Prevalence and characteristics of extended-spectrum β-lactamases-producing *Escherichia coli* from broiler chickens at different day-age

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ABSTRACT Commensal *Escherichia coli* from the poultries have been considered as reservoirs of extended-spectrum β-lactamases (**ESBL**)-encoding genes. Between May 2018 and March 2019, a total of 340 *E. coli* isolates were obtained from apparently healthy broiler chickens from 20 to 40 D old, distributed in 17 small-scale commercial farms. Finally, 45 isolates (8 from 20-day-old broiler chickens, 14 from 30-day-old ones, and 23 from 40-day-old ones) were identified as ESBL producers, which were further investigated to shed light on the virulence gene profiles, phylogenetic groups, and multilocus sequence types and to detect the ESBL plasmid-mediated quinolone resistance determinant (**PMQR**) genes as well as the mutations in the quinolone resistance-determining regions (**QRDR**) of *gyrA* and *parC*. Molecular analysis showed that phylogenic group A and B1 accounted for 66.7% of the ESBL producers. The overall occurrence of virulence genes ranged from 5.1% (*cva*) to 86.7% (*papC*). Twenty (44.4%) ESBL producers were considered as biofilm producers with moderate or heavy biofilm formation. The most predominant specific CTX-M subtype was $bla_{\text{CTX-M-14}}$ (n = 19), followed by $bla_{\text{CTX-M-9}}$ (n = 17), $bla_{\text{CTX-M-55}}$ (n = 9), $bla_{\text{CTX-M-15}}$ (n = 6), $bla_{\text{CTX-M-1}}$ (n = 5), and $bla_{\text{CTX-M-65}}$ (n = 4). Additionally, PMQR genes were identified in 86.7% of ESBL producers, *qnrS* (n = 21) was the most dominant PMQR gene, followed by the *aac(6′)-Ib-cr* (n = 15), *qnrB* (n = 12), and *qnrA* (n = 9), and all of them co-expressed with β-lactamase genes. All PMQR-positive isolates harbored simultaneously at least 1 mutation in the QRDR of *gyrA* and *parC*. Forty-five ESBL producers were assigned to 33 sequence types, and the most frequent sequence types (**STs**) was ST10 (n = 5) and followed by ST95 (n = 3). Additionally, ST302, ST88, ST410, ST187, and ST23 were represented by 2 ESBL producers, respectively, and the remaining ones exhibited diverse ST. Moreover, the prevalence of ESBL producers, the biofilm-forming ability, and the occurrence of the QRDR mutations among the *E. coli* isolates were characterized by gradually increased with advancing age of broiler chickens.

Key words: *Escherichia coli*, ESBL, PMQR, broiler

2020 Poultry Science 99:3688–3696
https://doi.org/10.1016/j.psj.2020.04.015

INTRODUCTION

The poultry industry has become one of the largest and fastest growing industry in the agrifood sector worldwide, and chickens are the most common sources of poultry meat (Ledergerber, et al., 2003; Bolan, et al., 2010; Shah, et al., 2017). Usually, antimicrobials were used to prevent and treat diseases or to promote their growth in many poultry farms, especially in the small-scale farms. However, frequent and uncontrolled use of antibiotics in food animals has become the primary driving force for the development and dissemination of resistant bacteria (Miles, et al., 2006), and the antibiotic administration also has profound effects on indigenous microbes of animal feces, leading to changes in microbial community structure. Monitoring the antimicrobial resistance development in bacteria isolated from animals is necessary to assess risk, facilitate proper use of antimicrobials, and prolong their useful lifespan (Thitaram, et al., 2016).

Escherichia coli (***E. coli***) is a normal inhabitant in the intestines of animals and humans, whereas some *E. coli* isolates are pathogenic and cause a variety of disease,

Received November 11, 2019.
Accepted April 10, 2020.

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and it is considered as one of the principal cause of morbidity and mortality in poultry (Kathayat, et al., 2018). Commensal bacteria is important because they can be reservoirs of resistance determinants and because they are all more ubiquitous than pathogens (Schaefer, et al., 2011). The high prevalence of extended-spectrum β-lactamases (**ESBL**)-producing *E. coli* in commensal isolates from apparently healthy animals suggests that commensal *E. coli* may play a significant role in serving as a resistance gene reservoir (Li, et al., 2016). Extended-spectrum β-lactam antibiotics, especially the third and fourth-generation cephalosporins, are very important antimicrobial agents for human and animals health. However, ESBL-producing *Enterobacteriaceae* have posed serious challenges in clinical practices, especially for broiler fattening farms. The rapid and wide dissemination of ESBL producers among the broiler chickens is considered a potential risk for humans because of the possible transfer of ESBL-producing isolates or ESBL genes to human (Gundran, et al., 2019).

The impact of food animals as reservoirs and disseminators of ESBL producers into the food production chain should be assessed. Unfortunately, there are no data available concerning the ESBL producers from broiler chickens in Shaanxi Province, China. Hence, the goal of the present study was to investigate the prevalence and drug resistance characteristics of ESBL producers in healthy broiler chickens at different day-age in Shaanxi Province. The results will be helpful for us to optimize a therapeutic scheme to avoid the spread and aggregation of antibiotic resistance.

