Full Length Research Paper

Antimicrobial susceptibility and virulence factors of *Escherichia coli* isolates obtained from faeces samples of chickens in east China

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Accepted 11 January, 2012

The study was conducted to determine the antibiotic resistance and virulence factors profile of *Escherichia coli* isolated from chicken faeces sample. Differential and selective media were used to isolate a total of 164 *E. coli* strains from 180 faeces samples at different chicken farm in east China. These strains were tested against 15 antibiotics using the agar disc diffusion. The isolates showed high resistance rates to sulfamethoxazole (91.5%), nalidixic acid (86%), tetracycline (85.4%), streptomycin (80.5%), ampicillin (79.9%), Trimethoprim-sulfamethoxazole (74.4%), Enrofloxacin (67.1%), Ciprofloxacin (56.7%), Norfloxacin (54.9%) and Chloramphenicol (51.2%). Rates of resistance to Amoxicillin/clavulanic acid, Gentamicin and Florfenicol were moderate (36, 37.8 and 14.6%), while low rates were recorded for Amikacin (9.2%) and Cefalexin (5.5%). Among the 164 isolates, 36 (23.7%) were haemolytic, 42 (27.6%) were hydrophobic, 132 (86.8%) were serum resistant and only four were positive for protease. Eleven virulence-associated genes, including *iucA*, *tsh*, *iss*, *fimH*, *sitA*, *hlyF*, *iron*, *traT*, *cdtB*, *ibeA*, and *cvaC*, were screened for via polymerase chain reaction (PCR) amplification. The *fimH* gene was the most prevalent with a detection rate of 86.6%, followed by *traT* (58.5%), *iron* (29.3%), and *iss* (25%) in these avian isolates.

Key words: *Escherichia coli*, antimicrobial resistance, virulence, chickens.

INTRODUCTION

*Escherichia coli* is a major pathogen of commercially produced poultry causing colibacillosis all over the world (Barnes et al., 2008). It is a major cause of respiratory and septicemic diseases in broiler chicken causing mortality, less than 5% and morbidity over 50% and also affects layers resulting failure of productivity and fertility of eggs (Barnes et al., 2008), which may result in significant economic losses to the poultry industry. Virulence factors for avian pathogenic *Escherichia coli* (APEC) include adhesins, ferric transport system, hemolyzation, toxin-production, exotoxins, type 1(F1A) and P(F11) fimbriae, curli, aerobactin, K1 capsule, and temperature-sensitive hemagglutinin (Tsh) and drug resistance plasmid (La Ragione et al., 2002; Mellata et al., 2003; Yaguchi et al., 2007).

Approaches to prevent and control APEC infections in the poultry industry includes, improved hygienic methods, vaccination, use of competitive exclusion products, and the introduction of novel immunopotentiators. However, each of these practices have had limited success (Gomis et al., 2003; La Ragione et al., 2004). This has necessitated the use of antimicrobial chemotherapy to control outbreaks of colibacillosis. Antibiotics are used by the poultry industry and poultry veterinarians to enhance growth and feed efficiency and reduce disease. This practice leads to the inevitable selection of antimicrobial...
resistance among commensals in the intestinal tracts of food animals, which poses a public health threat. For instance, antimicrobial-resistant bacteria from food animals may colonize the human population via the food chain, contact through occupational exposure, or waste runoff from animal production facilities (Witte, 1998). Food animals, in particular mature cattle, which may be asymptomatic carriers of \textit{E. coli} O157, including STEC, when exposed to antimicrobial agents in the animal production environment, may serve as a reservoir of antimicrobial-resistant bacteria.

The present study retrospectively examined antimicrobial susceptibility profiles and virulence factors among a collection of \textit{E. coli} recovered from different poultry farms (at least 5 km apart) between April, 2009 and September, 2010 located in eastern China, to provide a baseline of antimicrobial resistance among these pathogens for future studies.

\textbf{MATERIALS AND METHODS}

\textbf{Collection of fecal samples}

Fresh fecal samples from healthy or diseased chicken were collected aseptically on different poultry farms (Figure 1) (at least 5 km apart) between April, 2009 and September, 2010 located in eastern China. Not more than five samples were taken from the same farm of origin. All the specimens were kept in CITOSWAB medium (CITOTEST LABWARE MANUFACTURING Co., Ltd., Haimen City, China) at 4°C during transportation to the laboratory for \textit{E. coli} isolation within 2 days. Information on the use of antimicrobial agents at the farms where samples were collected for this study was not available.

