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Original Article

Antimicrobial Resistance Profiles and Clonal Relationship among Non-ESBL Avian Pathogenic *Escherichia coli* Isolates and ESBL Producing *E. coli* Isolates from Human Urinary Tract Infections

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Abstract

Background: We aimed to investigate antimicrobial resistance and clonal relationships among poultry *Escherichia coli* isolates from different broiler farms and their relationships with Extended-Spectrum Beta-Lactamase (ESBL) producing urinary pathogenic *E. coli* (UPEC) isolates from the same geographical area.

Methods: Twenty four *E. coli* isolates from six broiler farms with colibacillosis and 97 ESBL producing human UPEC isolates were investigated for resistance to critically important antimicrobials in human medicine in Shiraz, central Iran in 2015-16. In addition, clonal relationships of these isolates were investigated with Pulse Field Gel Electrophoresis (PFGE).

Results: As expected, cephalosporins and imipenem resistance were significantly higher in ESBL producing human *E. coli* isolates in comparison with non-ESBL avian pathogenic *E. coli* (APEC) isolates. In addition, significantly higher percentages of gentamycin and trimethoprim-sulfamethoxazole resistance were seen in human isolates. In contrast, nitrofurantoin resistance was significantly higher in APEC isolates. Based on PFGE patterns, five clusters were identified in APEC isolates. Isolates from each farm were closely related to each other by PFGE patterns. However, different PFGE restriction profiles were seen among the *E. coli* isolates from different broiler farms. Comparison of PFGE patterns among APEC and UPEC isolates showed two closely related PFGE patterns.

Conclusion: There were clonally related *E. coli* isolates caused the outbreaks of colibacillosis within broiler farms. Some of these isolates had closely related PFGE patterns with human UPEC isolates which suggest avian pathogenic *E. coli* strains as a potential zoonosis.

Keywords: *Escherichia coli*; Beta-lactamases; Avian colibacillosis; Urinary tract infection

Introduction

Escherichia coli is a part of the normal poultry intestinal microflora. However, there are certain strains nominated as avian pathogenic *E. coli* (APEC) which have capability to invade into dif-

ferent internal organs and cause the systemic avian colibacillosis (1). The efficacious treatment of avian colibacillosis mostly depends on the use of broad-spectrum antimicrobials which increased antibiotic resistance to critically important antimicrobials such as cephalosporins and fluoroquinolones (2). Massive use of antibiotics can lead to potential survival of specific *E.coli* isolates in poultry production chain (3) and potential transmission of these isolates to human or transmission of their antimicrobial resistance to human pathogens (2).

Similar to APEC strains, some human pathogenic *E. coli* strains, such as uropathogenic *E. coli* (UPEC) have evolved to colonize extraintestinal sites and termed as extraintestinal *E. coli* (ExPEC) (4). Among these extraintestinal infections, urinary tract infections (UTIs) are the most prevalent healthcare-associated infections in hospitalized patients and there are rising concerns about antibiotic therapy failures and substantial mortalities due to these infections (5).

Both APEC and human ExPEC contained some common virulence markers which suggested APEC as a potential source of UPEC causing UTIs or other infections in human (6,7). The potential relationship between APEC and human ExPEC strains has been evaluated with some genomic approaches such as PCR-based phylotyping, Multilocus Sequence Typing (MLST), Multilocus Variable Number Tandem Repeat Analysis (MLVA), Enterobacterial Repetitive Intergenic Consensus Sequence 2 PCR (ERIC2 PCR) fingerprinting, genomic subtractive hybridization, Random Amplified Polymorphic DNA (RAPD) analysis and Pulsed-Field Gel Electrophoresis (PFGE) (6,8-13). Among these methods, PFGE is commonly considered as a gold standard method for the epidemiological evaluations and molecular subtyping of microorganisms (14).

There are many reports about rising UTIs due to ESBL-producing *Enterobacteriaceae* in different parts of the world (15,16). Among the livestock and animal products, poultry and poultry products had the highest prevalence of ESBL-producing *Enterobacteriaceae* (17) and there was some evidence about the rule of poultry products in dissemination of ESBL producing *E. coli* in human UTIs (18).

