strains, the reference strain *E. coli* ATCC 25922 was included as control.

Antibiotics and reagents

The following antimicrobials were obtained from Shanghai Anpu experimental technology Co., Ltd. (Shanghai, China): ampicillin, cotrimoxazole, aztreonam, ofloxacin, norfloxacin, kanamycin, ciprofloxacin and gentamicin. Antimicrobial drugs were dissolved to a concentration of 5120 µg/ml, sterilized using a 0.22 µm filter, in accordance with the Clinical Laboratory Standards Institute (CLSI) guidelines. Antibiotic stock solution was kept at -20°C and used within 12 hours. The diluent used for the antibiotics used in this test is shown in the Table S3.

Identification and phylogenetic group analysis of *E. coli*

To isolate and identify different strains of *E. coli*, the samples were cultured on the MacConkey (MAC) agar at 37°C for 24 hours. Pick the single red colony grown on MAC medium and streak it on *E. coli* chromogenic medium for purification and subsequent identification. Identifications of *E. coli* isolates were performed by PCR amplification of the 16S rRNA, which generates a 433-bp fragment. Multiplex-PCR was used to determine the phylogenetic group of *E. coli* isolates following previously published protocols (Wang *et al*., 2016). The strains were assigned to phylogenetic groups based on the presence of the genes chuA and yjaA and TspE4.C2, where group A was $chuA^-$ and $TspE4.C2^-$, group B1 was $chuA^-$ and $TspE4.C2^+$, group B2 was $chuA^+$ and $yjaA^+$ and group D was $chuA^+$ and $yjaA^-$.

Biofilm formation assay

Biofilm formation was assessed using Microtitre plate method, a quantitative assay based on crystal violet staining and absorbance reading at 595 nm (A_{595}) as described previously (Wang *et al*., 2016). The wells of sterile 96-well flat-bottomed polystyrene microplates (Greiner, Germany) were filled with 100µL of LB medium and 100 µl of *E. coli* inoculum cultured overnight (diluted to OD600 of 0.2 in fresh LB medium) was added to each well and incubatedat 37°C for 24 h without shaking. The sample wells were stained with 1% crystal violet of 200 µl for 10 min after removing the media and washing of wells with phosphate buffer saline (PBS). Samples were then treated with 95% ethanol and the absorbance value at 595nm was measured with a Tecan GENios Plus microplate reader (Tecan, Austria). The A_{595} value measured at 595 nm in an automatic spectrophotometer was used as the negative control for the critical point Ac. The strain's ability to form biofilm was scored as follows: $A < Ac$, non-producers, $Ac \leq A < 1.5\times Ac$, weak producers, $1.5Ac \leq A < 2\times Ac$, moderate producers and $A \geq 2\times Ac$, strong producers. All isolates were tested in triplicate and the results were presented as average of the triplicates.

Determination of antimicrobial susceptibility for planktonic and biofilm cell

The antibiotic susceptibility for planktonic cells of *E. coli* was detected by broth micro dilution method as per the CLSI standards. Briefly, Antibiotic stock solution (ampicillin, cotrimoxazole, aztreonam, ofloxacin, norfloxacin, kanamycin, ciprofloxacin and gentamicin) were diluted to 1280 µg/ml. Add 100 µl of antibiotic in order that serial dilutions antibiotics to 0.62, 1.25, 2.5, 5, 10, 20, 40, 80,160 and 320 µg/ml in 96-well plates containing 100 µl LB broth, in the final volume of 100 µl. For planktonic cell, *E. coli* were added at 5×10^5 CFU/ml to the 96 wells plate, in the final volume of 200 µl. For biofilm cell, the method of culturing biofilms as described in the "Biofilm formation assay" in the previous test. The biofilms formed on the wells were washed by sterile saline phosphate-buffered saline (PBS) to remove planktonic cells. Then, add 100 µl of diluted antibiotic (640-1.25 µg/ml) and 100 µl of LB broth to the 96-well plate, in the final antibiotic concentrations range from 320-0.62 µg/ml.

