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Phylogenetic Group, Biofilm Formation and Drug Resistance of *Escherichia coli* Isolated from Goose and Fish in Henan, China

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ABSTRACT

Background: *Escherichia coli* (*E. coli*) is a pathogen that sickens both humans and animals. There are relatively few reports on the correlation between drug resistance and biofilm formation ability of *E.coli* isolated from goose and fish in water environment of China. This study investigated the genetic typing, antibiotic sensitivity and biofilm formation ability of *E. coli* strains isolated from goose and fish.

Methods: The phylogenetic clustering, drug resistance and biofilm formation ability of *E.coli* were tested by triple PCR, crystal violet microplate and Microdilution method.

Result: The results showed the prevalence of group B2 and D in fish-derived *E.coli* accounted for 59% and 12%, goose-derived *E.coli* accounted for 48% and 9%, respectively. Some strains could form biofilm and biofilm formation ability was associated with the drug resistance. The MIC values of strains growing in biofilm were 2-16 times higher than those of corresponding planktonic bacteria. The antibiotic resistance among biofilm-forming isolates was significantly higher than that strains unable to form biofilm ($p<0.05$).

Key words: Antibiotic resistance, Biofilm, *Escherichia coli*, Fish, Goose.

INTRODUCTION

Escherichia coli (*E.coli*) is a pathogen that sickens both humans and animals, often causing pneumonia, meningitis and sepsis in humans. Geese infected with *E.coli* show septicemia, balloon inflammation and granuloma and other lesions (Yeh *et al*. 2017; Yu *et al*. 2018). Fish infected with *E.coli* mainly show ulcers and congestion in the viscera (Assefa *et al*. 2019). At present, the prevention and treatment of *E.coli* disease is mainly based on antibiotics. However, with the continuous use of antibiotics, *E.coli* has developed strong drug resistance. Because the infection is difficult to cure and the spread of drug resistance, *E.coli* disease has become one of the most prevalent and widespread diseases in the world, which also poses a serious threat to human health (Geurtsen *et al*. 2022).

Bacterial biofilms are bacterial congregate membrane-like substance formed by bacteria and their exocytic secretions, which can help bacteria resist various harsh environments. Numerous studies have shown that the biofilm condition of bacteria is more resistant to drug and more able to evade the attack by the immune system than the planktonic conditionÿand this is one of the most important causes of bacterial infections (Yi *et al*. 2019). There are relatively few reports on the correlation between drug resistance and biofilm formation ability of *E.coli* from goose and fish in water environment of China. Therefore, in this study, the clinical isolates of *E.coli* from goose and fish were used as the research objects to explore the relationship between biofilm formation and drug resistance, in order to provide scientific basis for the prevention and control of *E.coli* disease from the perspective of anti-biofilm.

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MATERIALS AND METHODS

The experiment was conducted from January 2019 to August 2021 at College of Life Science, Luoyang Normal University.

Bacterial strains

There were 100 strains of goose-derived *E.coli* isolates and fish-derived *E.coli* isolated respectively, which were isolated in four different cities of Luoyang, Xinyang, Shangqiu and Zhengzhou in Henan province. The quality control strain was *E.coli* (ATCC25922). The strains were grown in Luria-Bertani broth (LB).

Reagent

Norfloxacin (NOR), Florfenicol (Nuflo), Gentamicin (GEN), Spectinomycin (SPT), Tilmicosin (TILM), Tylosin (TYL), Chlortetracycline (CTC), Tetracycline (TE), Amoxicillin (AMX), Doxycycline (DOX), Enrofloxacin (ENR) and

Trimethoprim (TMP) were purchased from Soleibao Technology Co., LTD.

Phylogenetic group identification of *E. coli*

Through the reference's methods(Clermont *et al*. 2000), three pairs of primers used for phylogenetic grouping of *E. coli*, chuA, yiaA and TSPE4.C2, were designed (Table 1). Genomic DNA was extracted from the isolates and *E. coli* was identified by multiplex PCR.

Biofilm formation ability test

The crystal violet microplate method was used for determination (Li *et al*. 2021; Liu *et al*. 2020). The critical point ODc value for judging whether the biofilm can be formed is 2 times the absorbance (OD) value of the negative control well (Wang *et al*. 2016; Yi *et al*. 2020).