In the present study, antibiotic resistance, production of ESBLs and clonal relationships were evaluated in APEC isolates from six farms. In addition, clonal relationships between these isolates and ESBL producing UPEC isolates from the same geographical area have been investigated with PFGE.

Methods

Sample collections and E. coli isolation

Six broiler chicken flocks with typical necropsy signs of avian colibacillosis were included in this cross-sectional study during Sep 2015-May 2016. These flocks from different geographical regions of Shiraz County were referred to Poultry Diseases Research Center, Shiraz University, Shiraz, Iran. After necropsy of each flock, four pericardial swabs from four different chickens with visible signs of pericarditis were obtained and collected in a sterile tube containing tryptic soy broth (TSB). The samples were cultured on EMB and MacConkey agar and 24 bacterial isolates identified as *E. coli* by IMViC test (19). In addition, 97 inpatient and outpatient ESBL producer *E. coli* isolates from UTIs obtained from microbiology laboratory of referral Shahid Faghihi hospital in Shiraz city, Iran. These isolates had recently isolated from patients after obtaining informed consent and identified as ESBL producing *E. coli* (unpublished data).

Antimicrobial susceptibility test and detection of ESBL production

Resistance of 24 APEC and 97 UPEC isolates against 12 antimicrobial agents (Table 1) was investigated using the disk diffusion method (MAST antibiotic discs, MAST, UK) in Mueller-Hinton agar (Merck, Darmstadt, Germany) following Clinical and Laboratory Standards Institute (CLSI) guidelines (20). ESBL production was detected using the double-disk diffusion (DDD) method using the following antibiotic disks (MAST, UK): cefotaxime (30 µg), cefotax-

ime/clavulanic acid (30/10 µg), ceftazidime (30 µg) and ceftazidime/clavulanic acid (30/10 µg) (21). Quality control was performed using the *E. coli* ATCC 25922 reference strain.

Pulse Field Gel Electrophoresis

All of the broiler and human isolates were typed by PFGE technique based on PulseNet One-Day (24–28 h) Standardized Laboratory Protocol (22). Briefly, Few pure colonies of *E. coli* isolates and *Salmonella ser. Braenderup* H9812 (standard marker) were embedded within agarose plugs. XbaI restriction endonucleases (10 U/µl-1) were used for digestion of genomic DNA according to the manufacturer recommendations (Thermo Scientific, Waltham, MA). Obtained restriction fragments separated by the CHEF-DRR III System (Bio-Rad Laboratories, Hercules, CA) using 1% X174 Agarose LFTM (Amresco, Solon, OH, USA), with switch times of 6.7–35.4 sec, a voltage of 6 V and an involved angle of 120° at 14°C for 19–20 h. Gels were stained with GelRed™ (Biotium Inc., UK) and imagined with UVItec Gel Documentation System (UVItec Ltd., Cambridge, UK). PFGE profiles were analyzed by Bionumerics software version 7.1 (Applied Maths, Sint-Martens-Latem, Belgium). The Dice similarity coefficient with a UPGMA dendrogram was produced based on 1.5% tolerance windows and 1.5% optimization. Eighty percent cutoff lines were considered to analyze genetic relatedness.

Statistical analyses

The chi-square test and Fisher's exact test were applied to evaluate antimicrobial susceptibility between chicken and human isolates. All results were statistically analyzed at the $P<0.05$ level of confidence using the SPSS 16.0 statistical software (SPSS Inc., Chicago, Illinois).

Ethics Approval

The study was approved by the Ethics Committee of the Faculty of Medicine, Shiraz University.

Results

Antimicrobial susceptibility test and detection of ESBL production

Antimicrobial resistance and susceptibility of 24 APEC and 97 UPEC isolates against 12 important antibiotics in UTIs were shown in Table 1. Low resistance to beta-lactams and aminoglycosides and high resistance to Nalidixic acid, Ciprofloxacin, Nitrofurantoin and Trimethoprim-Sulfamethoxazole were observed in APEC isolates. High percentages of antibiotic resistance were seen in UPEC isolates.