The 96-wells plates were incubated at 37°C for 24 h. The MIC was defined as the lowest concentration of antibiotic showing no visible growth. MBC was determined by sub-culturing the test dilutions on LB agar plates. The highest

dilution that yielded no bacterial growth on solid medium was taken as MBC. The tests were performed in triplicates. The reference strain *E. coli* ATCC 25922 was used as a control.

Biofilm imaging-confocal laser scanning microscopy

Biofilm were examined by Confocal Laser Scanning Microscopy (Hoiby *et al*., 2019) (CLSM) (Carl Zeiss LSM800, Germany). *E. coli* biofilms were incubated on round coverslip in a 24-well plate according to the above conditions. After incubation, the round coverslip was gently washed three times in PBS to remove planktonic cells. After drying normothermic, the biofilm was labelled with SYTO 9 as per the instruction manual from LIVE/DEAD BIOFILM (ABI L10316, Invitrogen, USA). The stained cells were analyzed by CLSM equipped and magnification at 630×.

Statistical analysis

The chi-square test was performed on the clinical data of clinical isolates of *E. coli* using Whonet 5.6 and SPSS 23.0 software. The relationship between drug resistance profile and BF phenotype was analyzed according to the difference of biofilm formation ability.

RESULTS AND DISCUSSION

We analyzed 204 samples recovered from diseased and healthy pigs in central China from 2014 to 2017 and isolated 185 *E. coli* strains on chromogenic media. 16S rRNA sequences were used to identify the bacterial species of the isolated strains.

Detection of biofilm formation ability of isolates

We used crystal violet staining to quantify the formation of biofilm in *E. coli* isolates and grouped isolated based on the A595 values of the stained biofilm as non-adherent, weakly, moderately, or strongly adherent (Fig 1 A). Out of the 185 samples, 56 (30.27%) had strong biofilm-forming ability, 54 (29.19%) had moderate ability, 52 (28.11%) showed weak ability and 23 (12.43%) could not form biofilm (Fig 1 B). This paper analyzed the biofilm-formation ability of *E. coli* from pigs in central China. Among the 185 strains of *E. coli* isolates, 162 strains can form biofilms and according to the biofilm-forming ability. This indicates that the biofilm formation rate of *E. coli* biofilms in central China is high. This may be caused by the long-term use of low-concentration antibiotics as growth promoters in pigs during the feed process (Chakraborty *et al.*, 2020; Pandey *et al.*, 2014). Under the pressure of low concentrations of antibiotics, *E. coli* that form biofilms were screened out, resulting in a high rate of *E. coli* biofilm formation.

Evaluation of Biofilm by Confocal Laser Scanning Microscopy (CLSM) in an Isolate

An *E. coli* isolate classified as strongly biofilm-forming ability on 96-wells polystyrene plates was selected for CLSM analysis of biofilm. As shown in Fig 2, the green fluorescence

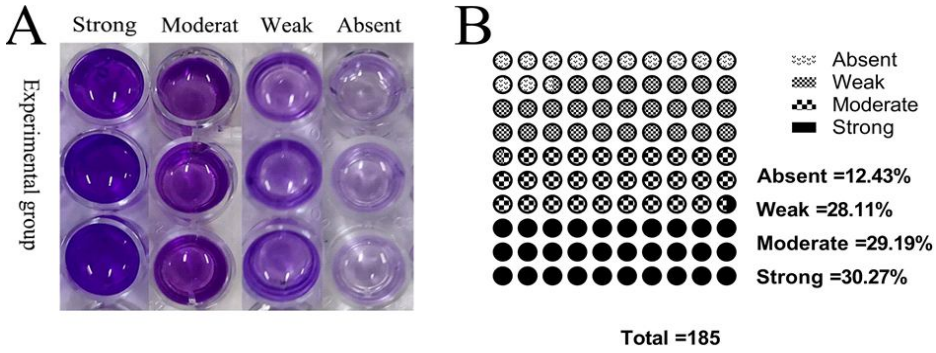

Fig 1: Biofilm-forming ability of *E. coli*. (A) The *E. coli* isolates biofilm-forming ability were classified as "strong, moderate, weak and absent" by crystal violet staining analysis. (B) Profile of biofilm-forming ability of *E. coli*.