\textbf{Isolation and identification of \textit{Escherichia coli}}

Feces were directly plated on Chromogenic \textit{E. coli} coliform (CECC) agar (Oxoid Ltd., Basingstoke, Hants, England) and incubated at 37°C overnight. Purple colonies picked from the CECC agar plates and typical of \textit{E. coli} were streaked onto eosin methylene blue (EMB) agar (Beijing Aoboxing Bio-tech Co. Ltd, Beijing, China) which were incubated at 37°C for 18 to 20 h. Colonies with a metallic sheen on EMB agar were picked and streaked onto MacConkey agar (Beijing Aoboxing Bio-tech Co. Ltd, Beijing, China). After overnight incubation at 37°C, one or two typical pink colonies were selected from each MacConkey agar plate and the isolates were tested by the indole, methyl red, Voges–Proskauer, and Simmons citrate (IMViC) tests for confirmation of \textit{E. coli}. API 20 E test strips (bioMerieux, Shanghai, China) were also used to confirm presumptive isolates as \textit{E. coli}.

\textbf{Antimicrobial susceptibility testing}

The antimicrobial sensitivity test of each isolate was carried out by the Kirby-Bauer disc diffusion method (Bauer et al., 1966) as recommended by the National Committee for Clinical Laboratory Standards (NCCLS, 2002). The following antimicrobials were tested: Nalidixic (NAL), Tetracycline (TE), Sulfamethoxazole (SMZ), Trimethoprim-sulfamethoxazole (SXT), Ampicillin (AM), Amoxicilne (AMX), Streptomycin (S), Chloramphenicol (C), Gentamicin (GM), Kanamycin (K), Cefalexin (CF), Amoxicilne-clavulanic acid (AN) and Florenicol (FFN), Norfloxacin, Ciprofloxacina, Ernoloxacin. Cartridges of antimicrobial containing discs were obtained from Shanghai Yihua Medical Science and Technology Co., Ltd. (Shanghai, China), stored between 4 and -20°C, and allowed to come to room temperature prior to use. Isolates were subcultured from the bank onto Miller's LB agar flasks and incubated for 18 to 24 h before being transferred to 5 ml sterile 0.9% saline to match the '0.5' MacFarland standard (Shanghai Ruqi Biological Technology Co. Ltd, Shanghai, China). A sterile cotton-tipped swab was used to streak air-dried Mueller-Hinton II plates within 15 min of adjustment of turbidity. Subsequently, antimicrobial discs were added and plates were incubated aerobically at 35±2°C for 16 to 18 h. The diameter of the zone of inhibition surrounding the antimicrobial discs was measured to the nearest mm. Isolates were deemed resistant only when the zone of inhibition was less than or equal to the resistance breakpoint recommended by the NCCLS guidelines. Quality control was performed as recommended using \textit{E. coli} strain ATCC 25922.

\textbf{Virulence factors determinations}

\textbf{Cell surface hydrophobicity}

The cell surface hydrophobicity of \textit{E. coli} was determined by salt aggregation test (SAT) (Raksha et al., 2003). Briefly, one loopful (10 µL) of bacterial suspension made in phosphate buffer was mixed with equal volume of ammonium sulphate solution of different molarity, that is, from 0.3125 m through 5.0 m, on a glass slide and observed for 1 min while rotating. The highest dilution of ammonium sulphate solution giving visible clumping of bacteria was scored as the salt aggregation test (SAT) value. Strains showing aggregation in 0.002 m phosphate buffer alone (pH 6.8) were considered autoagglutinative. \textit{E. coli} strains that had SAT value ≤1.25 m were considered hydrophobic (Raksha et al., 2003).

\textbf{Haemolysin production}

Haemolysin was detected by the presence of a zone of lysis around the colony on Blood agar plate (Siegfried et al., 1994). Briefly, the bacteria were inoculated onto 5% sheep blood agar and incubated overnight at 37°C. Haemolysin production was detected by the presence of a zone of complete lysis of the erythrocytes around the colony and clearing of the medium.

\textbf{Serum resistance}

Serum resistance was studied according the the procedures of Siegfried et al. (1994), overnight cultures of \textit{E. coli} grown at 37°C on Mueller-Hinton agar (MHA) were harvested and the cells were suspended in Hank’s Balanced Salt Solution (HBSS). The wells of microtitration plate were used for incubation of bacterial suspension with serum. 0.05 mL of bacterial suspension and 0.05 mL of serum were added to each well. Control wells contained 0.05 mL of HBSS instead of serum. The plate was placed on shaker water bath and rotated for 3 h at 37°C. 10 µl of each sample was withdrawn and spread on MHA plates simultaneously, 10 µl of bacterial suspension without serum was spread onto MHA plates and incubated for 18-24 h at 37°C and viable count was determined. Strains were termed serum sensitive if the viable count dropped to 1% of the initial value and resistant if >90% of organism survived after a 3 h incubation period.