Table 1: antimicrobial resistance of 24 non- ESBL APEC and 97 ESBL human UTI isolates against 12 routinely used antibiotics in UTIs

	Chicken isolates (non-ESBL)		*Human isolates (ESBL)*		
	Susceptible	*Resistant*	*Susceptible*	*Resistant*	
Antimicrobial agents	**No. (%)**	*No. (%)*	*No. (%)*	*No. (%)*	**P** *value*
Cefixime	21 (87.5)	3 (12.5)	3 (3.1)	94 (96.9)	< 0.001
Cefotaxime	18 (75)	6 (25)	1 (1.0)	96 (99.0)	< 0.001
Ceftriaxone	21 (87.5)	3 (12.5)	2 (2.1)	95 (97.9)	< 0.001
Ceftazidime	24 (100)	0 (0)	12 (12.4)	85 (87.6)	< 0.001
Cephalexin	19 (79.2)	5 (20.8)	1 (1.0)	96 (99.0)	< 0.001
Imipenem	21 (87.5)	3 (12.5)	13 (13.4)	84 (86.6)	< 0.001
Nalidixic acid	0 (0)	24 (100)	11 (11.3)	86 (88.7)	0.119
Ciprofloxacin	1 (4.2)	23 (95.8)	21 (21.6)	76 (78.4)	0.072
Amikacin	17 (70.8)	7 (29.2)	59 (60.8)	38 (39.2)	0.481
Gentamycin	24 (100)	0 (0)	64 (66.0)	33 (34.0)	< 0.001
Nitrofurantoin	1 (4.2)	23 (95.8)	72 (74.2)	25 (25.8)	< 0.001
Trimethoprim-Sulfamethoxazole	4 (16.7)	20 (83.3)	4 (4.1)	93 (95.9)	0.049

So that, cephalosporins, imipenem, gentamycin, nitrofurantoin and trimethoprim-sulfamethoxazole resistance were significantly higher in ESBL producing human UTIs *E. coli* isolates in comparison with APEC isolates (P<0.05). None of the APEC isolates were phenotypically ESBL producer.

Pulse Field Gel Electrophoresis

Twenty four chicken *E. coli* isolates were clustered into five PFGE groups with 80% similarity. The main cluster included 5 isolates and also there were 6 single clones. *Escherichia coli* isolates from same farm showed high clonal relationships together. In contrast, most of the isolates from different farms were unrelated *E. coli* clones (Fig. 1). Comparison of chicken and human *E. coli* isolates showed a large clonal variety. However, there was a chicken isolate with 80% similarity and another isolate with 78% similarity to a human inpatient UTI isolate (Fig. 2).

