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The Pathogenicity of Chicken Pathogenic *Escherichia coli* Is Associated with the Numbers and Combination Patterns of Virulence-Associated Genes

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Abstract

Various virulence-associated genes or pathogenicity island are responsible for determining the pathogenicity of *Escherichia coli* strains. However, the correlation of the number and combination patterns of virulence-associated genes in *Escherichia coli* strains with their pathogenicity remains largely unknown. In this work, 581 chicken *Escherichia coli* strains were isolated from 1045 liver samples of dead chickens from 50 chicken farms at four provinces in China during 2007-2012. Based on the pathogenic test of SPF chickens, 320 chickens pathogenic *Escherichia coli* isolates were identified as highly (n = 193), intermediate (n = 98) and low pathogenic (n = 29) strains, respectively. Furthermore, the number of virulence genes in the 320 chicken pathogenic and 50 non-pathogenic *Escherichia coli* strains was examined. Our results reveal that thirteen virulence genes in *Escherichia coli* strains were detected, and all strains carried at least two or more than two virulence-associated genes. This study also suggests that highly pathogenic *E. coli* strains simultaneously carried at least 8 to13 virulence genes while intermediate pathogenic strains carried at least 5 to 8 virulence genes. The number of virulence-associated genes detected in highly pathogenic strains showed there were more significant differences than that in low pathogenic strains ($P < 0.01$). The detection rate of genes *irp*2, *fyuA*, and *colV* in high pathogenic strains was significantly higher than that in low and non-pathogenic strains ($P < 0.01$). Nine virulence-asso-

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ciated genes *irp*2, *fyuA*, *iucA*, *iucD*, *iutA*, *papC*, *iss*, *tsh*, and *colV* were more often detected in highly and intermediate pathogenic *E. coli* strains. Taken together, our results provide evidences demonstrating that the pathogenicity of *Escherichia coli* strains is closely associated with the number and combination patterns of virulence-associated genes.

Keywords

Avian Pathogenic *Escherichia coli*, Pathogenicity, Virulence-Associated Genes

1. Introduction

The avian pathogenic *Escherichia coli* (APEC) infection of avian respiratory tract causes respiratory tract lesions and septicemia. This disease is referred to as air-sacculitis, pneumonitis, septicemia, and colibacillosis or coli septicemia. Colibacillosis is an important bacterial infectious disease against poultry industry [1]. In China, APEC causes broiler colibacillosis which results in serious economic losses to the poultry industry. Previous studies have suggested that virulence-associated genes and pathogenicity islands of bacteria play an important role in the pathogenicity of bacteria and that they are important parameters to clarify the mechanism of bacterial pathogenicity [2] [3]. To date, more than 25 virulence genes were detected in bacteria, such as the locus of enterocyte effacement (LEE), *ColV* and *ColBM* plasmid [4]-[7], high pathogenicity island (HPI) [8], and hemolysin A and temperature-sensitive hemagglutinin [9].

Various virulence-associated genes or pathogenicity island are responsible for determining the pathogenicity of *Escherichia coli* strains. In recent years, studies mainly focus on the types and distribution of virulence-associated genes as well as the relationship between distribution of virulence-associated genes and O serotypes. The aim of this study was to determine the relationship between pathogenicity of chicken pathogenic *Escherichia coli* and the number and combination patterns of virulence-associated genes. In the present study, 581 chicken *Escherichia coli* strains were isolated from 1045 liver samples of dead chickens from 50 poultry farms at Shaanxi, Henan, Hebei and Shanxi provinces in China during 2007-2012. Among 581 *Escherichia coli* strains tested, they were classified into high, intermediate, low pathogenic, and non-pathogenic groups. Among 320 pathogenic and 50 non-pathogenic *Escherichia coli* examined, 18 virulence-associated genes were identified by polymerase chain reaction (PCR) and sequence analysis. Our results reveal that the number and combination patterns of virulence-associated genes in *Escherichia coli* strains correlate with pathogenicity.

2. Materials and Methods

2.1. Source of Chicken *E. coli* Isolates

In the present study, a total of 581 chicken *Escherichia coli* strains were isolated from 1045 liver samples of sick and dead chickens from 50 chicken farms at Shaanxi, Henan, Hebei and Shanxi provinces in China during 2007-2012 (**Table 1**). Samples were collected from enlarge livers of sick and dead chicken in sterile condition. In the present study, 581 chicken *Escherichia coli* strains were cultured on MaConkey agar plates, and incubated at 37˚C for 18 h, and then single bacterial colony was picked up and purified. All 581 chicken *E .coli strains* were then sent to China Institute of Veterinary Drug Control for serotyping.

Table 1. Information concerning the sample collection and isolation.