MATERIALS AND METHODS

Sample Collection

Between May 2018 and March 2019, a total of 528 nonduplicate cloacal swabs were collected randomly from 17 small-scale commercial broiler farms (5,000 to 20,000 birds) distributed in 6 counties in Shaanxi Province, including Fuping, Fufeng, Jingyang, Danfeng, Pucheng, and Wugong county. Individual swabs were collected from broiler chickens ranging in age from 20 to 40 D. All samples were directly transported to the laboratory and processed immediately.

E. coli Isolation and Identification

All samples were immediately seeded on MacConkey agar plates. After incubation at 37°C for 18 to 24 h, 3 colonies with typical *E. coli* morphology (bright pink with a dimple) were randomly selected and transferred to eosin methylene blue agar for further purification, and then, the suspect *E. coli* isolates on eosin methylene blue agar (green colonies with a metallic sheen) were subjected to biochemical tests (indole, methyl red, oxidase, citrate, and triple sugar iron) as described previously(Liu, et al., 2017). Isolates were further identified as *E. coli* by 16S rDNA analysis. Finally, a total of 340 nonduplicate *E. coli* were obtained: 76 from 20-day-old broiler chickens, 116 from 30-day-old broiler chickens, and 148 from 40-day-old broiler chickens. All confirmed *E. coli* isolates were stored at −80°C in tryptic soy broth medium containing 30% glycerol until further analysis.

Antimicrobial Susceptibility Testing

All *E. coli* isolates were tested by a standardized microdilution method for antimicrobial susceptibility against amoxicillin–clavulanic acid, cefotaxime, ceftazidime, ceftrizxone, meropenem, enrofloxacin, florfenicol, tetracycline, doxycycline, oxytetracycline, colistin, gentamicin, amikacin, spectinomycin, and sulfamethoxazole/trimethoprim according to Clinical and Laboratory Standards Institute guidelines and were clinically categorized with breakpoints (CLSI, 2011). Finally, 45 isolates (8 from 20-day-old broiler chickens, 14 from 30-day-old ones, and 23 from 40-day-old ones) of the 340 *E. coli* isolates recovered in the study (13.2%) were identified as ESBL producers.

Biofilm Formation

Biofilm formation of the 45 ESBL producers was determined according to previously described protocols with slight modification (Stepanovic, et al., 2000; Kern, et al., 2018). Briefly, overnight grown isolates were cultured in 96-well flatbottom microtiter plates for 48 h. The wells were washed with 200 μL of 1% PBS 3 times, and then, the biofilms were stained with crystal violet. After several washes, 33% acetic acid was added, and the OD_{570} was measured with a microplate reader (ELx808, BioTek Instruments Inc., Winooski, VT). Furthermore, *E. coli* ATCC 25922 was used as negative control. Each isolate was evaluated in triplicate, and the mean was determined by averaging the proportion of each isolate individually (Stepanovic, et al., 2000).

Phylogenetic Typing and Virulence Genes Detection

Genomic DNA of the isolates was extracted using a bacterial genomic DNA extraction kit (Tiangen Ltd., Beijing, China) according to the manufacturer's instructions. The distribution of phylogenetic groups among the ESBL producers was determined by the new quadruplex PCR as recently described by Clermont et al. (Clermont, et al., 2013). Moreover, all ESBL producers were investigated for the presence of 10 virulence genes (*iutA*, *iss*, *papC*, *iucD*, *tsh*, *irp*-2, *hlyF*, *iroN*, *cva,* and *astA*), which are associated with colibacillosis. Sterilized deionized water was used as a negative control. The specific primers were showed in Supplementary Table 1.

Characterization of ESBL, plasmid-mediated quinolone resistance determinant (**PMQR**) genes, and screening for mutations within quinolone resistance-determining region (**QRDR**) of DNA gyrase and topoisomerase IV.

Table 1. The occurrence of virulence genes and the resistance profile in ESBL-producing *E. coli* isolates from broiler chickens of different ages.