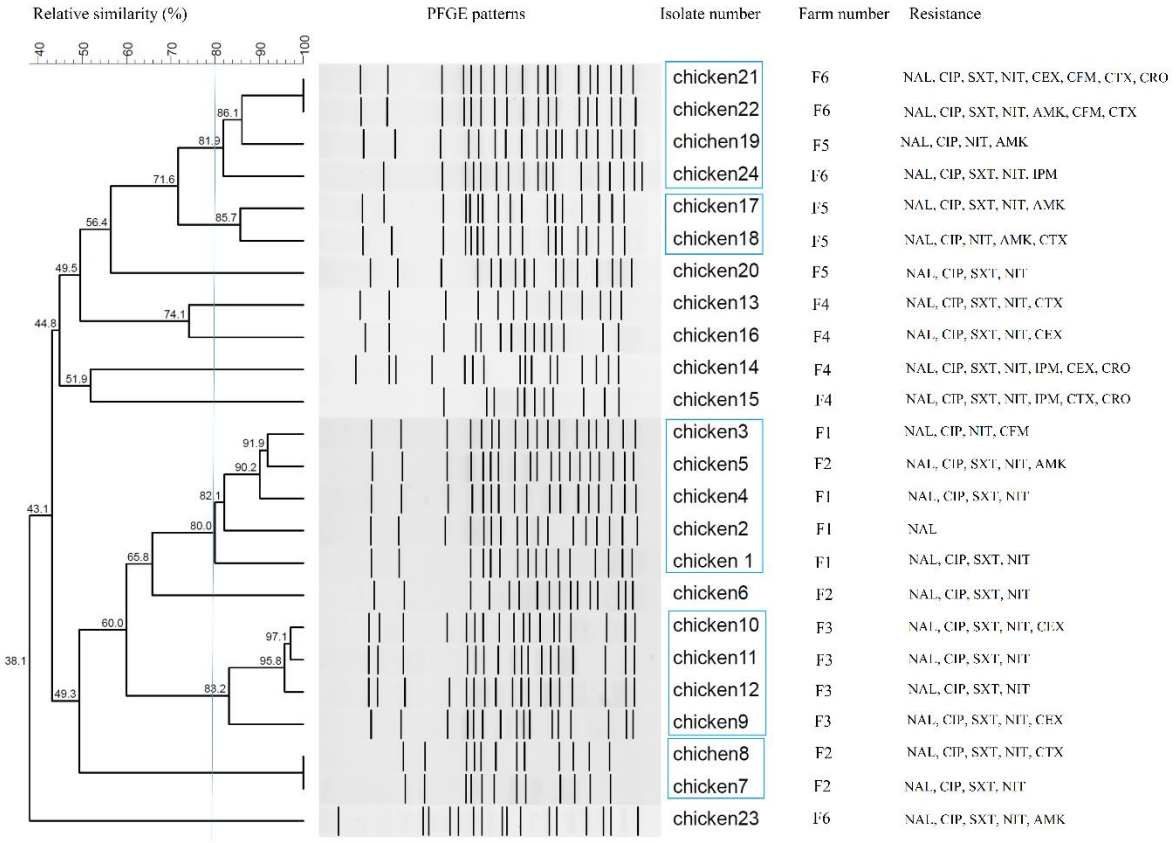

Fig. 1: PFGE patterns and dendrogram of APEC isolated from broiler chickens with colibacillosis. Boxes indicate clusters (isolates with >80% similarity by Dice coefficient similarity analysis)

