Virulence-Associated Genes and Genetic Diversity of Avian Pathogenic (APEC) and Fecal (AFEC) E. Coli Isolates from Chickens

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Research

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Abstract

Background: Avian pathogenic E. coli (APEC) is the etiologic agent of serious colibacillosis and causes extensive economic losses. To examine the genetic background of APEC, we characterized the serotypes, virulence genes, phylogenetic classification and MLST of 392 APEC and 586 AFEC strains isolated from infected chickens.

Results: The results showed that the most predominant serotypes were O78 (13.47%), O2 (9.16%), O18 (5.39%), O20 (4.42%) and O25 (4.09%). The major serotypes O78 (13.47%) and O2 (9.16%) were significantly higher in the APEC isolates than in the AFEC isolates. Among the 16 analyzed virulence-associated genes (VAGs), *iroN* (100%), *ompT* (100%), *fimC* (92.46%), *iss* (77.91%) and *irp2* (71.98%) were the most frequently identified. Over half (54.85%) of the strains possessed > 8–13 VAGs, and 85.23% of the strains carried *iroN-ompT-fimC-iss/irp2* VAG patterns. According to the phylogenetic analysis, phylogroups A (32.11%) and B2 (31.36%) proved to be the most prevalent phylogenetic groups in the AFEC and APEC isolates, respectively. The strains that belonged to phylogroup B2 were associated with more VAGs. Based on MLST, 46 STs belonging to 15 different clonal complexes were identified, and 4 were novel. ST88 (10.67%) was found to be the most dominant ST, and it possessed at least 9 VAGs and belonged to phylogroups B2 or D. Furthermore, the isolates belonging to B2-O78/O2-ST88 were the most likely APEC isolates to be associated with epidemics, and they carried more VAGs than the other strains.

Conclusions: Our findings have enriched our knowledge of the molecular characteristics of APEC isolates from chickens, which will be important for the prevention and control of avian colibacillosis.

Introduction

Avian pathogenic Escherichia coli (APEC) has been reported as an etiologic agent of colibacillosis in poultry worldwide [1–3]. APEC isolates can cause severe respiratory and systemic infections that lead to a significant economic burden in the poultry industry due to increased mortality, medication costs, and condemnation of carcasses in the prevention and control of the disease [4]. Avian colibacillosis is characterized by yolk sac infection, swollen-head syndrome, septicemia, and inflammation, such as pericarditis, perihepatitis, airsacculitis, salpingitis, arthritis, and peritonitis, of different organs or a combination of these syndromes [5].

APEC infects all ages of commercial poultry and is considered a major or minor pathogen that accounts for 3–4% of the mortality of birds on a farm; however, the mechanisms underlying infection and systemic displacement by APEC are ill defined. Elucidation of the underlying molecular mechanisms of APEC pathogenicity is essential for controlling avian colibacillosis.

Recently, multiple virulence-associated genes (VAGs) have been identified that contribute to APEC pathogenesis [6]. These VAGs are involved in different steps of infection and/or adaptation, and their functions can be classified as adhesins, invasins, toxins, iron acquisition systems, and protectins/serum resistance [7–8], thereby protecting the pathogen from the host immune response and enabling its extraintestinal existence [7]. The nature and the combination of the VAGs of APEC could determine the degree of virulence and their potential to cause specific diseases in specific hosts. However, the importance and interaction of specific VAGs that determine the pathogenesis of APEC infections are still poorly understood [9]. Furthermore, some avian fecal E. coli (AFEC) isolated from healthy poultry may also carry certain VAGs reflecting their virulence potential.

Previous observational studies have shown that a large number of different serotypes of E. coli, such as O1, O2, O5, O6, O7, O9, O18, O24, O25, O36, O54, O78, O100, and O115, are known to be detected in poultry [10–11]. Although several APEC serotypes are responsible for avian colibacillosis, the most commonly encountered APECs in different countries and on different farms are O1, O2, and O78 [12].

Other techniques, including phylogroup and multilocus sequence typing (MLST), are established tools to type APEC. E. coli isolates may be classified into four main phylogroups: A, B1, B2, and D. Among them, strains from the B2 and D phylogroups are most frequently found in APEC, while commensal intestinal strains commonly belong to the A and B1 groups [13]. MLST is a top-level genetic tool for differentiating E. coli that allows the assignment of closely related strains in clonal groups or complexes as a sequence type (ST) [14]. These standardized classifications have facilitated the identification and monitoring of pandemic strains that cause nosocomial and community outbreaks [15–16].

Several studies have described APEC strains in the literature; however, little information is available on the characteristics of avian E. coli, especially their serotype level, VAGs and molecular characterization. Therefore, the objective of this study was to determine the genetic background of highly pathogenic E. coli isolates of avian colibacillosis outbreaks and compare this information with that of AFEC strains obtained from healthy poultry.

Results

Isolate collections

In this study, a total of 928 *E. coli* strains were used to study the VAGs and genetic diversity among APEC and AFEC isolates. Among them, 392 APEC isolates were collected from tissues of freshly dead chickens with suspected colibacillosis, and 536 AFEC isolates with VAGs were collected from fresh chickens.

Serotyping of the isolates

All confirmed E. coli isolates were serotyped, and 28 different serotypes were identified among the E. coli strains using multiplex PCR (Table 1). Nevertheless, 6.12% and 9.70% of the E. coli isolates were not typeable as APEC and AFEC serotypes, respectively. All 28 serotypes were found in the AFEC strains, while O21, O36 and O91 were absent in the APEC strains.

