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Full Length Article

Detection of Resistance Phenotype and Gene of Avian *Escherichia coli* to β-lactam Antibiotics

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Abstract

Understanding and detecting β-lactam antibiotics resistance and resistance genes and elucidating the drug-resistant mechanism is of significance to prevention and treatment of avian *Escherichia coli* disease. In this study, the ESBLs (Extended Spectrum β-Lactamases) producing strains were screened from 41 *E. coli* isolates by double paper disk test; the β-lactam antibiotics resistance was investigated by Kirby–Bauer (K-B) drug susceptibility test; and three ESBLs genes including TEM, SHV and CTX-M were tested by Polymerase Chain Reaction. The results showed that there were 31 (75.6%) ESBLs producing strains. The *E. coli* had highest resistance to penicillin and first generation cephalosporin followed by to second and third generation cephalosporins and monocyclic lactams. PCR revealed 73.2% (30/41) of *E. coli* isolates carrying ESBLs gene. The positive rate of TEM, CTX-M and SHV were 63.4% (26/41), 31.7% (13/41) and 4.9% (2/41), respectively. There were 10 strains (24.4%) carrying more than two kinds of resistance genes. In conclusion, the drug resistance is directly related to the clinical use time and range of β-lactam antibiotics. The antibiotics resistance *of E. coli* isolated from avian to β-lactam in North China is mainly mediated by TEM and CTX-M genes. © 2017 Friends Science Publishers

Keywords: Avian *Escherichia coli*; β-lactam antibiotics; ESBLs gene; PCR

Introduction

In current animal husbandry, the prevalence of bacterial disease becomes more and more serious due to expansion of animal breeding scale, increase of raising density and stress factors. *Escherichia coli* is a major pathogen that can cause animal diarrhea and even lead to death of pigs and chickens. The antibiotics are often used for prevention and treatment of colibacillosis. The β-lactam antibiotic including penicillins, cephalosporins, cephamycins, monocyclic lactams and other atypical class refers to a large class of antibiotics containing the β-lactam ring structure. The β-lactam antibiotic is the most common antibacterial drug used by veterinarians. Due to the abuse of antibiotics and resistance gene transfer between bacteria, the drug resistance of bacteria is becoming more and more serious.

The main resistance mechanism of *E. coli* to β-lactam antibiotic is that it can produce ESBLs which can destroy the β-lactam ring through hydrolysis or non-hydrolysis and inactivate the antibiotic (George *et al.*, 1991). The production of ESBLs enzyme is mainly mediated by the resistance genes located on chromosome and plasmid. As a result of the ESBLs gene and other resistance genes coexisted in one plasmid, the ESBLs gene is often involved in bacterial multi-drug resistance (De Champs *et al.*, 2000). It has caused great difficulties to the clinical treatment of infectious diseases. At present, five types of ESBLs genes have been found including TEM, SHV, CTX-M, OXA and other type (Yuan *et al.*, 2010). The bacteria producing ESBLs have different genotypes in different regions. In the United States and South Korea，the TEM and SHV are the main genotypes; SHV-2 and SHV-5 are mainly in Germany; CTX-M is mainly in Europe; and TEM, SHV and CTX-M are mainly in China (Ye *et al.*, 2010). To monitor the β-lactam antibiotics resistance and to find out the mechanisms as well as to guide clinical medication, this study detected the β-lactam antibiotics resistance and ESBLs gene of avian *E. coli* isolated from North China.

Materials and Methods

Reagents

MHA medium and MacConkey medium, drug-sensitive paper discs were purchased from Hangzhou Tianhe Microorganism Reagent Co., Ltd (Hangzhou, China). PCR kits, *Taq* DNA polymerase, DNA Markers, dNTPs, agarose, Gold-view dye and other reagents were purchased from TaKaRa Biotechnology (Dalian) Co., Ltd. (Dalian, China).

Isolation and Identification of *E. coli*

The livers, feces, pericardial fluid and other pathological materials of dead chickens were collected from North China

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under sterile conditions, including Beijing, Hebei and Shanxi regions. The collected specimens were diluted with sterile saline and then cross-inoculated in MacConkey medium. After culturing for 12 to 16 h at 37℃, the single pink colony was picked and stained using Gram staining. After purification repeatedly, there were 41 isolates identified as avian *E. coli* by biochemical tests.

Screening and Confirmatory Tests for ESBLs

The screening of ESBLs producing strains was performed on the MHA medium by using disc diffusion method following Clinical and Laboratory Standards Institute (CLSI, 2011). Control strain *E. coli* ATCC25922 was supplied by China Institute of Veterinary Drugs Control (Beijing, China). The ceftazidime (CAZ), aztreonam (AZT), cefotaxime (CTX) and ceftriaxone (CRO) were used for initial screening test. The inhibition zone diameter of CAZ ≤ 22 mm, AZT ≤ 27 mm, CTX ≤ 27 mm and CRO ≤ 25 mm indicated the ESBLs production. The ESBLs phenotype was also confirmed by phenotypic confirmatory test. Two groups of drug-sensitive paper discs included (1) CAZ and CAZ plus clavulanic acid (CLA); (2) CTX and CTX plus CLA (single drug for 30 μg/piece; compound drug for 30/10 μg/piece). An increase of ≥ 5 mm for inhibition zone diameter of either CAZ or CTX in the presence of CLA confirmed the ESBLs production.