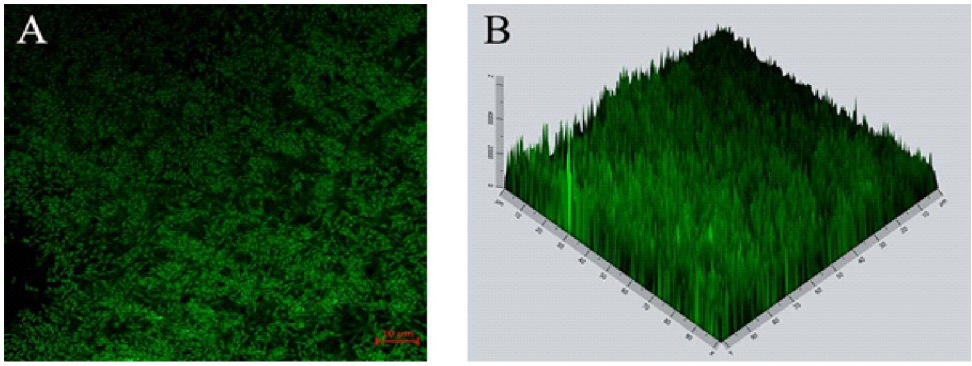

Fig 2: Confocal Laser Scanning Microscopy showing the structure (The bacterial biofilm structure is labeled as a green fluorescent band by the SYTO 9) of biofilm in *E. coli*. (A) 2D; (B) 2.5D. Magnification: 630×.

(SYTO 9) indicates the presence of biofilm and the biofilm structure and bacterial survival produced by *E. coli* isolated from a sample of diarrhea piglets. Observed by CLSM the isolates showed that the bacteria with strong biofilm-forming ability were dense clumps. In the biofilm state, the density of bacteria is high and the space between the bacteria is narrow and its three-dimensional structure can effectively protect the bacteria inside the biofilm. The extracellular matrix and the narrow space between the strains become a barrier preventing antibiotics from penetrating the biofilm.

Relationship between biofilm formation ability and phylogenetic groups of isolates

According to the biofilm formation ability, the *E. coli* strains were further classified into the following phylogenetic groups (Fig 3), strong (56): group A (10.71%, 6/56), group B1 (5.36%, 3/56), group B2 (35.71%, 20/56) and group D (48.21%, 27/56); moderate: group A (14.81%, 6/54), group B1 (7.41%, 3/54), group B2 (33.33%, 20/54) and group D (44.44%, 27/54); weak: group A (23.08%, 12/52), group B1 (19.23%, 10/56), group B2 (30.77%, 16/56) and group D (26.92%, 14/56).

We found that the biofilm formation ability of 162 isolates was different from each other and biofilm-forming ability may correlate with phylogenetic group. *E. coli* strains can be classified into four phylogenetic groups: “A” “B1” “B2” and “D” (Nowrouzian *et al*., 2005). We found that 40.12% (65/162) of the isolates belonged to group D, 33.33% (54/162) to group B2, 16.05% (26/162) to group B1 and 10.49% (17/162) to group A. These groupings served as a helpful reference for the ecological distribution and genetic diversity of *E. coli* in central China. Phylogenetic groups B2 and D are classified as pathogenic *E. coli* (Deshpande *et al*., 2015). Pathogenic strains belonging to group B2 and, to a lesser extent, group D, more frequently carry virulence factor genes compared to group A and B1 (Nowrouzian *et al*., 2005). The B2 group includes important pathogens, such as extra intestinal pathogenic, adherent-invasive and uropathogenic strains (Deshpande *et al*., 2015). In accordance to this, we found that *E. coli* isolated from dead and/or sick pigs mainly belonged to groups B2 and D (data not shown). In addition, the results of the phylogenetic group analysis showed that group B2 and D accounted for 83.93% (47/56) of the strong biofilm-forming ability group. Therefore, we can speculate that the formation of strong biofilms may be related to their strong adhesion and persistence ability.