Table 1
Serogroup distribution of the APEC and AFEC strains.

Serogroup	Total n = 928		APEC n = 392		AFEC n = 536		p-value
	Number	Percentage	Number	Percentage	Number	Percentage	
O1	26	2.80%	18	4.59%	8	1.49%	0.005
O2	85	9.16%	72	18.37%	13	2.43%	< 0.001
O4	30	3.23%	1	0.26%	29	5.41%	< 0.001
O5	29	3.13%	4	1.02%	25	4.66%	0.002
O6	24	2.59%	13	3.32%	11	2.05%	0.231
O8	26	2.80%	21	5.36%	5	0.93%	< 0.001
O9	35	3.77%	1	0.26%	34	6.34%	< 0.001
O11	22	2.37%	2	0.51%	20	3.73%	0.001
O15	17	1.83%	8	2.04%	9	1.68%	0.685
O18	50	5.39%	45	11.48%	5	0.93%	< 0.001
O20	41	4.42%	26	6.63%	15	2.80%	0.005
O21	31	3.34%	0	0.00%	31	5.78%	< 0.001
O24	31	3.34%	1	0.26%	30	5.60%	< 0.001
O25	38	4.09%	1	0.26%	37	6.90%	< 0.001
O36	18	1.94%	0	0.00%	18	3.36%	< 0.001
O45	17	1.83%	4	1.02%	13	2.43%	0.115
O54	18	1.94%	2	0.51%	16	2.99%	0.007
O65	31	3.34%	11	2.81%	20	3.73%	0.53
O78	125	13.47%	106	27.04%	19	3.54%	< 0.001
O86	24	2.59%	1	0.26%	23	4.29%	< 0.001
O91	11	1.19%	0	0.00%	11	2.05%	0.004
O100	21	2.26%	2	0.51%	19	3.54%	0.002
O103	21	2.26%	2	0.51%	19	3.54%	0.002
O115	20	2.16%	9	2.30%	11	2.05%	0.801
O128	9	0.97%	6	1.53%	3	0.56%	0.136
O147	29	3.13%	4	1.02%	25	4.66%	0.001
O149	20	2.16%	6	1.53%	14	2.61%	0.263
O157	3	0.32%	2	0.51%	1	0.19%	0.392
Not typeable	76	8.19%	24	6.12%	52	9.70%	0.041

The most predominant serotypes were O78 (13.47%), O2 (9.16%), O18 (5.39%), O20 (4.42%) and O25 (4.09%) (Fig. 1A). However, the most prevalent serotypes among the APEC and AFEC strains were significantly different. Of the serotypes tested, O78 and O2 were the most frequently observed in the APEC strains, accounting for 27.04% and 18.37%, respectively. O18 (11.48%), O20 (6.63%) and O8 (5.36%) were the next most frequent. These serotypes in the APEC stains were significantly higher than those in the AFEC stains (Fig. 1B). However, O25 (6.90%), O9 (6.34%), O21 (5.78%), O4 (5.41%) and O24 (5.41%) were the top five prevalent serotypes among AFEC strains and were significantly higher in the AFEC isolates than in the APEC isolates ($P < 0.0001$) (Fig. 1C).

Prevalence of virulence-associated genes

The frequencies and combinations of 16 VAGs in 928 E. coli strains (392 APEC and 536 AFEC) were assessed by PCR. The prevalence of each gene in the APEC and AFEC isolates is shown in Table 2. The iron acquisition system gene *iroN* and protectin/serum resistance gene *ompT* were carried by all of the detected strains. More than 70% of the isolates also carried *fimC* (92.46%), iss (77.91%) and *irp2* (71.98%). Gene *ibeA* was not detected in any strain, and *papC* and *neuC* were detected less frequently, with 1.94% and 4.74%, respectively. Compared with AFEC, all the VAGs, except *iroN*, *ompT* and *ibeA*, were significantly more prevalent in the APEC strains ($P < 0.01$).

Table 2
Prevalence of virulence-associated genes among the APEC and AFEC strains.

virulence-associated genes		Total (n = 928)		APEC(n = 392)		AFEC(n = 536)		p-value
		Number	Percentage	Number	Percentage	Number	Percentage	
Adhesins	*fimC*	858	92.46%	375	95.66%	483	90.11%	0.002
	papC	18	1.94%	17	4.34%	1	0.19%	<0.001
	tsh	579	62.39%	292	74.49%	287	53.54%	<0.001
	mat	576	62.07%	316	80.61%	260	48.51%	<0.001
Invasins	*ibeA*	0	0.00%	0	0.00%	0	0.00%	-
Toxins	*astA*	198	21.34%	115	29.34%	83	15.49%	<0.001
	vat	166	17.89%	111	28.32%	55	10.26%	<0.001
	hlyF	211	22.74%	123	31.38%	88	16.42%	<0.001
Iron acquisition systems	*iroN*	928	100.00%	392	100.00%	536	100.00%	-
	fyuA	482	51.94%	285	72.70%	197	36.75%	<0.001
	iucD	448	48.28%	251	64.03%	197	36.75%	<0.001
	irp2	668	71.98%	342	87.24%	326	60.82%	<0.001
Protectins/Serum resistance	*iss*	723	77.91%	344	87.76%	379	70.71%	<0.001
	neuC	44	4.74%	37	9.44%	7	1.31%	<0.001
	ompT	928	100.00%	392	100.00%	536	100.00%	-
	cva/cvi	135	14.55%	85	21.68%	50	9.33%	<0.001

Based on the different combinations of VAGs, the E. coli isolates were divided into 27 virulence gene profile types (VTs) (Table 3). Each strain possessed at least 3 different VAGs, and over half (54.85%) of the strains possessed > 8–13 VAGs.