Isolates ID	PG	Broiler chickens	Virulence genes	Resistance profiles
A1807011	A	20 D of age	*iroN*, *iss*, *iutA*, *tsh, cva*, *iucD*, *astA*	AMC, CTX, TEC, OXY, GEN
D1807024	A	20 D of age	*iroN*, *tsh*, *irp-2*, *iucD*, *astA*, *papC*	AMC, CTX, OXY, GEN
E1807028	A	20 D of age	*iucD*, *astA*, *papC*	AMC, CAZ, GEN, SXT
H1807032	B1	20 D of age	*iutA*, *iucD*, *astA*, *papC*	AMC, CTX, CRO, TEC, OXY
I1807050	B1	20 D of age	*iroN*, *iss*, *astA*, *papC*	AMC, CAZ, ENR, TEC, OXY, SXT
K1807055	B1	20 D of age	*iutA*, *iucD*, *astA*, *papC*	AMC, CAZ, ENR, FFC, TEC, OXY, SXT
L1807069	B2	20 D of age	*iutA*, *iucD*, *astA*, *papC*	AMC, CTX, CAZ, ENR, TEC, OXY, GEN, SXT
N1807126	D	20 D of age	*iroN*, *iss*, *astA*, *papC*	AMC, CTX, CRO, ENR, TEC, OXY, GEN, SXT
A1809023	A	30 D of age	*iroN*, *iss*, *tsh*, *cva*, *irp-2*, *iucD*, *papC*	CTX, CAZ, FFC, TEC, DOX, OXY, GEN, SXT
E1808013	A	30 D of age	*iroN*, *iss*, *tsh*, *iucD*, *papC*	CTX, TEC, GEN, SPM, SXT
B1809006	A	30 D of age	*iss*, *iutA*, *tsh*, *cva*, *iucD*	AMC, CTX, CAZ, ENR, TEC, DOX, GEN
C1800025	A	30 D of age	*iss*, *iucD*, *astA*, *papC*	AMC, CTX, CAZ, ENR, FFC, TEC, OXY, GEN, SPM
F1810051	A	30 D of age	*iroN*, *tsh*, *irp-2*, *iucD*, *astA*, *papC*	AMC, CTX, CAZ, ENR, FFC, TEC, OXY, SXT
H1807028	B1	30 D of age	*iroN*, *iss*, *iutA*, *iucD*	AMC, CTX, CAZ, ENR, DOX, OXY, GEN, SPM, SXT
H1903025	B1	30 D of age	*iss*, *iutA*, *iucD*, *astA*, *papC*	CTX, CRO, ENR, FFC, TEC, DOX, OXY, GEN, SPM, SXT
I1809016	B1	30 D of age	*iroN*, *tsh*, *iucD*, *astA*, *papC*	AMC, CAZ, CRO, ENR, FFC, TEC, OXY, GEN, AMK, SXT
J1811009	B2	30 D of age	*iss*, *iutA*, *tsh*, *cva*, *iucD*	AMC, CTX, CRO, ENR, FFC, TEC, DOX, SPM, SXT
K1902052	B2	30 D of age	*iroN*, *irp-2*, *iucD*, *astA*, *papC*	AMC, CTX, ENR, FFC, TEC, DOX, OXY, GEN, SXT
G1808083	C	30 D of age	*iroN*, *astA*	AMC, CTX, CRO, ENR, FFC, TEC, DOX, OXY, GEN, AMK
L1809103	D	30 D of age	*iroN*, *iucD*, *astA*, *papC*	AMC, CTX, CAZ, ENR, FFC, TEC, OXY, GEN, SPM, SXT
N1812066	D	30 D of age	*iroN*, *astA*, *papC*	AMC, CTX, CAZ, ENR, TEC, OXY, GEN, AMK
P1805027	E	30 D of age	*iroN*, *iss*, *tsh*, *irp-2*, *iucD*, *astA*, *papC*	AMC, CTX, ENR, FFC, TEC, OXY, SPM, SXT
A1807013	A	40 D of age	*iroN*, *irp-2*, *astA*, *papC*	AMC, CTX, CRO, ENR, FFC, TEC, DOX, SXT
A1811079	A	40 D of age	*tsh*, *irp-2*, *astA*, *papC*	AMC, CAZ, CRO, ENR, FFC, TEC, DOX, OXY, GEN, SPM, SXT
B1807025	A	40 D of age	*iroN*, *iss*, *tsh*, *iucD*, *astA*, *papC*	AMC, CTX, CAZ, CRO, ENR, FFC, DOX, OXY, AMK, SXT
C1808068	A	40 D of age	*iss*, *tsh*, *irp-2*, *astA*, *papC*	AMC, CTX, CAZ, ENR, TEC, DOX, OXY, GEN, SPM, SXT
C1902037	A	40 D of age	*iroN*, *iutA*, *tsh*, *iucD*, *astA*, *papC*	AMC, CTX, CAZ, CRO, ENR, FFC, TEC, OXY, SXT
D1806105	A	40 D of age	*astA*, *papC*	AMC, CAZ, CRO, ENR, FFC, TEC, DOX, OXY, GEN, SPM, SXT
E1809028	C	40 D of age	*iroN*, *iutA*, *tsh*, *iucD*, *astA*, *papC*	AMC, CTX, CAZ, ENR, FFC, TEC, DOX, OXY, AMK, SPM, SXT
F1809009	C	40 D of age	*iroN*, *iutA*, *tsh*, *iucD*, *astA*, *papC*	AMC, CTX, CAZ, ENR, FFC, TEC, OXY, GEN, AMK, SPM, SXT
K1808124	C	40 D of age	*astA*, *papC*	AMC, CTX, CAZ, CRO, ENR, FFC, TEC, OXY, GEN, AMK, SPM, SXT
G1903014	B1	40 D of age	*irp-2*, *iucD*, *astA*, *papC*	AMC, CTX, CRO, ENR, FFC, TEC, DOX, OXY, GEN, SXT
H1806023	B1	40 D of age	*iroN*, *iss*, *iutA*, *iucD*, *astA*, *papC*	AMC, CAZ, CRO, ENR, FFC, TEC, OXY, GEN, SPM
H1903053	B1	40 D of age	*iroN*, *iss*, *iutA*, *iucD*, *astA*, *papC*	AMC, CTX, CAZ, CRO, ENR, FFC, DOX, OXY, AMK, SPM
I1807041	B1	40 D of age	*iroN*, *iutA*, *iucD*, *astA*, *papC*	CTX, CAZ, CRO, ENR, TEC, DOX, OXY, SPM, SXT
G1808037	B1	40 D of age	*iroN*, *tsh*, *irp-2*, *astA*, *papC*	AMC, CTX, CAZ, CRO, ENR, FFC, TEC, OXY, GEN, SPM, SXT
K1808086	B1	40 D of age	*iss*, *iutA*, *tsh*, *cva*, *astA*	AMC, CTX, CRO, ENR, TEC, OXY, GEN, AMK, SXT
L1812044	B2	40 D of age	*iroN*, *iss*, *tsh*, *irp-2*, *astA*, *papC*	AMC, CTX, CAZ, CRO, MEM, ENR, FFC, TEC, DOX, OXY, GEN, AMK, SPM, SXT
M1903026	B2	40 D of age	*iroN*, *iutA*, *tsh*, *iucD*, *astA*, *papC*	AMC, CTX, CAZ, CRO, ENR, FFC, TEC, DOX, OXY, GEN, AMK, SPM, SXT