Drug sensitivity test

Antibacterial activity was detected by using the microdilution method according to the CLSI standards (Andrews. 2001; Bhatia and Sharma. 2015). Among them, one strain was randomly selected from the biofilm-positive strains for analyzed the drug sensitivity of biofilm and planktonic state. The final drug concentration of wells 1-10 was 128, 64, 32, 16, 8, 4, 2, 1, 0.5 and 0.25 μg/ml. The 11th well and the 12th well were used as medium control and bacterial liquid control respectively. In addition, according to the results of drug susceptibility tests, the antimicrobial resistance spectrum of biofilm-positive strains and biofilm-negative strains was statistically analyzed.

Table 1: Primers used for phylogenetic group of *E. coli*.

Primer	Primer sequence	Primer size
ChuAP 1	5′-GACGAACCAACGGTCAGGAT-3′	279
ChuAP 2	5′-TGCCGCCAGTACCAAAGACA-3′	
YiaAP 1	5′-TGCCGCCAGTACCAAAGACA-3′	211
YiaAP 2	5′-ATGGAGAATGCGTTCCTCAAC-3′	
TspE4.C2P 1	5′-GAGTAATGTCGGGGCATTCA-3′	152
TspE4.C2P 2	5′-CGCGCCAACAAAGTATTACG-3′	

RESULTS AND DISCUSSION

Bacterial clustering

The results showed fish-derived *E. coli* isolates were classified into phylogenetic four groups: group A (8%), group B1 (21%), group B2 (59%), group D (12%). The goose-derived isolates were classified into phylogenetic four groups: group A (10%), group B1 (33%), group B2 (48%), group D (9%). The fish-derived and goose-derived *E.coli* isolates were mainly B2+D.

Phylogenetic clustering is an important method for typing *E.coli*. According to previous reports, phylogenetic clustering of *E.coli* can be divided into four groups: A, B1, B2 and D, among which B2 and D are considered to be the main pathogenic groups (Javed *et al*. 2021; Lee *et al*. 2016). Pathogenic strains belonging to group B2 and, to a lesser extent, group D, are known to carry more virulence factor genes than strains of groups A and group B1 (Nowrouzian *et al*. 2005). More specifically, the B2 phylogenetic group of *E. coli* includes important pathogens such as extraintestinal pathogenic, adherent-invasive and uropathogenic strains (Deshpande *et al*. 2015). Zhuge *et al*. discovered avian pathogenic *E. coli* and human extraintestinal infection of large intestine Most of the bacilli belong to group B2 (Zhu Ge *et al*. 2014). In this study, it was found that the *E. coli* isolated from goose and fish were dominated by group B2 and D. These data provide a helpful reference about the ecological distribution and genetic evolution of *E. coli* in the area. The results were consistent with the results of dominant evolutionary grouping of avian pathogenic *E.coli* reported by Wang Yang *et al*.(Wang *et al*. 2016). However, it was different from the research results of other researchers (Higgins *et al*. 2007; Kuczkowski *et al*. 2016), which may be due to the different animal sources, sampling areas and feeding environment.

Table 2: Relationship between phylogenetic group and drug sensitivity of *E. coli* from fish.

Antibiotics	Number of resistance strains						
	Total	A (n=8)	B1 (n=21)	B2 (n=59)	D (n=12)	A+B1 (n=29)	B2+D (n=71)
Norfloxacin/NOR	94	6	19	58	11	25	69
Florfenicol/Nuflor	94	7	20	57	10	27	67
Gentamicin/GEN	92	7	18	57	10	25	67
Spectinomycin/SPT	98	8	20	58	12	28	70
Tilmicosin/TILM	98	8	20	59	11	28	70
Tylosin/TYL	98	7	21	59	11	28	70
Chlortetracycline/CTC	90	5	18	58	9	23	67
Tetracycline/TE	84	5	16	55	8	21	63
Amoxicillin/AMX	90	7	17	57	9	24	66
Doxycycline/DOX	98	8	21	57	12	29	69
Enrofloxacin/ENR	98	7	20	59	12	27	71
Trimethoprim/TMP	100	8	21	59	12	29	71

Table 3: Relationship between phylogenetic group and drug sensitivity of *E. coli* from geese.