Although all the VTs were present in the APEC and AFEC strains, there were obvious differences in the distribution. Most VTs (VT14, VT18, VT20, VT22, VT24, VT26 and VT27) that carried ≥ 9 VAGs were significantly higher in the APEC isolates than in the AFEC isolates ($p < 0.01$). In contrast, VTs (VT1-VT4) carrying ≤ 4 VAGs were significantly more prevalent in the AFEC isolates ($P < 0.05$). However, a clear difference in VT6, VT7, VT8, VT10, VT13, VT19 and VT23 prevalence between the APEC and AFEC isolates was not found ($P > 0.1$).

Table 3
The prevalence of virulence gene types in the APEC and AFEC strains.

VT	Virulence genes profiles	Total (n = 928)		APEC(n = 392)		AFEC(n = 536)		p-value
		Number	Percentage	Number	Percentage	Number	Percentage	
1	*iroN-ompT-tsh*	21	2.26%	3	0.775%	18	3.36%	0.009
2	*iroN-ompT-fimC-iss*	117	12.61%	20	5.10%	97	18.10%	<0.001
3	*iroN-ompT-fimC-irp2*	68	7.33%	21	5.36%	47	8.77%	0.049
4	*iroN-ompT-fimC-tsh*	84	9.05%	15	3.83%	69	12.87%	<0.001
5	*iroN-ompT-irp2-tsh*	32	3.45%	9	2.30%	23	4.29%	0.1
6	*iroN-ompT-fimC-iss-iucD-hlyF*	21	2.26%	7	1.79%	14	2.61%	0.403
7	*iroN-ompT-iss-mat-iucD-hlyF*	17	1.83%	5	1.28%	12	2.24%	0.28
8	*iroN-ompT-fimC-iss-irp2-tsh-mat*	39	4.20%	13	3.32%	26	4.85%	0.25
9	*iroN-ompT-fimC-iss-irp2-mat-cva/cvi*	20	2.16%	4	1.02%	16	2.99%	0.042
10	*iroN-ompT-fimC-iss-irp2-tsh-mat-astA*	9	0.97%	2	0.51%	7	1.31%	0.222
11	*iroN-ompT-fimC-iss-irp2-tsh-fyuA-iucD*	9	0.97%	1	0.26%	8	1.49%	0.057
12	*iroN-ompT-fimC-iss-irp2-mat-fyuA-iucD*	22	2.37%	3	0.775	19	3.54%	0.006
13	*iroN-ompT-fimC-iss-irp2-mat-hlyF-vat*	6	0.65%	1	0.26%	5	0.93%	0.203
14	*iroN-ompT-fimC-iss-irp2-tsh-mat-fyuA-iucD*	107	11.53%	65	16.58%	42	7.84%	<0.001
15	*iroN-ompT-fimC-iss-irp2-tsh-mat-iucD-hlyF*	12	1.29%	7	1.79%	5	0.93%	0.256
16	*iroN-ompT-fimC-iss-irp2-mat-fyuA-iucD-hlyF*	11	1.19%	2	0.51%	9	1.68%	0.104
17	*iroN-ompT-fimC-iss-irp2-mat-fyuA-iucD-astA*	67	7.22%	37	9.44%	30	5.60%	0.026
18	*iroN-ompT-fimC-iss-irp2-tsh-mat-fyuA-iucD-hlyF*	26	2.80%	19	4.85%	7	1.31%	0.001
19	*iroN-ompT-fimC-iss-irp2-tsh-mat-fyuA-hlyF-cva/cvi*	5	0.54%	1	0.26%	4	0.75%	0.313
20	*iroN-ompT-fimC-iss-irp2-tsh-mat-fyuA-astA-vat*	79	8.51%	52	13.27%	27	5.04%	<0.001

VT	Virulence genes profiles	Total (n = 928)		APEC(n = 392)		AFEC(n = 536)		p-value
		Number	Percentage	Number	Percentage	Number	Percentage	
21	*iroN-ompT-fimC-iss-irp2-tsh-mat-fyuA-iucD-hlyF-vat*	28	3.02%	11	2.81%	17	3.17%	0.748
22	*iroN-ompT-fimC-iss-irp2-tsh-mat-fyuA-iucD-hlyF-cva/cvi*	32	3.45%	23	5.87%	9	1.68%	0.001
23	*iroN-ompT-fimC-iss-irp2-tsh-mat-fyuA-iucD-astA-cva/cvi*	25	2.69%	10	2.55%	15	2.80%	0.828
24	*iroN-ompT-fimC-iss-irp2-tsh-mat-fyuA-iucD-astA -neuC*	18	1.94%	14	3.57%	4	0.75%	0.002
25	*iroN-ompT-fimC-iss-irp2-tsh-mat-fyuA-iucD-hlyF-vat-cva/cvi*	9	0.97%	7	1.79%	2	0.37%	0.03
26	*iroN-ompT-fimC-iss-irp2-tsh-mat-fyuA-iucD-hlyF-vat-cva/cvi-neuC*	26	2.80%	23	5.87%	3	0.56%	<0.001
27	*iroN-ompT-fimC-iss-irp2-tsh-mat-fyuA-iucD-hlyF-vat-cva/cvi-papC*	18	1.94%	17	4.345	1	0.19%	<0.001