(continued on next page)

Table 1. (*continued*)

Isolates ID	PG	Broiler chickens	Virulence genes	Resistance profiles
M1903059	B2	40 D of age	*iroN*, *iss*, *tsh*, *cva*, *irp-2*, *iucD*, *papC*	AMC, CTX, CAZ, CRO, MEM, ENR, FFC, TEC, OXY, GEN, SXT
N1811075	D	40 D of age	*iroN*, *iss*, *iucD*, *astA*, *papC*	AMC, CTX, CRO, ENR, TEC, DOX, OXY, AMK, SXT
O1811039	D	40 D of age	*iutA*, *tsh*, *iucD*, *astA*, *papC*	AMC, CTX, CAZ, CRO, ENR, FFC, TEC, OXY, GEN, SPM, SXT
P1810048	D	40 D of age	*iucD*, *astA*, *papC*	AMC, CTX, CAZ, ENR, FFC, TEC, OXY, GEN, AMK, SXT
P1812056	F	40 D of age	*iroN*, *iss*, *iutA*, *iucD*, *papC*	AMC, CTX, CRO, ENR, FFC, TEC, DOX, OXY, AMK, SPM, SXT
Q1812063	E	40 D of age	*iss*, *iutA*, *iucD*, *astA*, *papC*	AMC, CTX, CRO, ENR, FFC, TEC, OXY, GEN, AMK, SXT

Abbreviations: AMC, amoxicillin-clavulanic acid; AMK, amikacin; CAZ, ceftazidime; CTX, cefotaxime; COS, colistin; CRO, ceftriaxone; DOX, doxycycline; ENR, enrofloxacin; ESBL, extended-spectrum β-lactamases; FFC, florfenicol; GEN, gentamicin; MEM, meropenem; OXY, oxytetracycline; PG, phylogenetic group; SPM, spectinomycin; SXT, sulfamethoxazole-trimethoprim; TEC, tetracycline.

blaCTX-M

blaTEM and blaSHV types of β-lactamases were detected by PCR amplification. blaCTX-M group-specific primers for CTX-M-1, CTX-M-2, CTX-M-8, and CTX-M-9 groups were used to detect blaCTX-M genes. The primers used for PCR detection and sequencing were showed in Supplementary Table 1. The PCR products were sequenced by Sangon Biotech (Shanghai, China). Gene sequences were analyzed online using BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and identified using a β-lactamase database (http://www.lahey.org/Studies/). Meanwhile, all ESBL producers were characterized by PCR and sequencing to determine the prevalence of PMQR genes (*qnrA*, *qnrB*, *qnrD*, *qnrS*, *aac(6′)-Ib-cr*, *qepA*, and *oqxAB*), and the mutations within the QRDR of *gyrA* and *parC* as previously described (Liu, et al., 2012).

Multilocus Sequence Typing

All 45 ESBL producers were subjected to multilocus sequence typing (**MLST**). Internal fragments of 7 housekeeping genes (*adk*, *fumC*, *gyrB*, *icd*, *mdh*, *purA*, and *recA*) were amplified by PCR. The detailed scheme of the MLST procedure is available at the *E. coli* MLST database website (http://mlst.warwick.ac.uk/mlst/dbs/Ecoli).

Statistical Analyses

The biofilm formation are presented as a mean ± standard deviation. Student *t* test was used to compare the biofilm formation among the *E. coli* from broiler chickens at different day-age. All statistical analyses were performed using GraphPad Prism 6.0 (GraphPad Software Inc., La Jolla, CA). A *P*-values below 5% were interpreted as statistically significant.

RESULTS

Antimicrobial Susceptibility Testing

The antibiotic resistance profiles of 45 ESBL producers among 340 *E. coli* isolates collected from broiler chickens ranging in age from 20 to 40 D were showed in Table 1, and the prevalence of the ESBL producers was 10.5, 12.1, and 15.5%, respectively, depending on the age of the broiler chickens. Moreover, all ESBL producers expressed multidrug resistance profiles and showed high resistance to amoxicillin–clavulanic acid (91.1%), followed by tetracycline (88.9%), oxytetracycline (88.9%), cefotaxime (84.4%), enrofloxacin (86.7%), sulfamethoxazole–trimethoprim (80%), gentamicin (68.9%), florfenicol (66.7%), ceftazidime (64.4%), ceftriaxone (55.6%), spectinomycin (46.7%), doxycycline (44.4%), and amikacin (33.3%). On the other hand, the ESBL producers exhibited high susceptibility to meropenem (95.6%) and colistin (100%).