Place	No. flocks	Total No. samples	No. *E.coli* isolates	Distribution of O serotype
Shaanxi	18	310	165	O1, O2, O6, O14, O16, O17, O22, O38, O62, O70, O74, O78, O85, O88, O93, O98, O101, O114, O115, O123, O127, O128, O124, O161
Henan	12	238	133	
Hebei	9	255	145	
Shanxi	11	242	138	
Total	50	1045	581	

2.2. Pathogenic Test of SPF Chickens

581 chicken *Escherichia coli* strains were cultured in LB broth at 37˚C for 24 h. All tested *Escherichia coli* strains were individually suspended in sterile saline at a concentration of 10^8 CFU/mL. In the pathogenic test, 581 groups of one-day-old White Leghorn specific-pathogen-free chickens supplied by Yangling Green Square Biological Engineering Co. (China) were used. Each group included five one-day-old White Leghorn specific-pathogen-free chickens and was reared in separate cages with food and water. Each chicken was inoculated subcutaneously with 0.2 mL of *Escherichia coli* suspension (1×10^8 CFU/per mL). Deaths were recorded 4 times per day, and continuing for 7 days. Clinical signs of illness were recorded daily. The surviving chickens were killed at 7 day post-inoculation. The lesions were observed and bacteria were isolated and identified.

The pathogenicity of *Escherichia coli* strains was determined on the basis of lesions and mortalities as described previously [10]-[12]. The animal experiments were carried out in compliance with the regulations of the Guide for the Care and Use of Laboratory Animals prepared by the international animal welfare standards. All animal experiments in this study were approved by the committee on Research Animal Care of Northwest A and F University.

2.3. PCR Amplification of Virulence-Associated Genes in Chicken *E.coli* Strains

The different sets of primers (**Table 2**) used for PCR amplification were designed with Primer 5.0 software based on the previously published sequences [13] [14]. Primers were synthesized by Nanjing GenScript Biotechnology Co. (China).

All *Escherichia coli* strains and reference strains were grown on LB agar plates at 37˚C overnight. *Escherichia coli* colonies were suspended in 500 μL of deionized water and boiling for 10 min, followed by chilling on ice for 5 min and centrifugation at 10,000 ×g for 5 min, the supernatant was used as the DNA templates for PCR amplification. The PCR mixture contained 10 μL of 2 × PCR Master mix (including 2 × Taq DNA polymerase, 2 × PCR Buffer and 2 × dNTP mixture) (TaKaRa), 1 μL of primer pair, 4 μL of DNA template, and deionized water to a final volume of 25 μL. PCR was completed by an initial heat activation of 5 min at 95˚C; then 30 cycles of 30 s at 94˚C, 30 s at Tm, and 45 s at 72˚C; and an extension of 10 min at 72˚C. PCR products were separated by size by 1% agarose gel electrophoresis along with DL2000 DNA markers and visualized after staining with ethidium bromide on a UV transilluminator. PCR products amplified from the virulence genes in *Escherichia coli* strains were sequenced and their sequences were compared with previously published sequences by using DNAStar software.

2.4. Combination of Virulence-Associated Genes and Their Correlations with Pathogenicity

Chi-square test were used to analyze the numbers and combination of virulence-associated genes in 320 chicken pathogenic and 50 non-pathogenic *Escherichia coli* strains and to analyze the numbers and combination of virulence-associated genes and their correlations with pathogenicity.

3. Results

3.1. Pathogenic Test

Among 581 *Escherichia coli* strains examined, 320 strains caused the death of chicken, suggesting that they are pathogenic *Escherichia coli strains*; 261 strains did not cause the death of chicken, suggesting that they are non-pathogenic *Escherichia coli* strains. Pathogenic test indicated that 320 chicken *E. coli* strains were classified into high , intermediate , and low pathogenicity groups based on the lesions and mortality of experimentally infected chickens [12]. Pathogenic test shows that the percentage of high pathogenic, intermediate pathogenic and low pathogenic strains was 60.3% (193/320), 30.6% (98/320), 9.1% (29/320), respectively.

3.2. Detection of Virulence-Associated Genes in Pathogenic *Escherichia coli* Strains by PCR and Sequence Analysis

Among 18 virulence-associated genes examined, 13 virulence-associated genes *irp*2, *fyuA*, *iucA*, *iucD*, *iutA*, *FimA*, *FimC*, *papC*, *iss*, *hlyA*, *tsh*, *colV*, and *colBM* in 581 *Escherichia coli* strains were detected by PCR. The

Table 2. Primers used in this study.