The frequencies of VAGs in most VTs showed a regular and cumulative pattern, and most strains had similar evolutionary steps; 85.23% of the strains carried *iroN-ompT-fimC-iss/irp2* VAG patterns, and 62.21% of the strains possessed *iroN-ompT-fimC-iss-irp2-tsh/mat* VAG patterns. Hypothetical steps of VAG acquisition were illustrated according to VAG frequencies among the VTs (Fig. 2). The VT1 strains could evolve into VT4 and VT5 strains by acquiring *fimC* and *irp2*, respectively. VT2 and VT3 could evolve into VT8 by acquiring *irp2-tsh-mat* and *iss-tsh-mat*, respectively. VT8, by acquiring *fyuA-iucD*, could evolve into VT14, and VT11 and VT12 could also evolve into VT14 by acquiring *mat* and *tsh*, respectively. VT14 further evolved into VT18, VT21, VT22, VT25, VT26 and VT27 by obtaining corresponding VAGs. VT8 strains that had not acquired *iucD* later evolved into VT10, VT19 and VT20 through the acquisition of *astA*, *fyuA-hlyF-cva/cvi* and *fyuA-astA-vat*, respectively.

Phylogenetic Classification analysis

The phylogenetic analysis showed that phylogroups A and B2 proved to be the most prevalent phylogenetic groups, with 32.11% and 31.36%, respectively, followed by phylotypes D (18.64%) and B1 (17.89%). Within the APEC strains, phylogroup B2 was the largest and contained over half (59.95%) of the strains, while for the AFEC strains, 47.95% belonged to phylotype A and showed the most common groups (Table 4).

Table 4
Phylogenetic groups of APEC and AFEC strains.

Phylogroup	Total (n = 928)		APEC (n = 392)		AFEC (n = 536)		p-value
	Number	Percentage	Number	Percentage	Number	Percentage	
A	298	32.11%	41	10.46%	257	47.95%	P<0.001
B1	166	17.89%	22	5.61%	144	26.87%	P<0.001
B2	291	31.36%	235	59.95%	56	10.45%	P<0.001
D	173	18.64%	94	23.98%	79	14.74%	P<0.001

The strains that belonged to phylogroups B2 and D were associated with more virulence genes, presenting an average of 9.64 and 9.13 virulence genes for each strain, respectively, which was significantly higher than that for the B1 (5.68) and A (5.18) groups (Table S1).

MLST-based genotype analysis

MLST was performed to analyze the genotypic diversity of E. coli isolates based on 7 housekeeping genes. According to MLST, 928 strains were divided into 46 STs that belonged to 15 different clonal complexes, and 4 were novel and found in this study (Fig. 3, Table S2). ST88 (10.67%) was found to be the most dominant ST, followed by ST243 (8.94%), ST461 (7.76%), and ST142 (7.22%), while five STs, ST65, ST118, ST122, ST141, and ST298, were less prevalent and were represented in only one AFEC strain. ST142 was the most common ST in the AFEC strains but was absent in the APEC strains. ST461, ST88 and ST243 were the three most common ST types among the APEC strains, accounting for 11.48%, 10.97% and 9.95%, respectively. Furthermore, all of these strains possessed at least 9 VAGs and belonged to phylogroups B2 or D.

The serotype and STs of the strains exhibited epidemic preferences (Fig. 4). ST88 was the most predominant ST among the O78 and O2 serotypes, and all the APEC strains with ST88 belonged to these two serotypes. Eleven different STs of O78 strains were found, and the top five STs were ST88 (29.60%), ST243 (20.00%), ST461 (15.20%), ST131 (10.40%), and ST85 (8.80%), which were also the most dominant STs of the APEC strains. Eighty-five O2 strains were divided into 17 STs, and ST254 (18.82%) and ST855 (12.94%) were the most common STs, except ST88. ST254 was the most common ST in the APEC strains but was missing in the AFEC strains among the O2 serotypes.

Discussion

Colibacillosis is caused by APEC and is considered one of the serious threats to the poultry industry and public health. APEC infections in birds cause many different kinds of clinical manifestations, ranging from respiratory tract infections to swollen head syndrome, which leads to death [12]. Although previous studies have reported the epidemic characteristics and pathogenic mechanism of APEC strains, detailed data on serotype, VAGs and molecular characteristics are often unavailable in many regions of China. Given that the zoonotic potential of APEC strains is still questionable, a considerable number of strains were isolated from chickens affected with colibacillosis, and several characteristics were compared between APEC and AFEC isolates in the present study.

APEC isolates often have diverse serotypes, and some overlapping serogroups are commonly detected in AFEC isolates. Although different O serogroups have been associated with colibacillosis, certain specific serogroups (O78, O2, and O1) are more frequently reported than others [11, 17]. Here, O78 was the most predominant serogroup, followed by O2, which is similar to the results from previous studies. O1, however, was the sixth most frequently observed serotype among the

APEC isolates in this investigation, and it was significantly higher than that in the AFEC isolates. O18 and O8 were also major serogroups, although the results have been controversial in different studies [12, 18].