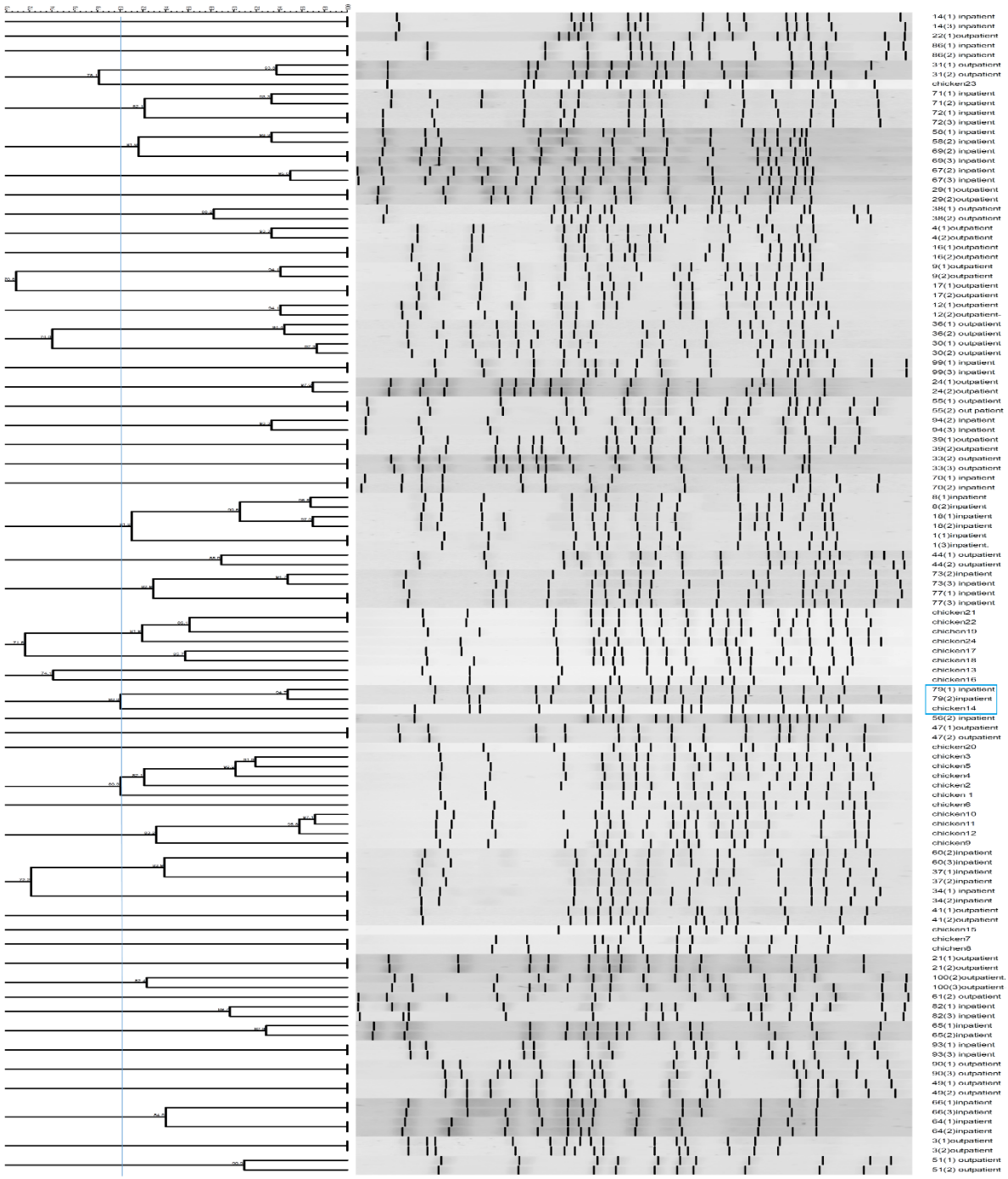

Fig. 2: PFGE patterns and dendrogram of 24 APEC and 97 UPEC isolates. Boxes indicate clusters (isolates with >80% similarity by Dice coefficient similarity analysis)

Discussion

In this study, there were high antimicrobial resistance against fluoroquinolones (nalidixic acid and ciprofloxacin) and trimethoprim-sulfamethoxazole in APEC and UPEC isolates. Fluoroquinolones such as flumequine, enrofloxacin and norfloxacin were widely used in poultry

farms in Iranian poultry industry (23). The high prevalence of quinolone-resistant *E. coli* isolates in broiler chickens coincides with high prevalence of fluoroquinolone resistance among human clinical *E. coli* isolates and broiler chickens could be considered as a source of human fluoroquinolone-resistant *E. coli* (24, 25). There were similar trends about trimethoprim-sulfamethoxazole, which massively used in Iranian poultry farms (26, 27).

Easy transfer of trimethoprim-sulfamethoxazole resistance factor has been shown among chicken *E. coli* isolates (28). Food-producing animals could be an important reservoir for integrons carrying trimethoprim-sulfamethoxazole resistance determinants (29). Nitrofurantoin resistance was also high in *E. coli* isolated from broilers in our study. Nitrofurans are forbidden for poultry usage in Iran. However, there was evidence about high illegal use of furazolidone, a banded member of this antibiotic family for animal usages, in Iranian poultry farms (27). Nitrofuran metabolites as residues via poultry products could produce mutagenic or carcinogenic side effects and may transfer antibiotic resistance to human microbial pathogens (30). In contrast with chicken *E. coli* isolates, nitrofurantoin resistance was low in UPEC isolates in this study. Nitrofurantoin could be used as one of the first drug of choice for the prophylaxis of recurrent urinary tract infections and treating uncomplicated UTIs. However, due to its inability to achieve therapeutic blood concentrations, this antibiotic is used only for the treatment of uncomplicated UTIs and not recommended for complicated UTIs and systemic infections (31).