Phylogenetic Groups and the Virulence Genes Distribution

Phylogenetic analysis showed that the 45 ESBL producers were composed of phylogenetic groups A (n = 14), B1 (n = 12), D (n = 6), B2 (n = 6), C (n = 4), E (n = 2), and F (n = 1) (Table 1). Subgroups A and B1 were the main phylogenetic groups. Generally, 9 of 10 virulence genes tested were determined among the 45 ESBL producers. The prevalence of individual virulence gene ranged from 5.1% (*cva*) to 86.7% (*papC*), *iucD*, *iroN*, *iucD*, *iss*, *iutA*, *tsh*, and *irp-2* were detected in 38 (84.4%), 31 (68.9%), 30 (66.7%), 23 (51.3%), 20 (44.4%), 18 (40%), and 10 (22.2%) isolates, respectively. *vat* gene was not detected in any isolate.

Detection of Biofilm Formation

The results obtained for 45 ESBL producers were presented in Table 2. Most of the ESBL producers (55.6%) exhibited a weak adherence ability to abiotic surfaces, whereas 19 (42.2%) isolates were classified as moderate biofilm producers, and 1 (2.2%) isolate was heavy biofilm producers. Moreover, all the ESBL producers from 40-day-old broiler chickens had moderate and heavy biofilm-forming abilities.

Table 2. Comparison of in vitro biofilm formation for 45 ESBL-producing *E. coli* isolates from broiler chickens of different ages.

Isolate ID	Broiler chickens	Phylogenetic group (PG)	Biofilm formation	
			Mean	SD
A1807011	20 D of age	A	0.09	0.04
D1807024	20 D of age	A	0.10	0.06
E1807028	20 D of age	A	0.09	0.08
H1807032	20 D of age	B1	0.07	0.03
I1807050	20 D of age	B1	0.03	0.03
K1807055	20 D of age	B1	0.08	0.05
L1807069	20 D of age	B2	0.02	0.02
N1807126	20 D of age	D	0.09	0.07
A1809023	30 D of age	A	0.42	0.12
E1808013	30 D of age	A	0.42	0.22
B1809006	30 D of age	A	0.31	0.08
C1800025	30 D of age	A	0.13	0.02
F1810051	30 D of age	A	0.22	0.06
H1807028	30 D of age	B1	0.11	0.03
H1903025	30 D of age	B1	0.14	0.03
I1809016	30 D of age	B1	0.26	0.04
J1811009	30 D of age	B2	0.07	0.01
K1902052	30 D of age	B2	0.06	0.03
G1808083	30 D of age	C	0.06	0.02
L1809103	30 D of age	D	0.18	0.04
N1812066	30 D of age	D	0.14	0.12
P1805027	30 D of age	E	0.28	0.07
A1807013	40 D of age	A	0.64	0.06
A1811079	40 D of age	A	0.87	0.14
B1807025	40 D of age	A	0.38	0.10
C1808068	40 D of age	A	0.89	0.11
C1902037	40 D of age	A	0.41	0.07
D1806105	40 D of age	A	0.71	0.06
E1809028	40 D of age	C	0.57	0.04
F1809009	40 D of age	C	0.70	0.17
K1808124	40 D of age	C	0.17	0.03
G1903014	40 D of age	B1	0.17	0.11
H1806023	40 D of age	B1	0.52	0.08
H1903053	40 D of age	B1	0.78	0.31
I1807041	40 D of age	B1	0.19	0.07
G1808037	40 D of age	B1	0.15	0.04
K1808086	40 D of age	B1	0.31	0.07
L1812044	40 D of age	B2	0.15	0.07
M1903026	40 D of age	B2	1.26	0.16
M1903059	40 D of age	B2	0.32	0.05
N1811075	40 D of age	D	0.44	0.10
O1811039	40 D of age	D	0.22	0.02
P1810048	40 D of age	D	0.31	0.07
P1812056	40 D of age	F	0.35	0.1
Q1812063	40 D of age	E	0.63	0.06
ATCC 25922			1.00	

Abbreviation: ESBL, extended-spectrum β-lactamases.

Identification of β-lactamases and PMQR Genes As Well As the Mutations in QRDR

As shown in Table 3, 42 (93.3%) isolates carried $bla_{\text{CTX-M}}$ genes, and the most predominant specific CTX-M subtype identified was $bla_{\text{CTX-M-14}}$ (n = 27), followed by $bla_{\text{CTX-M-9}}$ (n = 13), $bla_{\text{CTX-M-55}}$ (n = 7), $bla_{\text{CTX-M-15}}$ (n = 6), $bla_{\text{CTX-M-1}}$ (n = 5), $bla_{\text{CTX-M-65}}$ (n = 2), $bla_{\text{CTX-M-74}}$ (n = 1), and $bla_{\text{CTX-M-25}}$ (n = 1), and they were divided into 4 specific groups. Other β-lactamase genes bla_{TEM} and bla_{SHV} were detected in 16 and 22 ESBL producers, respectively. Furthermore, 39 of 45 (86.7%) ESBL producers showing resistance to enrofloxacin (minimum inhibitory concentration [**MIC**] ≥16 μg/mL) harbored at least 1 PMQR gene, which was co-located in the ESBL-producing isolates with β-lactamase genes. *qnrS* (n = 21) was the most dominant PMQR gene, followed by the *aac(6′)-Ib-cr* (n = 15), *qnrB* (n = 13), and *qnrA* (n = 9), whereas the *qnrD*, *qepA,* and *oqxAB* genes were not detected in any isolate. It is noteworthy that PMQR genes were seldom detected in the ESBL producers from the broiler chickens at 20 D of age, whereas they were more prevalence in the ESBL producers from the broiler chickens at 30 and 40 D of age. Moreover, point mutations in QRDR of *gyrA* or *parC* were detected among 44 out of 45 ESBL producers showing decreased susceptibility or resistance to enrofloxacin (Table 3, the MIC were not showed), and a single *gyrA* mutation (Ser83Leu) was detected in the ESBL producers expressing reduced susceptibility to enrofloxacin (enrofloxacin MICs 0.5–2 μg/mL), and double *gyrA* mutations (Ser83Leu and Asp87Asn) were found in the