Gene	Description	PCR products (bp)	Primer sequence (5′to 3′)[a]
		pTJ100-related genes	
iucA	Aerobactin production	236	F-CGCGAGCGGCTCATACAGG R-TCGTCGGGCAGCGTTTCT
iucD	The aerogenes gene	400	F-AGTTCTATCGCTTCCTTAC R- GAGACCCAGTTTATTTCC
iutA	Ferric aerobactin receptor gene	424	F-AACAAACCGATGATGAAACG R-GTGCCAGCCTCAAACTCC
iss	Increased serum survival gene	756	F-GTTCTCCGTCGGGCTACT R-GCTCTGCGTGATGATGTT
tsh	Temperature-sensitive hemagglutinin gene	488	F-ACGGTCAATAATGAACTCG R-CAGGAATATGCACCTCCC
		Iron-related genes	
*irp*2	Iron repressible gene	236	F-CGCGAGCGGCTCATACAGG R-TCGTCGGGCAGCGTTTCT
fyuA	Gene of the pesticin receptor of Yersinia	235	F-ACCGTTATCGCCATTCTG R-CTGTGAAGTCTGGGCATTAG
		Adhesins-related genes	
papA	Genes encoding parts of the P pilus	374	F-GCTCCAACTATTCCACAG R-TTCAGGGTATTAGCATCAC
papC	Genes encoding parts of the P pilus	234	F-GGGCGTGATAACGATTC R-ATTTGCCAGCGGACTAC
eaeA	Encoding outer membrane protein intimin	827	F-GCGTTACATTGACTCCC R-CATTGCTACCACCTTGC
FimA	Genes encoding parts of the F1 pilus	352	F-CAGGTTCGTACCGCATCG R-TCGCATCCGCATTAGCAG
FimC	The Type 1 fimbrial adhesin	337	F-GCCGATGGTGTAAAGGAT R-CCGTCAGGTAATAGGGTGT
		Toxins-related genes	
vat	Toxin gene	861	F-TAAATGAGGTGGGCTGTG R-AGGATGCCTCCGTAAACT
stx1	Shiga toxin 1	374	F-GCCATTCGTTGACTACTT R-CTCATCAGATGCCATTCT
*stx*2	Shiga toxin 2	240	F-ACTGTCTGAAACTGCTCC R-TGACATTCTGGTTGACTCT
hlyA	Transport gene of the hemolysin operon	290	F-TTGGGATACGCTGATAGG R-CACCCTTGACTAATAACTCG
		Other factors	
colV	ColV production	307	F-ACGGATGCTCAGTTTCT R-TGTGCTTGGCGTCATAG
colBM	Colicins B and colicins M	226	F-GAGCCTGCTGTCACCCTT R-GTTCAGATAATGCCCGATG

[a]F: forward; R: reverse.

specific PCR products of expected sizes were revealed by agarose gel electrophoresis (**Figure 1** and **Figure 2**). The sequences of these virulence-associated genes were determined and deposited with GenBank accession numbers, as shown in **Table 3**. Sequence analysis revealed that these 13 virulence-associated gene sequences detected in this study show high identity with those of published genes (**Table 3**).