\textbf{Protease test}

Protease production was tested using milk agar (Alane et al., 1996).
Detection of virulent related genes

The isolates were then assigned to investigation for 11 virulence related genes by polymerase chain reaction assays (the primer are list in Table 2), as previously described. Chromosomal DNA from *E. coli* isolates was prepared using a Wizard genomic DNA purification kit (Takara, Dalian, China). The PCR was performed in a 50 µl volume consisting of a 0.25 mM concentration of each deoxyribonucleotide, 1.5 mM MgCl₂, 1U of Gold Taq DNA polymerase, and 50 pmol of each primer. The PCR was carried out using a thermal cycler (Perkin-Elmer, Vienna, Austria) with an initial denaturing cycle at 95°C for 10 min; followed by 30 cycles of 94°C for 30 s, 55°C for 45 s, and 72°C for 45 s; and a final extension step at 72°C for 7 min (Yang et al., 2004). The amplification products were resolved by electrophoresis in a 1.0% agarose gel and visualized under UV light.

RESULTS

Antimicrobial susceptibility

A total of 164 *E. coli* strains were isolated from 180 faeces sample. All the 164 isolates were tested for their susceptibility to antimicrobial agents of human and veterinary importance in accordance with NCCLS methods (Table 1). All the isolates demonstrated high rates of resistance to Sulfamethoxazole (91.5%), Nalidixic acid (86%), Tetracycline (85.4%), Streptomycin (80.5%), Ampicillin (79.9%), Gentamicin (37.8%). Due to the high incidence of quinolone resistance, all isolates were also tested against a panel of veterinary-use and human-use fluoroquinolones. Among these isolates, fluoroquinolone resistance ranged between 54.9 to Norfloxacin, 56.7% to Ciprofloxacin, and 67.1% to Enrofloxacin. *E. coli* isolates displayed elevated levels of resistance to all fluoroquinolones tested (Table 1).

Prevalence of virulence-associated genes in *E. coli* isolates

Virulence factors such as haemolysin, surface hydrophobicity, serum resistance and protease were studied for all the isolates. The most common virulence factor identified was serum resistance in 138 (84.2%) isolates. Haemolysin was produced by 40 (24.4%) isolates, 48 strains (29.3%) were hydrophobic and only 15 (9.2%) produced protease.

The prevalence of virulence-associated genes in 164 isolates is shown in Table 2. Among 13 virulence associated genes tested, the most prevalent is *fimH* (86.6%) and the least is *tsh* (4.9%). Among the 164 *E. coli* isolates, 6 isolates harbored 10 virulence-associated genes, 132 isolates (80.5%) had two or more virulence-associated genes (Table 2).

DISCUSSION

Epidemiological surveillance of antimicrobial resistance is indispensable for empirical treatment of infections,
implementing control measures, and preventing the spread of antimicrobial resistant microorganisms (Goosens and Sprenger, 1998). In this study, we examined antimicrobial resistance in *E. coli* isolates from chicken farms in the east of China. Because of the geographic sampling of the avian isolates, this surveillance provides a representative sample of the resistance trends in the east Chinese poultry industry. Similar to the findings of previous studies in China (Dai et al., 2008; Lei et al., 2010), most *E. coli* isolates of avian origin described here were highly resistant to Sulfamethoxazole, Nalidixic acid, Tetracycline, Streptomycin, Ampicillin, Trimethoprim-sulfamethpxazole, Enrofloxacin, Ciprofloxacin, Norfloxacin and Chloramphenicol. The frequent use of antimicrobials as therapy or feed additives in animals selected high antimicrobial resistance in bacteria. It was noteworthy that our study revealed very high rates of Nalidixic acid (86%), Ciprofloxacin (56.7%) and Norfloxacin (54.9%) resistance in *E. coli* isolates recovered from poultry. The high resistance to fluoroquinolone drugs had also been reported in other studies in China, where more than 60% of *E. coli* isolated from animals and human were resistant to different fluoroquinolone drugs (Yang et al., 2004; Dai et al., 2008). This may reflect the generally more extensive use of quinolones as feed additives and for treatment of bacterial diseases. The high rates of fluoroquinolones resistance strains detected are indeed of public health concern. Though, there was no direct evidence to support the association, it is urgently needed to limit the usage of fluoroquinolone in animals especially as feed additives in China. Virulence factors enable *E. coli* to colonise selectively the mucosal epithelium, evoke an inflammatory reaction and eventually proceed to tissue invasion. The capacity of *E. coli* to produce many virulence factors contributes to its pathogenicity. These virulence factors enable some members of the normal flora to elicit an infection by overcoming the host defence mechanisms. Pilus is the main virulent factor involved in adhesion of pathogenic *E. coli* to the host cells, including type 1 pilus, P pilus, and curli. The subunit of type 1 pilus encoded by *fimH* gene is located at the tip of the fimbria as well as laterally in the fimbrial structure. *FimH* binds to mannose-containing glycoprotein receptors and can mediate bacterial attachment to a variety of different host cell types (Mulvey et al., 1998). In this experiment, PCR detection showed that 86.6% of the isolates contained *fimH* gene among 164 isolates tested, which was consistent with previous reports (Ewers et al., 2004). These data suggest that type 1 pilus is present in most of the isolates from the poultry farms studied. High pathogenicity islands (HPI) is related