Comparison of antibiotic resistance rates between planktonic condition and biofilms condition in different biofilm-forming ability groups (strong, moderate, weak)

To test whether the strains of different biofilm-forming ability groups in the planktonic state and the biofilm state have different resistance to antibiotic drugs, we conducted drug sensitivity assays using 8 different antibiotic drugs. In the planktonic state (Supplementary material, Table S1), there was no significant difference in the resistance rate between the different biofilm-forming ability groups and all isolates showed a low resistance rate to norfloxacin (8.02%), cotrimoxazole (26.54%), ofloxacin (33.95%), ciprofloxacin (39.51%). The resistance rate against ampicillin was 58.02% and the resistance rate for other antibiotics was between 40% to 55%. In the biofilm state (Supplementary material, Table S2), the resistance rates of the strong biofilm-forming

Table S1: Antibiotic resistance level of *E. coli* isolates in planktonic.

Antibiotics	Total (162)	resistance % (strains No)		
		Strong (n=56)	Moderate (n=54)	Weak (n=52)
Ampicillin	58.02 (94)	50.00 (28)	57.41 (31)	67.31 (35)
Aztreonam	53.09 (86)	46.43 (26)	51.85 (28)	61.54 (32)
cotrimoxazole	26.54 (43)	28.57 (16)	24.07(13)	26.92 (14)
Ofloxacin	33.95 (55)	30.36 (17)	35.19 (19)	36.54 (19)
Norfloxacin	8.02 (13)	8.93 (5)	7.41 (4)	7.69 (4)
Ciprofloxacin	39.51 (64)	37.50 (21)	42.59 (23)	38.46 (20)
Gentamicin	49.38 (80)	48.21 (27)	59.26 (32)	40.38 (21)
Kanamycin	40.74 (66)	44.64(25)	37.04 (20)	40.38 (21)

Table S2: Antibiotic resistance level of *E. coli* isolates in biofilm.

	Total (162)	resistance % (strains No)		
		Strong (n=56)	Moderate (n=54)	Weak (n=52)
Ampicillin	88.89 (144)	100.00 (56)	90.74 (49)	75.00 (39)
Aztreonam	81.48 (132)	92.86 (52)	85.19 (46)	65.38 (34)
cotrimoxazole	46.91 (76)	71.43 (40)	38.89 (21)	28.85 (15)
Ofloxacin	62.96 (102)	89.29 (50)	59.26 (32)	38.46 (20)
Norfloxacin	20.37 (33)	8.11 (20)	12.96 (7)	11.54 (6)
Ciprofloxacin	69.14 (112)	94.64 (53)	66.67 (36)	44.23 (23)
Gentamicin	83.33 (135)	96.43 (54)	88.89 (48)	63.46 (33)
Kanamycin	69.14 (112)	92.86 (52)	70.37 (38)	42.23 (22)

Table S3: The diluent used for antibiotics in this test.

Antibiotics	Diluents
Ampicillin	Phosphate Buffered Saline (PBS, PH=6.0)
Aztreonam	Phosphate Buffered Saline (PBS, PH=6.0)
Cotrimoxazole	Distilled water
Ofloxacin	Distilled water, 0.1 M NaOH
Norfloxacin	Distilled water, 0.1 M NaOH
Ciprofloxacin	Distilled water, 0.1 M NaOH
Gentamicin	Distilled water
Kanamycin	Distilled water

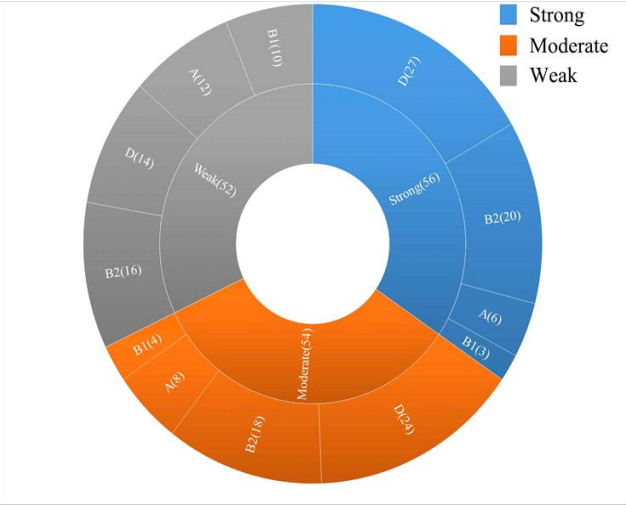

Fig 3: Phylogenetic groups of *E. coli*.Inner circle: the number of strains with different biofilm-forming ability groups (weak, moderate, strong) in the isolates; outer circle: the number of strains with different phylogenetic groups (A, B1, B2, D) in each biofilm-forming ability group.