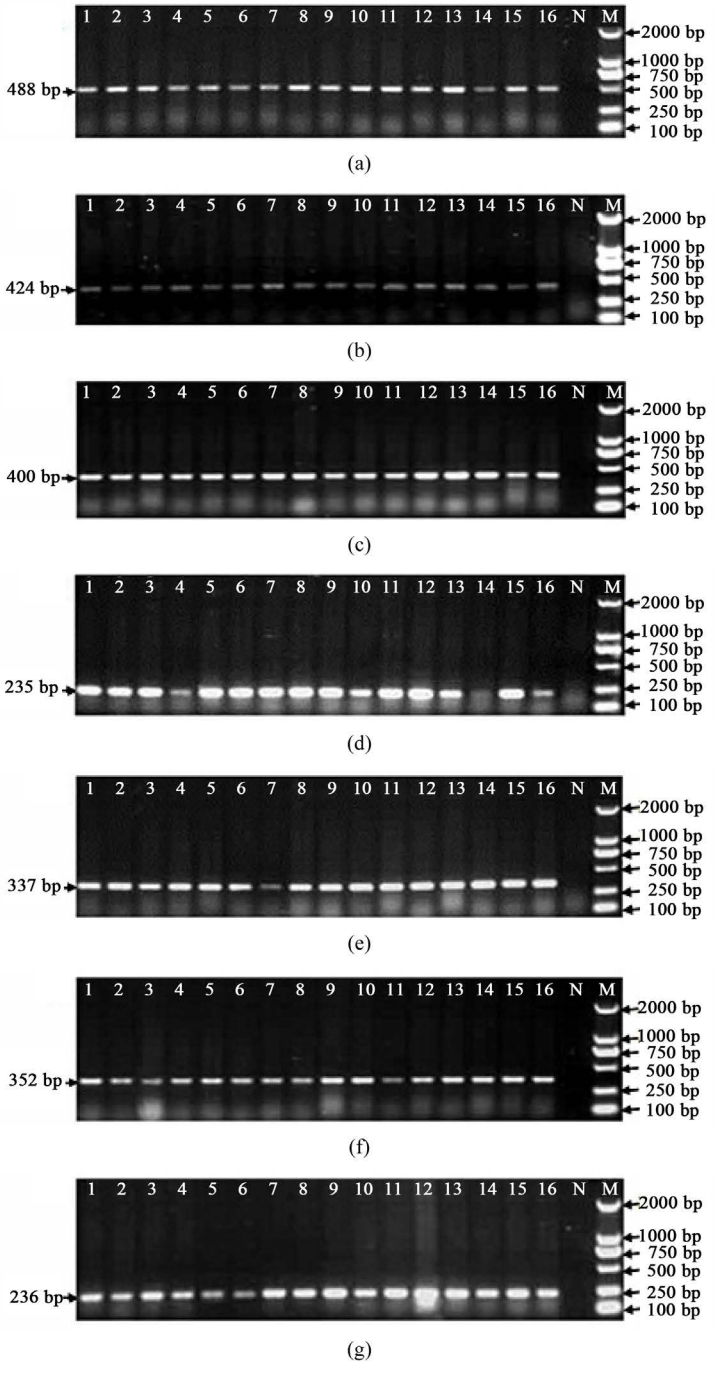

Figure 1. PCR amplification of 7 virulence-associated genes in *E. coli* strains. The specific PCR products of expected sizes were revealed by agarose gel electrophoresis. Representative electrophoretograms for PCR products amplified from each pathogen were shown. Lane M, DL 2000 marker; Lane N, Negative control. Panel (a), lanes 1 - 16: strains carried *tsh*; Panel (b) lanes 1 - 16: strains carried *iutA*; Panel (c), lanes 1 - 16: strains carried *iucD*; Panel (d), lanes 1 - 16: strains carried *fyuA*; Panel (e), lanes 1 - 16: strains carried *FimC*; Panel (f), lanes 1 - 16: strains carried *FimA*; Panel (g), lanes 1 - 16: strains carried *irp*2.