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### Table 1. Percentages of antimicrobial resistance in *Escherichia coli* by NCCLS disc diffusion methods.

<table>
<thead>
<tr>
<th>Antimicrobial agent</th>
<th>Amount/disc (µg)</th>
<th>Diffusion zone breakpoint (mm)</th>
<th>% Resistant Strains (n = 164)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Penicillins</strong></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Ampicillin</td>
<td>10</td>
<td>≤13</td>
<td>79.9 (131/164)</td>
</tr>
<tr>
<td>Amoxicillin/clavulanic acid</td>
<td>20/10</td>
<td>≤13</td>
<td>36 (59/164)</td>
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<tr>
<td><strong>Cephalosporins</strong></td>
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<td></td>
<td></td>
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<tr>
<td>Cefalexin</td>
<td>30</td>
<td>≤14</td>
<td>5.5 (9/164)</td>
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<tr>
<td><strong>Aminoglycosides</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Streptomycin</td>
<td>10</td>
<td>≤11</td>
<td>80.5 (132/164)</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>10</td>
<td>≤12</td>
<td>37.8 (62/164)</td>
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<tr>
<td>Amikacin</td>
<td>30</td>
<td>≤14</td>
<td>9.2 (15/164)</td>
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<tr>
<td><strong>Quinolones/fluoroquinolones</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nalidixic acid</td>
<td>30</td>
<td>≤13</td>
<td>86 (141/164)</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>5</td>
<td>≤15</td>
<td>56.7 (93/164)</td>
</tr>
<tr>
<td>Enrofloxacin</td>
<td>5</td>
<td>≤12</td>
<td>67.1 (110/164)</td>
</tr>
<tr>
<td>Norfloxacin</td>
<td>10</td>
<td>≤10</td>
<td>54.9 (90/164)</td>
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<tr>
<td><strong>Sulfonamides</strong></td>
<td></td>
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<tr>
<td>Sulfamethoxazole</td>
<td>250</td>
<td>≤12</td>
<td>91.5 (150/164)</td>
</tr>
<tr>
<td>Trimethoprim-sulfamethoxazole</td>
<td>1.25/23.75</td>
<td>≤10</td>
<td>74.4 (122/164)</td>
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<tr>
<td><strong>Phenicols</strong></td>
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</tr>
<tr>
<td>Chloramphenicol</td>
<td>30</td>
<td>≤12</td>
<td>51.2 (84/164)</td>
</tr>
<tr>
<td>Florfenicol</td>
<td>30</td>
<td>≤21</td>
<td>14.6 (24/164)</td>
</tr>
<tr>
<td><strong>Tetracyclines</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tetracycline</td>
<td>30</td>
<td>≤14</td>
<td>85.4 (140/164)</td>
</tr>
</tbody>
</table>
to ferric absorption and up-take. The *iucA* and *iron* are two iron-related genes. In our study, 40.3% isolates were *iron* and *iucA*-positive. The high prevalence and sequence homology of HPI genes confirm the existence of possible horizontal transmission mechanisms in pathogenic *E. coli* from different origins (Schmidt and Hensel, 2004). Haemolysin production is associated with pathogenicity of *E. coli*, especially the more severe forms of infection. In the present study, a few strains of *E. coli* (24.4%) produced haemolysin detected by blood agar plates, a possible source of multidrug resistant isolates in humans.

**REFERENCES**