APEC strains usually carry a large number of VAGs with different functions, and more VAGs in the same strain are often detected among E. coli from lesions [19–20]. Similar results were presented in our findings, and significantly more APEC isolates than AFEC isolates were collected from lesions. O-antigen is well proven to be the virulence factor of E. coli and can protect the bacteria from clearance by the neutrophils and macrophages of the host [21]. It is clear from the results of this study and previous evidence that serogroups O78 and O2 often possess more virulence and are considered virulent. However, different strains from the same serotype may vary in their virulence [18].

APEC strains are characterized by the possession of several VAGs, which enable these bacteria to survive an extraintestinal life and to cause colibacillosis [22–23]. In recent years, the mechanisms behind the pathogenesis and epidemic characteristics of VAGs of APEC strains have been extensively studied [12, 18]. Some essential virulence genes, *iroN* (siderophore), *ompT* (outer membrane protease), *iss* (serum survival) and *hlyF* (hemolysin), carried by plasmids are considered preferential molecular markers for APEC [12, 24]. APEC isolates from poultry clinically diagnosed with colibacillosis were positive for at least one of these VAGs, and the frequencies vary greatly in different studies [11].

Sixteen VAGs were detected in the current study, and the frequencies of most VAGs were similar to those in these previous reports [11]. Among those VAGs, the frequencies of *iroN* and *ompT* were higher, while the frequencies of *iucD*, *fyuA* and vat were lower. Subedi et al. [25] reported an identical result: the virulence genes *iroN* and *ompT* were harbored by each APEC isolate, but *iss* and *hlyF* were higher than those in the present study. We also found that all the AFEC isolates (carrying VAGs) were positive for both *iroN* and *ompT*.

It is speculated that the association of several different VAGs could increase the pathogenicity of bacteria [20]. The functions of virulence genes tested in the present study are well documented, and accumulation of these genes may be a potential risk factor for APEC infection. The epidemic characteristics of VAGs constitute unique VTs and evolution steps. Therefore, monitoring VTs with multiple VAGs in different hosts and understanding the evolution steps may be helpful for reducing economic losses in the poultry industry and the potential zoonotic risks of APEC strains[26].

Phylogenetic classification and MLST have several important advantages over PFGE, including shorter assay times, better standardization, and repeatability of data among laboratories. Epidemiological surveys in most previous studies have classified APEC strains as predominantly phylogroup B2, followed by phylogroup D, which are mainly responsible for extraintestinal infections and possess more VAGs [27]. The B2 group is closely related to pathogenicity and is frequently found among serogroups O78 and O2 isolates [28]. In the present study, the APEC isolates belonging to B2-O78/O2-ST88 were the most associated with epidemics and carried more VAGs than the other strains. Our studies have suggested that there is a relationship among different E. coli phylogenetic groups, STs, serogroups and the virulence capabilities of the strains.

In conclusion, this study demonstrated the prevalence of common VAGs in APEC and AFEC strains recovered from colibacillosis tissue and fresh tissue. Our studies suggested that different VAGs have accumulated in APEC strains. However, the presence of these VAGs in AFEC isolates poses a potential risk of causing colibacillosis. Furthermore, the identification of predominant serotypes and molecular characteristics, which are closely related to pathogenicity, may be particularly useful in the diagnostic approach. Thus, regular screening and monitoring of APEC strains is essential for implementing intervention programs to reduce the risk of colibacillosis.

Materials And Methods

Sample collection and bacterial isolation

Animal-based active surveillance was conducted for 1568 tissue swab samples (liver, heart, lung and spleen), and 1867 fresh samples from infected chickens with typical lesions of an E. coli infection were collected from 26 different farms in Hebei Province from 2018 to 2020. All the samples were cultured on MacConkey agar overnight at 37°C, and pink-colored suspected bacterial colonies were further isolated on LB agar. For each sample, only one colony was isolated and used for subsequent examination.

The pure and presumptive positive strains were then confirmed as E. coli via biochemical analysis using the API20E system (bioMerieux, Marcy-l'Etoile, France) according to the manufacturer's recommendations. The isolates were stored at − 80°C in 25% glycerol.

Preparation of DNA templates

The DNA templates for PCR (serotype, VAGs, phylogroup, MLST) were directly extracted from bacterial colonies using the boiled lysate method. Briefly, a single colony from an overnight culture at 37°C on LB agar was suspended in 30 μL sterile molecular grade water and boiled at 100°C for 10 min. The sample was immediately cooled on ice for 5 min and centrifuged at 13,000 g at 4°C for 10 min. The supernatant, containing DNA, was transferred to a fresh tube for use [29].

Serological characterization

Serotyping characterization was carried out using a multiplex PCR method for analyzing 162 different O antigens within 20 groups [30]. Multiplex PCR was performed as follows: each 30 μL reaction mixture contained 2 μL DNA template, 0.5 μL of each primer, 10 μL Taq MasterMix (Takara, Japan) and deionized water to a final volume of 30 μL.

Detection of virulence-associated genes

All strains were tested by PCR for the presence of 16 virulence-associated genes [8, 11, 25, 31, 32, 33], including adhesins (*fimC*, *papC*, *tsh*, *mat*), invasins (*ibeA*), toxins (*astA*, *vat*, *hlyF*), iron acquisition systems (*iroN*, *fyuA*, *iucD*, *irp2*), and protectins/serum resistance (*iss*, *neuC*, *ompT*, *cva/cvi*). PCRs were performed according to published protocols, and the primer sequences are listed in Table S3.