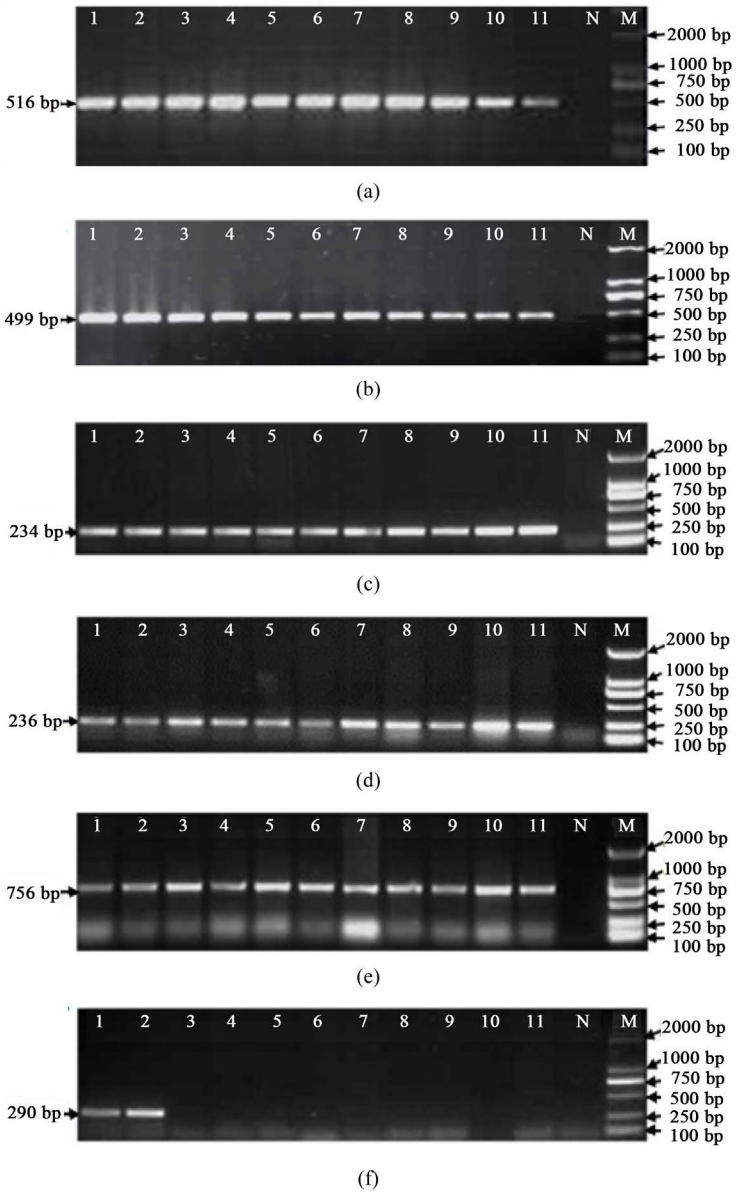

Figure 2. PCR amplification of 6 virulence-associated genes in *E. coli* strains. The specific PCR products of expected sizes were revealed by agarose gel electrophoresis. Representative electrophoretograms for PCR products amplified from each pathogen were shown. Lane M, DL 2000 marker; Lane N, Negative control. Panel (a), lanes 1 - 11: strains carried *colBM*; Panel (b), lanes 1 - 11: strains carried *colV*; Panel (c), lanes 1 - 11: strains carried *papC*; Panel (d), lanes 1 - 11: strains *iucA*; Panel (E), lanes 1 - 11: strains carried *iss*; Panel (f), lanes 1-2: strains carried *hlyA*; lanes 3 - 11, *hlyA* gene not detectable.

3.3. Detection of 13 Virulence-Associated Genes in Pathogenic and Non-pathogenic *Escherichia coli* Strains

Among 320 pathogenic and 50 non-pathogenic *Escherichia coli* strains examined, at least 2 to 13 genes were detected (**Table 4**). By examining 18 virulence-associated genes, the *papA*, *vat*, *eaeA*, *stx*1, and *stx*2 genes were not detectable in this study. Among the 13 virulence-associated genes detected, the most prevalent is *colBM* (100%) and the least one is *hlyA* (0.6%) (**Table 4**).

Our results reveal that the number of virulence genes in 193 high pathogenic *Escherichia coli* strains was significantly higher than that of 127 intermediate and low pathogenic strains ($P < 0.01$). Furthermore, the detection rates of the virulence genes for *irp*2, *fyuA*, *iutA*, *papC*, *iss*, *colV*, and *iucD* in 291 high and intermediate pa-

Table 3. Sequence homology of 13 virulence-associated genes in chicken *E. coli* strains compared with published sequences.

Virulence-associated genes	Isolate sequences (accession no.)[a]	Published sequences (accession no.)	Percentage of sequence homology (%)
*irp*2	JX485631	EU120030.1	98.9% - 99.4%
fyuA	JX466849	Z38064.1	99.1% - 99.4%
iucA	JX466845	X76100.1	98.5% - 99.3%
iucD	JX466843	NC009837.1	98.9% - 99.1%
iutA	JX466848	NC009837.1	99.2% - 99.8%
FimA	JX466846	NC000913.2	98.7% - 99.4%
FimC	JX466847	FJ866110.1	99.4% - 99.6%
papC	JX485632	HQ165752.1	98.4% - 99.5%
hlyA	JX466842	FM180012.1	99.2% - 99.6%
iss	JX466844	X52665.1	95.8% - 99.6%
Tsh	JX466850	AY280856.1	98.5% - 99.2%
colV	KC447292	AY545598.5	98.4% - 99.3%
colBM	KC447290	NC009837.1	98.4% - 99.3%

[a]Sequences determined in this study.

Table 4. Detection of virulence-associated genes in pathogenic and non-pathogenic strains.

Virulence genes	Detection of virulence genes (%) (No. of positive strains/ No. of total strains)				
	High pathogenic strains (n = 193)	Intermediate pathogenic strains (n = 98)	Low pathogenic strains (n = 29)	Total	Non-pathogenic strains (n = 50)
*irp*2	87.6 (169/193)	44.9 (44/98)	0.0	66.6 (213/320)	0.0
fyuA	90.2 (174/193)	70.4 (69/98)	20.7 (6/29)	77.8 (249/320)	0.0
iucA	68.4 (132/193)	38.8 (38/98)	34.5 (10/29)	53.1 (170/320)	30 (15/50)
iucD	80.8 (156/193)	70.4 (70/98)	34.5 (10/29)	73.8 (236/320)	20 (10/50)
iutA	85.5 (165/193)	54.1 (53/98)	24.1 (7/29)	70.3 (225/320)	20 (10/50)
FimA	77.2 (149/193)	46.9 (46/98)	41.4 (12/29)	64.7 (207/320)	60 (30/50)
FimC	92.7 (179/193)	86.7 (85/98)	75.9 (22/29)	90.9 (291/320)	70 (35/50)
papC	31.1 (60/193)	4.1 (4/98)	0.0	20.0 (64/320)	0.0
iss	72.5 (140/193)	34.7 (34/98)	13.8 (4/29)	55.6 (178/320)	0.0
hlyA	1.0 (2/193)	0.0	0.0	0.6 (2/320)	0.0
tsh	83.4 (161/193)	29.6 (29/98)	27.6 (8/29)	61.8 (198/320)	16 (8/50)
colV	85.5 (165/193)	50.0 (49/98)	0.0	66.9 (214/320)	0.0
colBM	100.0 (193/193)	100.0 (98/98)	100.0 (29/29)	100.0 (320/320)	100 (50/50)