ability group and the weak biofilm-forming ability group were the highest and the lowest, respectively. And the isolates showed a high resistance rate to ampicillin (88.89%), gentamicin (83.33%), aztreonam (81.48%). The resistance rate against norfloxacin was 20.37% and the resistance rate for other antibiotics was between 45% to 80%. Obviously, the antibiotic resistance among biofilm producing *E. coli* was significantly higher than those that did not produce biofilm ($p<0.05$) and the correlation between biofilm production and antibiotic resistance was statistically significant ($p<0.05$) for the antibiotics that were tested (Fig 4D).

Next, we investigated the change relationship of antibiotic resistance rates between planktonic condition and biofilms condition in different biofilm-forming ability groups (strong, moderate, weak) of *E. coli* isolates (Fig 4). For the strong biofilm-forming ability group (Fig 4C), when the isolate formed a biofilm, the resistance rate is significantly higher than that of the planktonic state. Among them, the resistance rate of ofloxacin increased by 58.93% and other antibiotics was increased rate between 40% to 58% besides for norfloxacin it was 26.79%. For the moderate biofilm-forming ability group (Fig 4 B), the resistance rate of norfloxacin increased by 5.56% and for other antibiotics therate increased was between 10% to 35%. As shown in Fig 4A, the weak biofilm-forming ability group had the least increase in drug resistance rate, all below 10% except gentamicin (23.08%).

The *E. coli* isolates from central China showed a high resistance rate to most antibiotics that were tested, with more than 92% of the strains showing MDR. We found that *E. coli* isolates were sensitive to quinolone and sulfonamide, such as norfloxacin and cotrimoxazole. However, the isolates showed high resistance against beta-lactam antibiotics, including ampicillin and aztreonam. The strains exhibited moderate resistance against aminoglycoside antibiotics (gentamicin, kanamycin). Tian *et al*. (2009) reported the increased prevalence of animals carrying *E. coli* isolates with reduced susceptibility to third generation cephalosporins and monobactams from 2002 to 2007 in pig farms in China. Gao *et al*. (2015) selected six pig farms located in different regions of Shandong Province, China and found that the extended-spectrum β-lactamases (ESBLs)-producing *E. coli* from all six pig farms were susceptible to amoxicillin/clavulanic acid (AMC), piperacillin/tazobactam (TZP), ampicillin/salbactam (SAM) and trimethoprim (TMP), but resistant to ampicillin (AMP) and cephalothin (CF) and highly resistant to tetracycline (TE) (Gao *et al*., 2015). *E. coli* strains collected from a large-scale swine farm in Xiamen of China were most frequently resistant to sulfonamide, trimethoprim, aminoglycoside, chloramphenicol, beta-lactam and tetracycline (Liu *et al*., 2015).

In this paper, the antibiotics susceptibility tested proved all biofilm producing *E. coli* had higher antibiotic resistance than non-biofilm producing *E. coli*. In addition, we found that *E. coli* producing strongly biofilm were significantly more resistant to antibiotics than moderately and weakly biofilm producing *E. coli* ($p < 0.05$). And the association between the ability of biofilm formation and antibiotic resistance was statistically significant ($p < 0.05$). Similarly, Pavlickova *et al*. found that 46 out of 66 antibiotic resistant isolates were able to form biofilm, showing a significant correlation between prevalence of antibiotic resistance and biofilm formation ability (Pavlickova *et al*., 2017).