Table 3. The distribution of β-lactamase genes, PMQR genes as well as the mutations in QRDR in ESBL-producing *E. coli* isolates from broiler chickens of different ages.

Isolates ID	PG	MLST	Broiler chickens	β-lactamase genes		PMQR genes	Mutation in QRDR
				CTX-M type	TEM, SHV types		
A1807011	A	ST10	20 D of age	-	TEM-1, SHV-12	-	
D1807024	A	ST10	20 D of age	-	SHV-12	-	Ser83Leu
E1807028	A	ST302	20 D of age	CTX-M-14	-	-	Ser83Leu
H1807032	B1	ST522	20 D of age	CTX-M-1	-	-	Ser83Leu
I1807050	B1	ST68	20 D of age	CTX-M-1	TEM-1	*qnrS*	Ser83Leu + Asp87Asn
K1807055	B1	ST155	20 D of age	CTX-M-55	-	*qnrS*	Ser83Leu + Asp87Asn
L1807069	B2	ST95	20 D of age	CTX-M-14	-	*qnrA*	Ser83Leu + Asp87Asn
N1807126	D	ST57	20 D of age	-	TEM-1, SHV-12	*qnrS*	Ser83Leu + Asp87Asn
A1809023	A	ST10	30 D of age	CTX-M-9	SHV-12	-	Ser83Leu
E1808013	A	ST1700	30 D of age	CTX-M-55	SHV-12	-	Ser83Leu
B1809006	A	ST175	30 D of age	CTX-M-1	-	*aac(6′)-Ib-cr*	Ser83Leu + Asp87Asn
C1800025	A	ST86	30 D of age	CTX-M-1, CTX-M-14	-	*qnrA*, *qnrS*	Ser83Leu + Asp87Asn
F1810051	A	ST93	30 D of age	CTX-M-14	-	*aac(6′)-Ib-cr*	Ser83Leu + Asp87Asn + Ser80Ile
H1807028	B1	ST88	30 D of age	CTX-M-14	SHV-12	*qnrS*	Ser83Leu + Asp87Asn + Ser80Ile
H1903025	B1	ST104	30 D of age	CTX-M-14	TEM-1	*qnrA*, *qnrB*	Ser83Leu + Asp87Asn
I1809016	B1	ST95	30 D of age	CTX-M-65	SHV-12	*qnrB*	Ser83Leu + Asp87Asn + Ser80Ile
J1811009	B2	ST69	30 D of age	CTX-M-15	SHV-12	*aac(6′)-Ib-cr*	Ser83Leu + Asp87Asn + Ser80Ile
K1902052	B2	ST95	30 D of age	CTX-M-15	-	*aac(6′)-Ib-cr*	Ser83Leu + Asp87Asn
G1808083	C	ST410	30 D of age	CTX-M-65	-	*qnrS*	Ser83Leu + Asp87Asn
L1809103	D	ST187	30 D of age	CTX-M-14	TEM-1	*qnrS*	Ser83Leu + Asp87Asn
N1812066	D	ST115	30 D of age	CTX-M-9	TEM-1, SHV-2	*qnrS*	Ser83Leu + Asp87Asn + Ser80Ile
P1805027	E	ST2375	30 D of age	CTX-M-9, CTX-M-14	-	*qnrA*	Ser83Leu + Asp87Asn + Ser80Ile + Glu84Val
A1807013	A	ST652	40 D of age	CTX-M-14	TEM-1, SHV-12	*qnrB*, *aac(6′)-Ib-cr*	Ser83Leu + Asp87Asn + Ser80Ile
A1811079	A	ST302	40 D of age	CTX-M-14, CTX-M-74	-	*qnrA. qnrB*, *aac(6′)-Ib-cr*	Ser83Leu + Asp87Asn + Ser80Ile + Glu84Val
B1807025	A	ST1152	40 D of age	CTX-M-14	TEM-1, SHV-12	*qnrB*	Ser83Leu + Asp87Asn
C1808068	A	ST10	40 D of age	CTX-M-9, CTX-M-14	-	*qnrS*	Ser83Leu + Asp87Asn + Ser80Ile
D1806105	A	ST10	40 D of age	CTX-M-14	TEM-1	*qnrB*, *aac(6′)-Ib-cr*	Ser83Leu + Asp87Asn + Ser80Ile
C1902037	A	ST124	40 D of age	CTX-M-9, CTX-M-55	SHV-12	*qnrS*	Ser83Leu + Asp87Asn
E1809028	C	ST23	40 D of age	CTX-M-9, CTX-M-14	SHV-5	*qnrA. qnrB*, *aac(6′)-Ib-cr*	Ser83Leu + Asp87Asn + Ser80Ile
F1809009	C	ST23	40 D of age	CTX-M-14, CTX-M-55	TEM-1	*aac(6′)-Ib-cr*	Ser83Leu + Asp87Asn
K1808124	C	ST410	40 D of age	CTX-M-9, CTX-M-14	SHV-12	*qnrS*	Ser83Leu + Asp87Asn
G1903014	B1	ST101	40 D of age	CTX-M-14	SHV-12	*qnrA. qnrB*, *aac(6′)-Ib-cr*	Ser83Leu + Asp87Asn + Ser80Ile
H1806023	B1	ST1148	40 D of age	CTX-M-9	TEM-1, SHV-12	*qnrS*	Ser83Leu + Asp87Asn
H1903053	B1	ST46	40 D of age	CTX-M-14, CTX-M-15	TEM-1	*qnrS*	Ser83Leu + Asp87Asn + Ser80Ile
I1807041	B1	ST88	40 D of age	CTX-M-9, CTX-M-14	-	*qnrS*	Ser83Leu + Asp87Asn + Ser80Ile + Glu84Val
G1808037	B1	ST77	40 D of age	CTX-M-9, CTX-M-14	TEM-5	*qnrB*, *aac(6′)-Ib-cr*	Ser83Leu + Asp87Asn
K1808086	B1	ST206	40 D of age	CTX-M-14, CTX-M-55	SHV-5	*qnrS*, *aac(6′)-Ib-cr*	Ser83Leu + Asp87Asn + Ser80Ile
L1812044	B2	ST131	40 D of age	CTX-M-1, CTX-M-15	TEM-30	*qnrS*, *aac(6′)-Ib-cr*	Ser83Leu + Asp87Asn + Ser80Ile
M1903026	B2	ST69	40 D of age	CTX-M-15, CTX-M-55	SHV-12	*qnrA*, *qnrB*, *qnrS*	Ser83Leu + Asp87Asn + Ser80Ile
M1903059	B2	ST555	40 D of age	CTX-M-9, CTX-M-14, CTX-M-15	-	*qnrA*, qnrS	Ser83Leu + Asp87Asn
N1811075	D	ST38	40 D of age	CTX-M-9, CTX-M-14	SHV-12	*qnrS*	Ser83Leu + Asp87Asn + Ser80Ile
O1811039	D	ST187	40 D of age	CTX-M-14	TEM-5, SHV-12	*qnrS*	Ser83Leu + Asp87Asn + Ser80Ile
P1810048	D	ST216	40 D of age	CTX-M-9, CTX-M-14, CTX-M-55	-	*qnrB*, *aac(6′)-Ib-cr*	Ser83Leu + Asp87Asn + Ser80Ile + Glu84Val
P1812056	F	ST501	40 D of age	CTX-M-14, CTX-M-25	TEM-1, SHV-12	*qnrS*, *aac(6′)-Ib-cr*	Ser83Leu + Asp87Asn + Ser80Ile + Glu84Val
Q1812063	E	ST350	40 D of age	CTX-M-14	TEM-5, SHV-12	*qnrB*	Ser83Leu + Asp87Asn + Ser80Ile