thogenic *Escherichia coli* strains were significantly higher than that of 29 low pathogenic and non-pathogenic *Escherichia coli* strains ($P < 0.01$). The detection rate of the virulence genes for *iucA*, *fimA*, and *tsh* in intermediate and low pathogenic *Escherichia coli* strains shows no significant difference ($P > 0.05$). The detection rate of virulence genes for *FimC* and *colBM* in three different pathogenic *Escherichia coli* strains shows no significant difference ($P > 0.05$). Interestingly, the hemolysin *hlyA* gene was detected only in highly pathogenic chicken *Escherichia coli*.

3.4. Combinations of Virulence-Associated Genes and Their Correlations with Pathogenicity

By examining 193 highly pathogenic *Escherichia coli* strains, it was found that all strains simultaneously carried at least 8 to13 virulence genes. As shown in **Table 5**, 14 strains (7.3%) simultaneously carried 12 virulence genes, causing the death of 4 - 5 chickens within 24 hours; 85 strains (44%) simultaneously carried 10 or 11 virulence genes, causing the death of 4 - 5 chickens within 72 hours. Among 13 virulence genes detected in 193 highly pathogenic *Escherichia coli* strains, a total of 51 different combinations of virulence-associated genes were observed. It was found that 134 of 193 (69.43%) highly pathogenic *Escherichia coli* strains belonged to 10 predominant patterns as indicated in **Table 5**. The most prevalence of combination patterns of virulence-associated genes (*irp*2 + *fyuA* + *iucA* + *iucD* + *iutA* + *fimA* + *fimC* + *iss* + *tsh* + *colV* + *colBM and rp*2 + *fyuA* + *iucA* + *iucD* + *iutA* + *fimA* + *fimC* + *papC* + *iss* + *tsh* + *colV* + *colBM*) in highly pathogenic *Escherichia coli* strains were 22.3% and 7.3%, respectively (**Table 5**).

By examining 98 intermediate pathogenic *Escherichia coli* strains, 12 virulence genes were detected in these strains which simultaneously carried at least 5-8 virulence genes. In the present study, 36 different combination patterns from these 12 virulence genes in 98 intermediate pathogenic *Escherichia coli* strains were found and 60.2% (59/98) of them belonged to the 7 predominant patterns (**Table 5**). The most prevalence combination patterns of virulence-associated genes (*irp*2 + *fyuA* + *iutA* + *fimC* + *iss* + *colV* + *colBM and irp*2 + *fyuA* + *iucD* + *fimA* + *fimC* + *colV* + *colBM*) in intermediate pathogenic *Escherichia coli* strains were 13.2% and 10.2%, respectively (**Table 5**).

By assessing 29 low pathogenic *Escherichia coli* strains, 9 virulence genes were detected in these strains which simultaneously carry at least 2 to 4 virulence genes. Twelve different combinations from these 9 virulence genes were observed, and 62% (18/29) of them belonged to 4 predominant patterns (**Table 6**). The most prevalence combination patterns of virulence-associated genes (*iucD* + *iutA* + *fimC* + *colBM* and *fimA* + *fimC* + *colBM*) in low pathogenic *Escherichia coli* strains were 20.7% and 17.2%, respectively (**Table 6**). Furthermore, there are 7 virulence genes that were detected in 50 non-pathogenic *Escherichia coli* strains, and each carries at least 2 to 4 virulence genes. Four different combinations from 7 virulence genes were seen, and 70% (14/20) of them belong to 2 predominant patterns (**Table 6**). The major combination patterns of virulence-associated genes (*colBM* + *fimA* and *colBM* + *fimC* + *tsh*) in non-pathogenic *Escherichia coli* strains were 20.7% and 30%, respectively (**Table 6**).

Table 5. The predominant patterns of virulence genes in 193 high pathogenic and 98 intermediate pathogenic strains.