Antibiotics	Number of resistance strains						
	Total	A (n=10)	B1 (n=33)	B2 (n=48)	D (n=9)	A+B1 (n=43)	B2+D (n=57)
Norfloxacin/NOR	80	6	22	38	14	28	52
Florfenicol/Nuflor	48	4	19	21	4	23	25
Gentamicin/GEN	73	4	21	42	6	25	48
Spectinomycin/SPT	100	10	33	48	9	43	57
Tilmicosin/TILM	98	10	33	47	8	43	55
Tylosin/TYL	98	9	33	48	8	42	56
Chlortetracycline/CTC	94	9	30	46	9	39	55
Tetracycline/TE	98	8	33	48	9	41	57
Amoxicillin/AMX	94	10	31	46	7	41	53
Doxycycline/DOX	100	10	33	48	9	43	57
Enrofloxacin/ENR	94	8	32	47	7	40	54
Trimethoprim/TMP	96	9	32	48	7	41	55

Table 4: MIC value of antibiotics to different types of *E. coli* strains (μg/ml).

Antibiotic	Bacterial type	Fish	Goose
Tetracycline	Biofilm	16/R	4/S
	Planktonic	8/I	2/S
Aureomycin	Biofilm	128/R	64/R
	Planktonic	16/R	8/I
Spectinomycin	Biofilm	>128/R	>128/R
	Planktonic	>128/R	>128/R
Tylosin	Biofilm	>128/R	>128/R
	Planktonic	>128/R	>128/R
Enrofloxacin	Biofilm	64/R	128/R
	Planktonic	8/S	32/I
Timicosin	Biofilm	>128/R	>128/R
	Planktonic	>128/R	>128/R
Florfenicol	Biofilm	>128/R	>128/R
	Planktonic	>128/R	>128/R
Doxycycline	Biofilm	8/I	128/R
	Planktonic	2/S	64/R
trimethoprim	Biofilm	8/S	16/R
	Planktonic	2/S	2/S
Norfloxacin	Biofilm	128/R	128/R
	Planktonic	8/I	64/R
Amoxicillin	Biofilm	>128/R	>128/R
	Planktonic	>128/R	>128/R
Gentamicin	Biofilm	128/R	>128/R
	Planktonic	64/R	128/R

Notes: S: Sensitivity; I: Intermediation; R: Resistance.

Biofilm formation ability

The results of this study showed that some strains of *E.coli* isolated from goose and fish could form biofilm. Among the fish-derived *E.coli* isolates, 69% strains had biofilm formation ability, including 36% with strong ability, 33% with weak ability and 31% without biofilm formation ability. Among the goose-derived *E.coli* isolates, 60% strains had biofilm formation ability, including 42% with strong ability, 18% with weak ability and 40% without biofilm formation ability. This indicates that the biofilm formation rate of *E. coli* from goose and fish in Henan, China is high. This may be due to the long-term use of low concentrations of antibiotics in feeding (Chakraborty *et al*. 2020; Li *et al*. 2021). Earlier, other workers also reported biofilm production by *E.coli* from chicken, pig, duck and chicken products (Li *et al*. 2021; Wang *et al*. 2011; Wang *et al*. 2016). However, no report could be traced in literature on the biofilm forming ability and drug resistance by *E.coli* isolated from goose and fish.

Antibiotic sensitivity

The results of the drug sensitivity test of *E. coli* isolated from fish were shown in Table 2. All isolates showed a high resistance rate (84% - 100%). The results of the drug sensitivity test of *E. coli* isolated from goose were shown in Table 3. All isolates also showed a high resistance rate (48% -100%).The drug-resistance rate of the phylogenetic groups B2 and D was higher than the resistance rate of the phylogenetic groups A and B1. This study found that *E. coli* isolated from fish and goose had high multiple drug resistance rates. The reason may be related to different breeding environments. From the point of view of the aquaculture environment when collecting samples, the aquaculture density of fish is highly concentrated and in order to ensure the water quality of fish farming, appropriate amount of fungicides will be regularly put into the water. Long-term use of large amounts of antibacterial drugs can easily lead to high resistance rate of *E. coli* from fish. In the breeding process of geese, some antibacterial drugs will be used regularly, which will increase the resistance of *E. coli*.