Abbreviations: CTX, cefotaxime; MLST, multilocus sequence typing; PG, phylogenetic group; PMQR, plasmid-mediated quinolone resistance determinant; QRDR, quinolone resistance-determining regions.

ESBL producers with enrofloxacin MIC ranged from 4 to 16 μg/mL. Moreover, a third mutation (Ser83Leu) (enrofloxacin MICs 32–64 μg/mL) and a fourth mutation (Asp87Asn) (enrofloxacin MICs ≥128 μg/mL) in *parC* were detected correlating with the level of enrofloxacin MIC in a stepwise manner. The ESBL producers from the broiler chickens at 20 D of age harbored no more than 2 mutations in the *gyrA* gene of the QRDR, and no mutation was found in the *parC* gene of the QRDR. The occurrence of QRDR mutations was significantly associated with the age of the broiler chickens. The majority of ESBL producers from broiler chickens at 40 D of age had 3 or 4 mutations in the QRDR of *gyrA* and *parC*. All PMQR-positive isolates harbored simultaneously at least 1 point mutation in the QRDR of *gyrA* and *parC* (Table 3).

MLST Profiles

Forty-five ESBL producers were assigned to 33 ST (Table 3), and all of them have been reported in chickens according to the MLST database. The most frequent sequence types were ST10 (n = 5) and followed by ST95 (n = 3). Additionally, ST302, ST88, ST410, ST187, and ST23 were represented by 2 ESBL producers, respectively, and the remaining ESBL producers exhibited diverse ST types.

DISCUSSION

Previous study have reported that *E. coli* in fecal samples of healthy animals could serve as potential reservoirs of resistant isolates and transferable resistance genes (de Vries, et al., 2011; Osman, et al., 2018). The current study identified a prevalence (13.2%) of ESBL-producing *E. coli* derived from the fecal samples of apparently healthy broiler chickens during 2018 to 2019 in Shaanxi Province. All ESBL producers demonstrated multidrug resistance profiles. It is higher than that found in previous similar studies in pigs (9.6%) and surface waters (2.8%) in Shaanxi Province (Liu, et al., 2018), whereas remarkably lower than that in dogs (24.2%) and retail foods (22.3%) in Shaanxi Province (Xi, et al., 2015; Liu, et al., 2016). In addition, among the *E. coli* analyzed in this study, the prevalence of ESBL producers (13.2%) is considerably lower than that previously found in chickens in Henan Province (60.8%) during 2007 to 2008 (Yuan, et al., 2009) and Northeast China (Heilongjiang, Jilin and Liaoning Province, 100%) during 2011 to 2013 (Tong, et al., 2015). Our study provided important information on the trends in the burden of infections because of ESBL producers in veterinary medicine in Shaanxi Province although our data are limited. Especially, the prevalence of ESBL producers increased in a stepwise manner from 10.5 to 15.5% with advancing age of broiler chickens.