193 high pathogenic strains		98 intermediate pathogenic strains	
Combination patterns	Detection (%) (No. of positive strains/No. of total strains)	Combination patterns	Detection (%) (No of positive strains/No. of total strains)
colBM + *irp*2 + *fyuA* + *iucA* + *iucD* + *iutA* + *fimA* + *fimC* + *tsh* + *colV* + *iss*	22.3 (43/193)	*colBM* + *irp*2 + *fyuA* + *iutA* + *fimC* + *iss* + *colV*	13.2 (13/98)
colBM + *irp*2 + *fyuA* + *iucA* + *iucD* + *iutA* + *fimA* + *fimC* + *tsh* + *colV* + *papC* + *iss*	7.3 (14/193)	*colBM* + *irp*2 + *fyuA* + *iucD* + *fimA* + *fimC* + *colV*	10.2 (10/98)
colBM + *irp*2 + *fyuA* + *iucA* + *iucD* + *iutA* + *fimA* + *fimC* + *tsh* + *colV*	6.7 (13/193)	*colBM* + *irp*2 + *fyuA* + *iucA* + *iucD* + *fimC*	8.2 (8/98)
colBM + *irp*2 + *fyuA* + *iucA* + *iucD* + *fimA* + *fimC* + *papC* + *iss* + *tsh* + *colV*	6.2 (12/193)	*colBM* + *iucD* + *iutA* + *fimC* + *tsh*	8.2 (8/98)
colBM + *irp*2 + *fyuA* + *iutA* + *fimA* + *fimC* + *papC* + *tsh* + *colV*	5.7 (11/193)	*colBM* + *fyuA* + *iucA* + *iucD* + *fimA* + *fimC* + *colV*	7.1 (7/98)
colBM + *irp*2 + *fyuA* + *iutA* + *iucD* + *fimA* + *fimC* + *iss* + *tsh* + *colV*	4.7 (9/193)	*colBM* + *fyuA* + *iucD* + *fimA* + *fimC* + *iss*	7.1 (7/98)
colBM + *irp*2 + *fyuA* + *iutA* + *iucD* + *fimA* + *fimC* + *iss* + *colV*	4.7 (9/193)	*colBM* + *irp*2 + *fyuA* + *iucA* + *fimC*	6.1 (6/98)
colBM + *irp*2 + *fyuA* + *iucA* + *iucD* + *iutA* + *fimC* + *papC* + *iss* + *tsh* + *colV*	4.1 (8/193)		
colBM + *irp*2 + *fyuA* + *iucD* + *fimA* + *fimC* + *iss* + *tsh* + *colV*	4.1 (8/193)		

Table 6. The predominant patterns of virulence genes in 29 low pathogenic and 20 non-pathogenic strains.

29 low pathogenic strains		50 non-pathogenic strains	
Combination patterns	Detection (%) (No. of positive strains/No. of total strains)	Combination patterns	Detection (%) (No. of positive strains/No. of total strains)
colBM + *fimC* + *iucD* + *iutA*	20.7 (6/29)	*colBM* + *fimC* + *FimA* + *iucA*	20.0 (10/50)
colBM + *fyuA* + *fimC* + *tsh*	10.3 (3/29)	*colBM* + *fimA* + *iucD* + *iutA*	10.0 (5/50)
colBM + *fimA* + *fimC*	17.2 (5/29)	*colBM* + *fimC* + *tsh*	30.0 (15/50)
colBM + *fimA* + *tsh*	13.8 (4/29)	*colBM* + *fimA*	40.0 (20/50)

Among 320 *Escherichia coli* strains examined, the detection rate of high pathogenicity island genes (*irp*2, *fyuA*, and *colV)* in high pathogenic strains were significantly higher than those in low and non-pathogenic strains ($P < 0.01$) (**Table 7**). Nine virulence-associated genes *irp*2, *fyuA*, *iucA*, *iucD*, *iutA*, *papC*, *iss*, *tsh*, and *colV* were significantly more often detected in high and intermediate pathogenic *E. coli* strains (**Table 7**). Furthermore, our results revealed that the combination patterns from five or more than five genes in high and intermediate pathogenic *E. coli* strains were highly correlated with its pathogenicity ($P < 0.01$).

4. Discussion

In recent years, virulence-related genes or pathogenicity island were found in many pathogenic bacteria, and this may be related to the evolution of bacterial virulence. For example, high pathogenicity island (HPI) is firstly discovered in *Yersinia*, but it also widely exists in *Escherichia coli* of human, pigs, cattle and rabbit [15]-[20]. Both *irp*2 and *fyuA* genes are closely associated with HPI, and can be used as the marker in HPI detection [21]. A previous report by Hu *et al*. suggested that the detection rate of *irp*2 in pathogenic *Escherichia coli* of human was 10.0% [22]. More recently, Smith *et al*. reported that the detection rate of *fyuA* in pathogenic *Escherichia coli* strains of pigs was 15.9% [23]. In the present study, the higher detection rates of genes for *irp*2 and *fyuA* (66.6% and 77.8%) in chicken pathogenic *Escherichia coli* were observed. Furthermore, our results reveal that chicken pathogenic *Escherichia coli* strains simultaneously carried *irp*2 *and fyuA* genes were up to 60.3% (193/320). The detection rate of these HPI gene combination in highly chicken pathogenic strains were 80.8% (156/193), showing significant difference ($P < 0.01$) than that in intermediate (37/98, 37.8%) and low (0/29, 0%) pathogenic strains. The present study provides evidences suggesting that HPI pathogenicity islands and its related genes are closely associated with the pathogenicity of chicken *Escherichia coli* strains.