Resistance against aminoglycosides and beta-lactam antibiotics was relatively low in APEC isolates in this study. Gentamicin and amikacin are not available for mass application in Iranian poultry farms. Then, absent of selection pressure for aminoglycosides could explain the low resistance against these antimicrobial agents in APEC *E. coli* isolates (32). Beta-lactam antibiotics such as cephalosporins and carbapenems were rarely used in Iranian poultry farms (26,27). However, there was evidence about presence of beta-lactamases genes in broiler chickens of Iran (33,34). Because of suggestive reports about potential transmission of ESBL genes and plasmids from poultry *E. coli* isolates to humans, public health concerns about this situation should not be overlooked (35,36).

Clonal relationship between antibiotic resistance patterns and identified PFGE clones has been shown in APEC isolates in previous studies (8,37). However, different patterns of antibiotic resistance has been shown in *E. coli* isolates of same cluster in our study. Based on PFGE data, five clusters were identified in chicken *E. coli* isolates, with an association observed between the origin of these isolates and the PFGE patterns. That is, most of the isolates assigned to the same farm had similar or very closely related PFGE patterns. *Escherichia coli* isolates of the same farm exhibiting identical PFGE patterns indicate the clonal spread of APEC strains in poultry farms. Clonal spread of *E. coli* isolates from broiler breeders to respective broiler progenies has been shown by PFGE (3). Poulsen et al (38) used PFGE for longitudinal study of *E. coli* transmission from broiler breeders to broilers and showed that specific pathogenic clones of *E. coli* originating from broiler breeders could transmitted to the broilers and horizontally transmission among the newly hatched chicks could multiply these special isolates in the hatcheries. In our study, clonal relationship between *E. coli* isolates from different farms has been found for two isolates in two different clusters.

Diversity of multi-drug resistant APEC was estimated in outbreaks of broiler colibacillosis in Spain and identified different PFGE restriction profiles among *E. coli* isolates of different farms (39). Only two epidemiologically related isolates were found in study which belonged to the same farm (39). Same PFGE patterns or clusters were also recovered from the same chicken farms in ESBL producing *E. coli* isolates in Chinese poultry farms (40).

There were experimental evidence, virulence gene similarities and comparative genomics between APEC and UPEC suggested the zoonotic potential of APEC (18). In this study, a non-ESBL

APEC isolate exhibited 80% PFGE profile similarity to two ESBL producing inpatient *E. coli* isolates. Moreover, another chicken isolate exhibited 78% similarity to two outpatient *E. coli* isolates. By contrast, no correspondence was identified at the 80% similarity level between outpatient and inpatient UTIs *E. coli* isolates (not shown). Similarity between human and chicken *E. coli* isolates was evaluated in relation to ciprofloxacin resistance status and found just one instance of a close match, by PFGE analysis (41). However, they emphasized on extensive genomic diversity within *E. coli* because of a close PFGE profile match between isolates from hosts with no apparent association (41). In contrast, there were other reports about distinction of ciprofloxacin-resistant *E. coli* strains from humans from ciprofloxacin-resistant avian strains, based on their phylogenetic backgrounds and Virulence Genotype (9). *E. coli* Strains isolated from children and chickens living in close contact were analyzed and showed that clusters of *E. coli* which were prevalent among the chicken flocks were distinct from those in children (11).

There were rising reports about chicken products as food reservoirs for human extra-pathogenic *E. coli* (ExPEC) infections in many countries such as United States (10), Iceland (25), Canada (13), Netherland (42), Italy (43) and Denmark (44). The continuous presence of specific human ExPEC strains in poultry or poultry products and rare observation of these strains in other animal products introduced the poultry as a potential reservoir for human ExPEC (18).

Conclusion

Genetically related strains caused the outbreaks of colibacillosis within each broiler farms. In contrast, there were different PFGE restriction profiles among the majority of *E. coli* isolates of different farms. Based on PFGE patterns, two *E. coli* isolates from chicken colibacillosis lesions had closely related PFGE patterns with human UTIs sampled during the same period and in the same geographic area as the chicken isolates. This result reminded the controversial hypothesis that avian colibacillosis *E. coli* strains could be considered a zoonosis.

Ethical considerations

Ethical issues (Including plagiarism, informed consent, misconduct, data fabrication and/or falsification, double publication and/or submission, redundancy, etc.) have been completely observed by the authors.

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Conflict of interest

The authors declare that there is no conflict of interests.

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