Phylogenetic Classification

The E. coli isolates were assigned to four phylogenetic groups (A, B1, B2, or D) based on three genes, *ChuA* and *YjaA* and an anonymous DNA fragment, *TSPE4.C2*, and a rapid and simple method as previously described [34], and the primers are shown in Table S4. The *chuA* and *TspE4.C2*-negative and *TspE4.C2*-positive E. coli strains were classified as groups A and B2, respectively, and *chuA*-negative and *TspE4.C2*-positive, *chuA*-positive and *yjaA*-negative E. coli strains were grouped into B1 and D (Fig S1), respectively.

Multilocus sequence typing (MLST)

MLST analysis of 7 housekeeping genes was performed for each isolate according to the protocols described on the EcMLST website (http://www.shigatox.net/ecmlst). The PCR amplification conditions of the 7 housekeeping genes were as follows: 95℃ for 5 min; 30 cycles of 94 ℃ for 30 s, 55 ℃ for 90 s, and 72 ℃ for 1 min; and 72 ℃ for 5 min with ExTaq DNA polymerase (Takara, Japan). The PCR products were bidirectionally sequenced, and the sequences of the 7 housekeeping genes were edited by using SeqMan 7.0. Each unique allele was assigned a different number, and the allelic profile (string of seven allelic loci) was used to define each isolate's sequence type (ST) [35]. Clustering analysis was used to infer relationships among the isolates using the fingerprint analysis software BioNumerics (version 7.1).

Statistical Analysis

The data for the APEC and AFEC isolates were analyzed using a chi-square test to find any significant differences. These differences were considered statistically significant when $P < 0.05$.

Declarations

Ethics approval and consent to participate

Ethical approval was granted for this study. Our study was conducted according to the Ethics Committee of Animal Experiments at the Institute of Husbandry and Pharmaceutical Sciences of Chinese Academy of Agricultural Sciences in Lanzhou, China. We gained consent from the owners of the animals for use in the study.

Consent for publication

All the authors agreed to the publication of the paper.

Availability of data and material

The data supporting the findings of this study are contained within the manuscript.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

Z.Z. and J.Y.Z. designed the study; Z.Z., M.Z.C., Q.Q.Z., Y.X.S., X.Z.Z., C.Y.W. and G.B.C. generated and provided the dataset; Z.Z., M.Z.C., W.W.W., Y.Y.Z. and G.B.C. performed the experiments, analyzed the data, and wrote the manuscript. All authors have read and approved the final manuscript.

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Figures

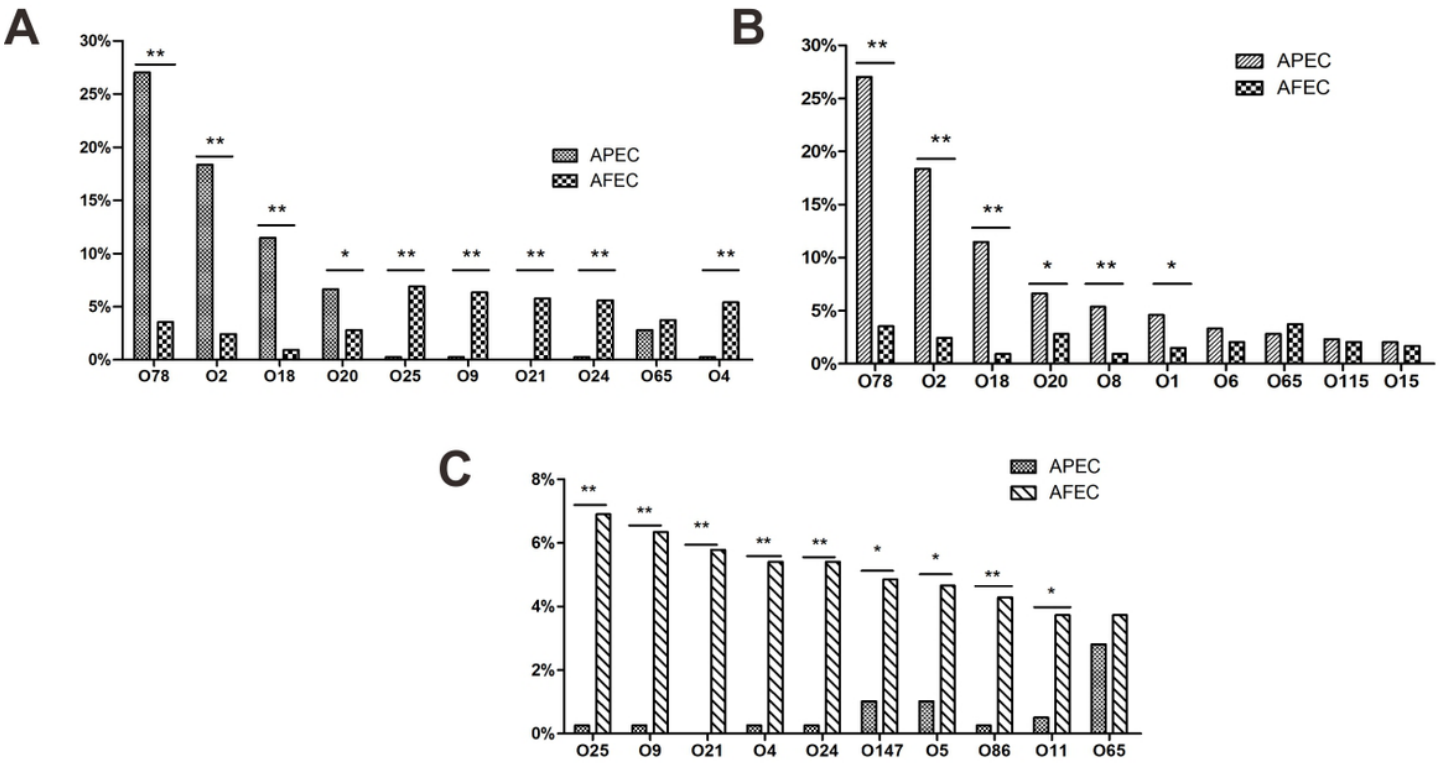

Figure 1

Distribution of the most predominant serotypes in E. coli. A: The 10 most predominant serotypes in total strains; B: The 10 most predominant serotypes in the APEC strains; C: The 10 most predominant serotypes in the AFEC strains.