Pilus is the main virulent factor that is involved in adhesion of pathogenic *Escherichia coli* to the host cells, including types I and P pilus. Previous study suggested that type I pili existed mainly in pathogenic *Escherichia coli* strains, and it existed more widely in chicken pathogenic *Escherichia coli* than P pili [24]. Both *FimA* and *FimC* genes are responsible for encoding essential protein of biosynthesis process of type I pili while *PapA* and *papC* are important functional genes of type P pili that encode the main component proteins of type P pili. A previous study by Galli *et al*. reported that the detection rate of *FimA* gene was 92.8% in bovine *E. coli* strains [25]. It was also reported that 60.0% of *FimC* gene were detected in chicken *E. coli* among isolates tested [26]. In the present study, the detection rate of *FimA* gene (64.4%) is lower than that reported by Galli *et al*. [25] whereas *FimC* gene (89.7%) is higher than that reported by Wang *et al*. 2004. A previous investigation found that 6.5% of chicken pathogenic *Escherichia coli* isolates carried *papC* gene [27]. It is interesting to note that the higher detection rate of this gene (15.9%) than that reported by Jin *et al*. [27] was seen, but the *papA* gene in chicken pathogenic *Escherichia coli* strains was not detected in all tested strains.

ColV plasmid widely exists in avian pathogenic *Escherichia coli* strains which correlate with the strain virulence. It contains a variety of virulence genes, such as gas bacillus gene *iucA*, *iucD* and *iutA*, Iss protein gene *iss*, hemolysin A gene *hlyA* and temperature-sensitive hemagglutinin gene *tsh*. They are important virulence genes related to pathogenicity of *Escherichia coli*, and mainly exist in the *ColV* plasmid [28]. *ColBM* plasmid is a newly discovered *E. coli* virulence factor, which may evolve from *ColV* plasmid, and it possesses virulence genes that are similar to *ColV* [29]. Our study reveals that the detection rate of *ColBM* gene (100%) in chicken pathogenic *Escherichia coli* strains is much higher than that of *ColV* gene (66.6%). The reason why the detection rate showing big difference needs to be further studied.

Table 7. Eight of the highest rate of virulence gene combinations in high pathogenic and intermediate pathogenic strains.

193 high pathogenic strains		98 intermediate pathogenic strains	
Combination patterns	Detection (%) (No of positive strains/No. of total strains)	Combination patterns	Detection (%) (No of positive strains/No. of total strains)
colBM + *irp2* + *fyuA* + *fimC* + *colV*	82.9 (160/193)	*colBM* + *fimC*	86.7 (85/98)
colBM + *irp2* + *fyuA* + *fimC* + *colV* + *iutA*	82.4 (159/193)	*colBM* + *fimC* + *fyuA*	68.4 (67/98)
colBM + *irp2* + *fyuA* + *fimC* + *colV* + *tsh*	81.3 (157/193)	*colBM* + *fimC* + *iucD*	66.3 (65/98)
colBM + *irp2* + *fyuA* + *fimC* + *colV* + *iucD*	79.3 (153/193)	*colBM* + *fimC* + *fyuA* + *iucD*	60.2 (59/98)
colBM + *irp2* + *fyuA* + *fimC* + *colV* + *fimA*	76.2 (147/193)	*colBM* + *fimC* + *iutA*	52.0 (51/98)
colBM + *irp2* + *fyuA* + *fimC* + *colV* + *iss*	71.5 (138/193)	*colBM* + *fimC* + *colV*	50.0 (49/98)
colBM + *irp2* + *fyuA* + *fimC* + *colV* + *iucA*	68.4 (132/193)	*colBM* + *fimC* + *irp2*	43.9 (43/98)
colBM + *irp2* + *fyuA* + *fimC* + *colV* + *iutA* + *iucD*	72.0 (139/193)	*colBM* + *fimC* + *fimA*	43.9(43/98)

At present, studies mainly focus on the types and distribution patterns of virulence-related genes [27]. In the present study, we show the correlations between the numbers and combination patterns of virulence-related genes and their pathogenicity in pathogenic *E. coli* strains. The number and combination patterns of virulence-related genes detected in highly and intermediate chicken pathogenic strains showed more significant difference than those in low and non-pathogenic strains ($P < 0.01$). Our results reveal that there are at least 8 to 13 virulence-related genes and 51 different combination patterns from these genes in the highly pathogenic chicken *E. coli* strains and at least 5 to 8 virulence-related genes and 36 different combination patterns from the genes in the intermediate pathogenic chicken *E. coli* strains, suggesting that the number and combination patterns of these virulence-related genes are significantly correlated with the pathogenicity ($P < 0.01$). This study provides an important insight into the correlation between the number and combination patterns of virulence-associated genes and *E. coli* pathogenicity.

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