It is well known that bacteria growing in a biofilm are intrinsically resistant to many antibiotics (Li *et al*., 2020). The improvement of bacterial resistance can cause the disease to prolong and the formation of biofilm is one of the important reasons for the improvement of drug resistance (Wang *et al*., 2019). Biofilm increases antibiotic resistance up to 1000 folds and high antimicrobial concentrations are required to inactivate organisms growing in a biofilm (Ciofu *et al*., 2017). This may be due to the failure of antibiotics to penetrate biofilms and the slow growth rate, altered

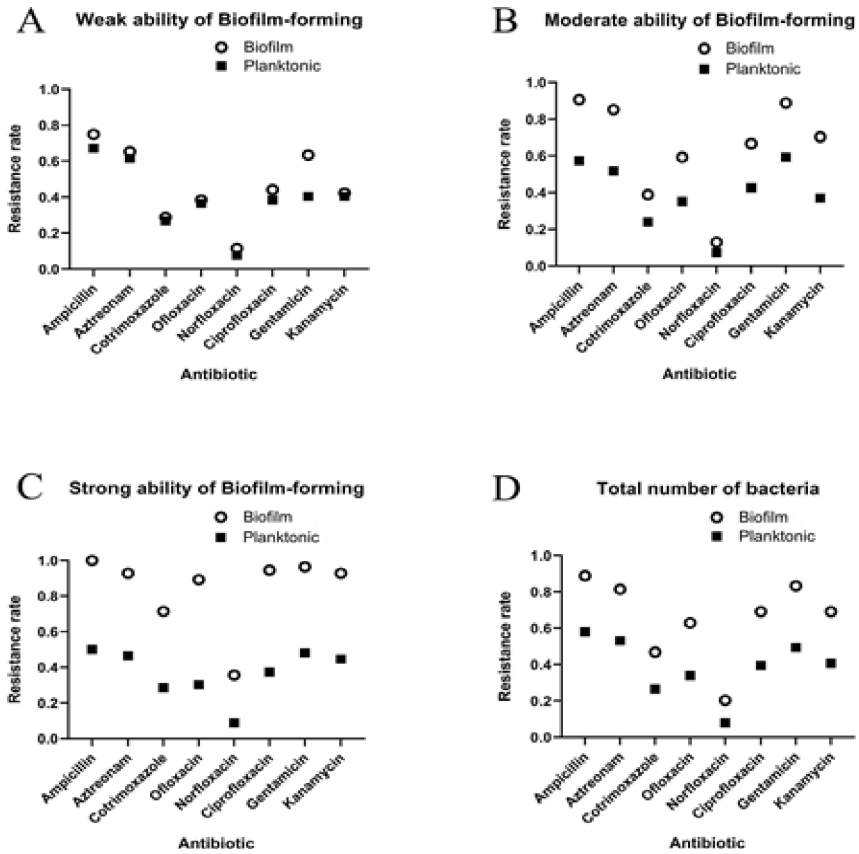

Fig 4: The relationship between antibiotic resistance and biofilm-formation ability. The antimicrobial resistance rate of isolates in different biofilm-forming ability groups (Weak, Moderate, Strong) in the planktonic state and biofilm state. In each group, the ordinate represents the drug resistance rate (the number of drug-resistant strains in this group / all strains in this group); the abscissa represents the antibiotics used in the test. (A) Antibiotic resistance rate of strains with weak biofilm formation ability. (B) Antibiotic resistance rate of strains with moderate biofilm formation ability. (C) Antibiotic resistance rate of strains with strong biofilm formation ability. (D) Antibiotic resistance rate of the total strains.

metabolism, persister cells, oxygen gradients and extracellular biofilm matrix (Venkatesan *et al*., 2015). Obviously, reducing the ability to form biofilms will be an important means to combat multi-drug resistant *E. coli*.

CONCLUSION

In this study, we investigated the correlation between biofilm production and antibiotic resistance of *E. coli* in central China. Antibiotic susceptibility testing revealed that more than 92% of the strains were MDR strains. A biofilm assay revealed that 87.57% of isolates could form biofilms. Furthermore, strongly biofilm producing *E. coli* had significantly higher antibiotic resistance than moderately and weakly producing *E. coli* ($p < 0.05$).

ACKNOWLEDGEMENT

This work was supported by the National Natural Science Foundation of China (31902309,31772761), Luoyang Normal University National project cultivation program (2014GJPY-5), Young Teacher Foundation of Luoyang Normal University (2018XJGGJS-13) and New Agricultural Research and Practice Reform Project of henan province (2020JGLX136). We are grateful to Prof. Daniel Grenier for critically read and corrected the manuscript

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