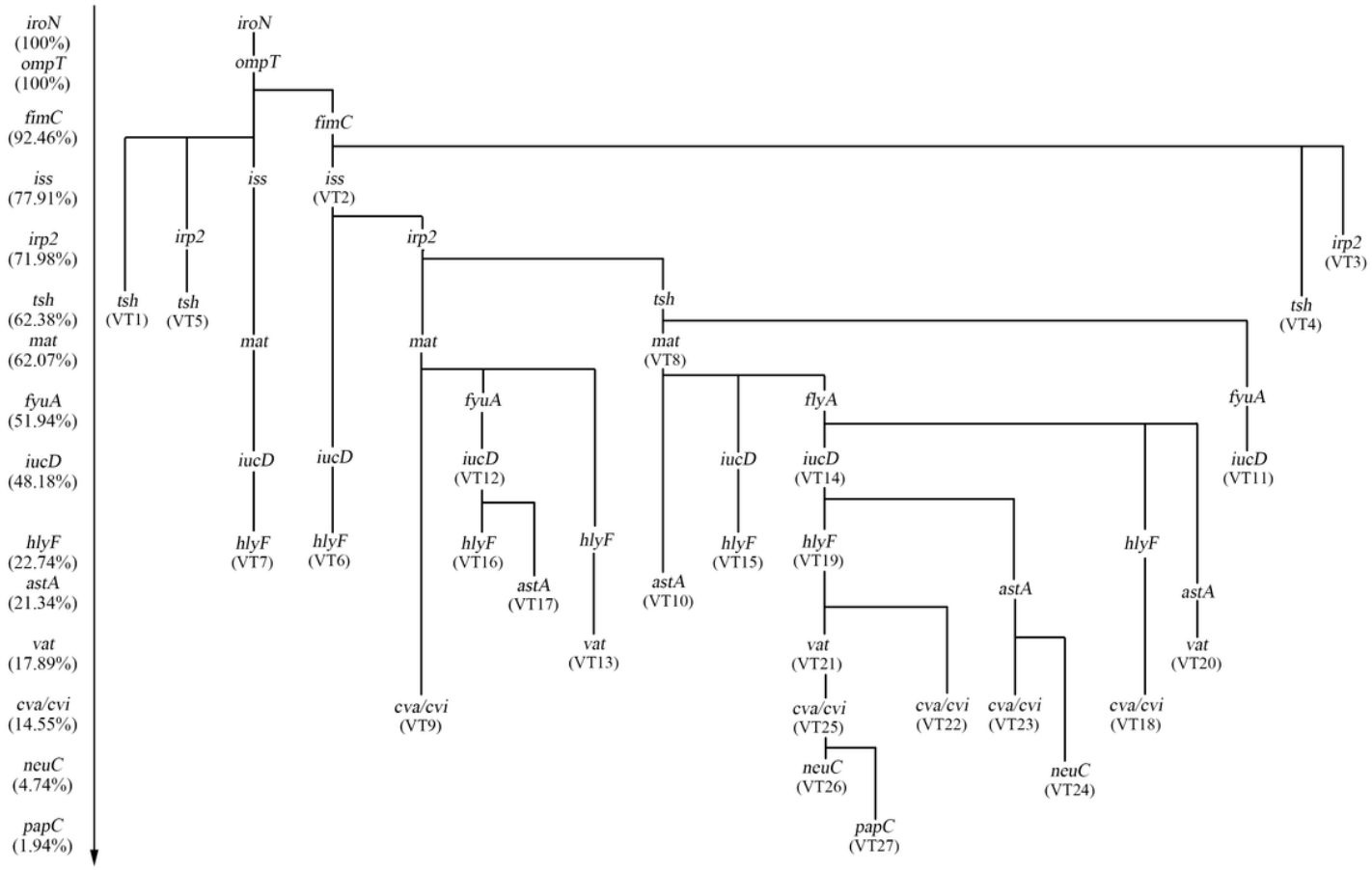

Figure 2

Accumulation of virulence genes and evolution of VTs in E. coli. According to the virulence gene frequencies among the VTs, the hypothetical steps of virulence gene acquisition were illustrated.