At present, most of the drug susceptibility test objects are planktonic bacteria. In this study, the MIC values against planktonic and biofilm *E.coli* were detected and the results showed that the MIC values would increase with the formation of biofilm. Compared with planktonic bacteria, the MIC values of tetracycline, aureomycin, enrofloxacin,

Table 5: Resistance spectrum of biofilm-positive strains and biofilm-negative strains to antibiotics.

Multiple drug resistance spectrum	No. of resistant strains		No. of biofilm-positive strains		No. of biofilm-negative strains	
	Fish	Goose	Fish	Goose	Fish	Goose
4 and below	0	0	0	0	0	0
5	0	1	0	0	0	0
6	0	1	0	1	0	1
7	10	7	7	5	3	2
8	6	6	4	4	2	2
9	9	8	7	5	2	3
10	8	19	7	10	1	9
11	20	38	12	22	8	16
12	47	20	32	13	15	7

doxycycline, TMP trimethoprim, norfloxacin and gentamicin against biofilm fish-derived *E.coli* were increased by 2, 8, 8, 4, 4, 16 and 2 times, respectively. The MIC values of tetracycline, aureomycin, enrofloxacin, doxycycline, TMP trimethoprim and norfloxacin against biofilm goose-derived *E. coli* were increased by 2, 8, 4, 2, 8 and 2 times, respectively (Table 4). Similarly, Significant differences were observed between MICs of planktonic cells and MICs of UPEC biofilms, indicating a higher level of bacterial tolerance in biofilm form (Rafaque *et al*. 2020). Wang *et al*.found that the *E. coli* minimum biofilm eradication concentrations were generally two times higher than the planktonic minimum inhibitory concentrations (Wang *et al*. 2020).They believed that MIC would significantly increase with the formation of biofilm, which further indicated that the formation of biofilm could increase the drug resistance of bacterial strains. Therefore, biofilm is of great significance for the prevention and treatment of clinical *E.coli*. It is necessary to evaluate the bacterial status of infection during clinical medication. Especially when the clinical treatment effect is poor, drug resistance and biofilm factors need to be considered.

Correlation analysis of biofilm formation ability and drug resistance

Table 5 indicated that the isolates showed multidrug-resistance (MDR) (100%). Among fish-derived *E.coli* isolates, 90 strains were resistant to more than 8 drugs and the biofilm-positive strains accounted for 69% (62/90) and the biofilm-negative strains accounted for 31% (28/90). Among goose-derived *E.coli* isolates, 91 strains were resistant to more than 8 drugs and the biofilm-positive strains accounted for 59% (54/91) and the biofilm-negative strains accounted for 41% (37/91). The antibiotic resistance of biofilm-forming *E. coli* isolates was found to be significantly higher than that of strains unable to form biofilm ($p<0.05$). The results of this study showed the number of drug resistance of biofilm-positive strains was higher than that of biofilm-negative strains, further proving that the biofilm-forming ability of bacteria is closely related to drug resistance. Similarly, Qian *et al*. (2022) revealing that the populations that exhibited more robust biofilm formation likely contained larger proportions of extensively drug-resistant (XDR) isolates. Dumaru *et al*. found that there was strong association between the MDR-status and biofilm-production in gut bacteria (Dumaru *et al*. 2019). Katongole *et al*. demonstrated a high prevalence of biofilm-forming Uropathogenic *E. coli* strains that are highly associated with the MDR phenotype (Katongole *et al*. 2020).

CONCLUSION

The biofilm formation ability was associated with the drug resistance. In this study, phylogenetic clustering detection, biofilm formation ability and drug resistance of *E. coli* from goose and fish were studied to provide reference for the pathogenic mechanism and prevention and control of *E.coli* from goose and fish and to lay a foundation for the subsequent research on the drug resistance mechanism of *E. coli*.

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