Our data showed that the majority (97.8%) of the ESBL producers had minimal or moderate biofilm-forming capability. Moreover, our results showed the diversity of biofilm formation in ESBL producers from different day-old broiler chickens. A positive linear association was found between the biofilm-forming abilities of the ESBL producers and the age of broiler chickens. Biofilm formation for ESBL producers from 40-day-old broiler chickens was significantly greater than that from 20-day-old and 30-day-old broiler chickens. The ESBL producers from 20-day-old broiler chickens produced weak biofilms, whereas 73.9% ESBL producers from 40-day-old broiler chickens had moderate, even heavy biofilm-forming abilities. This difference was statistically significant ($P < 0.01$). It is also indicated that biofilm formation is a dynamic process, depending on the age of the broiler chickens.

In China, CTX-M-type ESBL were the most common genotype of *E. coli* isolated from chickens (Tong, et al., 2015). Generally, a diversity of β-lactamase genes was detected within ESBL producers from broiler chickens in Shaanxi Province according to our results, and the $bla_{\text{CTX-Ms}}$ were represented by 8 $bla_{\text{CTX-M}}$ subtypes that mostly expressed $bla_{\text{CTX-M-14}}$. It is in accord with the previous studies that $bla_{\text{CTX-M-14}}$ is the most dominant $bla_{\text{CTX-M}}$ subtype in the ESBL-producing *E. coli* from pigs and dogs in Shaanxi Province (Liu, et al., 2016; Liu, et al., 2018). Second to $bla_{\text{CTX-M-14}}$, $bla_{\text{CTX-M-9}}$ was the most frequent variant identified in this study. Additionally, we identified 2 $bla_{\text{CTX-M-65}}$ positive isolates from 30 D and 40 D broiler chickens. $bla_{\text{CTX-M-65}}$ has been detected in the surface water in Shaanxi Province, and it has become one of the predominant $bla_{\text{CTX-M}}$ genes in ESBL-producing bacterial isolates from animals in China and has been frequently reported in other places in China (Yin, et al., 2009; Rao, et al., 2014; Yang, et al., 2014). Previous reports (Yuan, et al., 2009; Tong, et al., 2015) showed that $bla_{\text{CTX-M-15}}$, $bla_{\text{CTX-M-55}}$, and $bla_{\text{CTX-M-65}}$ were the most prevalent subtypes in the ESBL producers from chickens in Northeast China (Heilongjiang, Jilin, and Liaoning Provinces), Jiangsu, and Henan Province, respectively, and reflected the geographical variations in the prevalence of CTX-M cluster groups. $bla_{\text{CTX-M-55}}$ has been widely documented in food-producing animals and pets in China (Rao, et al., 2014).

Moreover, it is worthy to note that CTX-M-1 and CTX-M-9 group members were found to coexist in 9 isolates, which was similar to the results in other studies (Sun, et al., 2010). If 2 or more $bla_{\text{CTX-Ms}}$ belonged to 2 groups frequently coexist in the same isolate, which could promote the occurrence of other recombinant enzymes in the future.

We found a highly diverse population representing 33 ST in the ESBL producers from broiler chickens, and they showed higher genetic diversity than ESBL producers from dogs, pigs, and surface water in Shaanxi Province, with the corresponding ratio of the MLST types to the isolate number in broiler chickens, dogs, pigs, and surface water were 73.3, 55, 54.5, and 48.7%, respectively, combining with the previous studies in our laboratory.

A large number (57.8%, 26/45) of isolates changed designation from the original phylogenetic group A and group B1. However, the panel of virulence genes selected for this study was limited in number and represents only a subset of known virulence genes. Some important determinants of virulence may have been missed because of this limitation.

In conclusion, this study analyzed the molecular characteristics of the ESBL-producing *E. coli* isolates from different D-old broiler chickens. Our results revealed that the prevalence of ESBL producers from broiler chickens were 13.2%, and the most predominant virulence gene among the ESBL producers was papC, whereas blaCTX-M-14 and qnrS were the most prevalent β-lactamase and PMQR genes, respectively. Moreover, the prevalence of ESBL producers, the biofilm-forming abilities, and the occurrence of QRDR mutations among the *E. coli* isolates gradually increased with advancing age of broiler chickens. Moreover, the ESBL producers showed a highly diverse population representing 33 ST.

ACKNOWLEDGMENTS

The work presented in this study was funded by the Key Research and Development Program of Shaanxi Province of China (No. 2018NY-005).

Conflict of Interest Statement: The authors did not provide a conflict of interest statement.

SUPPLEMENTARY DATA

Supplementary data associated with this article can be found in the online version at http://doi.org/10.1016/j.psj.2020.04.015.

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