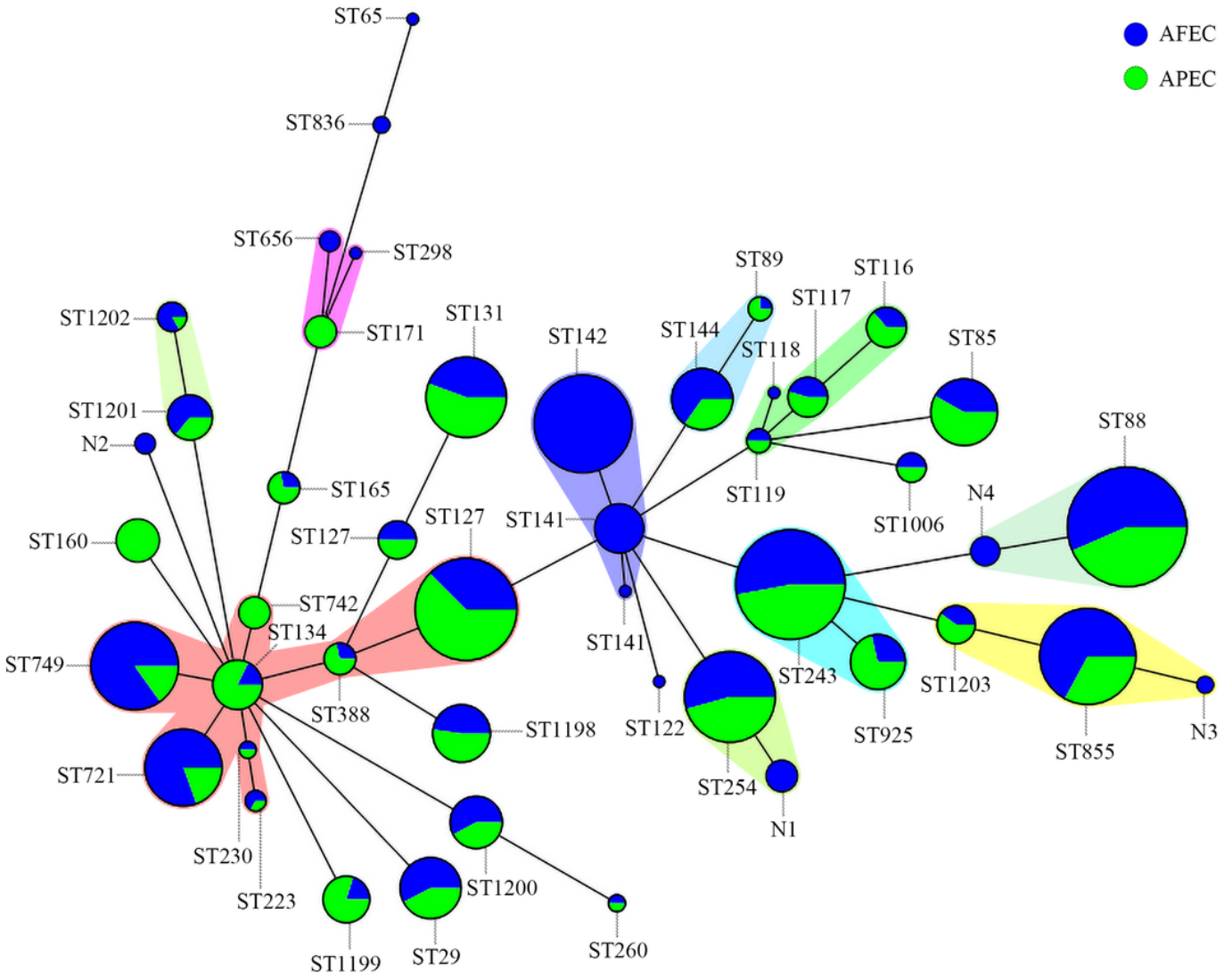

Figure 3

Minimum spanning tree of the 938 E. coli isolates from chickens based on multilocus sequence typing (MLST). The minimum spanning tree was constructed using the 7 identified STs obtained from the 938 isolates using BioNumerics Software. Each circle corresponds to a single ST. The shadow zones in different colors correspond to different E.coli (APEC or AFEC).

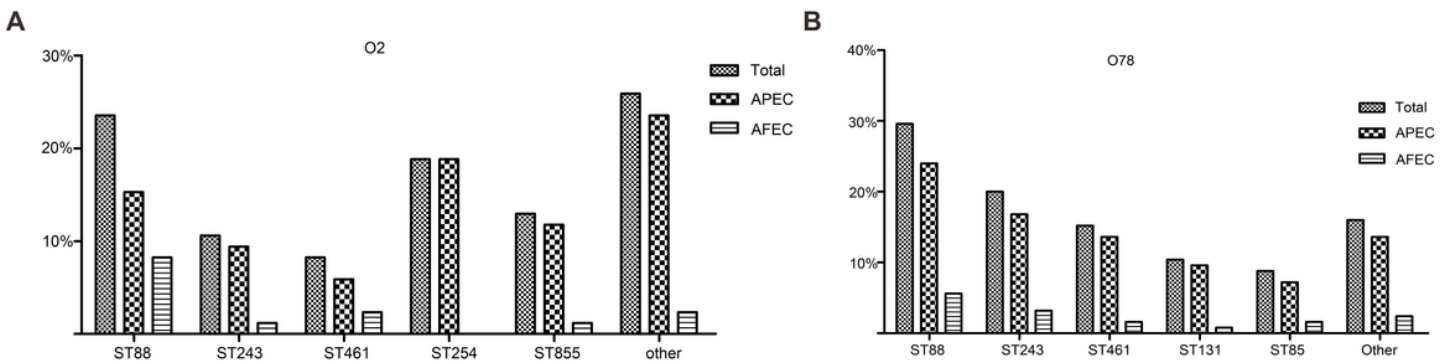

Figure 4

The major STs in O78 (A) and O2 (B) serotype strains.

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