Drug Susceptibility Test

The antibiotics investigated in the study included penicillins (ampicillin, carbenicillin and piperacillin), 1st generation cephalosporins (cephalothin), 2nd generation cephalosporins (cefuroxime, cefoxitin and cefaclor), 3rd generation cephalosporins (ceftriaxone, cefotaxime and ceftazidime), 4th generation cephalosporins (cefepime) carbapenems (imipenem) and monocyclic lactams (aztreonam). Drug susceptibility tests were done by using K-B method. The test liquid was coated on the MHA medium followed by placement of drug sensitive paper discs on the medium. After incubation for 24 h at 37°C, the inhibition zone diameters were measured and drug resistance was calculated following CLSI (2011).

PCR Amplification of β-lactam Antibiotic Resistance Genes

The primers were designed (Table 1) according to Ye *et al.* (2010) and synthesized by Beijing Sunbiotech Co. Ltd., (Beijing, China). PCR was carried out using MyCycler thermal cycler (Bio-Rad, Hercules, CA) with boiling method to get the bacterial chromosomal DNA (Sambrook and Russell, 2008). PCR reaction was conducted in 50.0 μL mixture containing 1.25 U *Taq* DNA polymerase, 0.8 m*M*/L dNTPs, 0.2 μ*M*/L each primer, 2.5 μL template DNA and ddH_2O, 5 μL 10× *Taq* buffer was added to make the final volume as 50 μL. The PCR thermal cycles were as follows: initial denaturation temperature at 94°C for 5 min; 32 cycles of denaturation at 94°C for 50 s, annealing at 55°C for 55 s, and extension at 72°C for 60 s; final extension temperature at 72°C for 5 min. The amplification products were stored at 4°C until use.

Gel Electrophoresis of PCR Products

A 1.0 μL 6× loading buffer were mixed with5.0 μL PCR products and subjected to electrophoresis on a 1.5% (wt/vol) agarose gel at 80 V for 50 min. The JY1000C universal electrophoresis power was purchased from Beijing Liuyi Instrument Factory (Beijing, China), GoLd-view was used as dye and DL-2000 DNA Marker was used as molecular weight standard. After electrophoresis, the gels were observed under WD-9413C UV gel imaging system (Beijing Sunbiotech Co. Ltd., Beijing, China). PCR products were sequenced and the sequences were analyzed by DNAStar (http://www.dnastar.com/).

Results

Screening of ESBLs Producing Strains

Results showed that the inhibition zone diameter of the control strain to each drug was in line with the CLSI standard. A total of 31 ESBLs producing strains were screened and confirmed from 41 *E. coli* isolates by screening and confirmatory tests with a detection rate of 75.6% (31/41).

Avian *E. coli* Isolates Resistance to β-lactam Antibiotic

Majority (92.7%) of the *E. coli* isolates demonstrated resistance to penicillins and first generation cephalosporins. The sensitive and resistance rates to 2nd and 3rd generation cephalosporins and monocyclic lactams were close, but resistance rate to cefoxitin was very low. The *E. coli* isolates were extremely sensitive to carbapenems and 4th generation cephalosporins. The sensitive rates to cefepime and imipenem were 87.8 and 100%, respectively (Table 2).

Distribution of β-lactam Antibiotic Resistance Genes in Avian *E. coli* Isolates

DNA templates of 41 *E. coli* isolates were prepared for ESBLs gene analysis. The ESBLs genes were amplified from 30 *E. coli* isolates (including carrying one and more than one resistance gene), and positive rate was 73.2% (30/41). Moreover, the TEM gene had the highest positive rate, up to 63.4% (26/41). The positive rate of CTX-M and SHV were 31.7% (13/41) and 4.9% (2/41). The electrophoresis of PCR products from some isolates is shown in (Fig. 1–3). The amplified ESBLs genes had higher than 98% nucleotide sequence similarity to the

Table 1: Characteristics of primer pairs specific for ESBLs genes

Resistance genes	Pimer sequence (5′→3′)	Size (bp)
TEM	Forward: CAGAAACGCTGGTGAAAGTA	719
	Reverse: ACTCCCCGTCGTGTAGATAA	
CTX-M	Forward: AGTGAAAGCGAACCGAATC	365
	Reverse: CTGTCACCAATGCTTTACC	
SHV	Forward: ATGCGTATATTCGCCTGTG	502
	Reverse: CCTCATTCAGTTCCGTTTCC	

Table 2: Resistance rate of avian *E. coli* isolates to β-lactam antibiotics

Antibiotics	Resistance rate (%)[a]		
	Resistant	Intermediate	Sensitive
Ampicillin	92.7 (38/41)	7.3 (3/41)	0 (0/41)
Carbenicillin	90.2 (37/41)	4.9 (2/41)	4.9 (2/41)
Piperacillin	78.1 (32/41)	12.2 (5/41)	9.8 (4/41)
Cephalotin	75.6 (31/41)	12.2 (5/41)	12.2 (5/41)
Cefuroxime	48.8 (20/41)	26.8 (11/41)	24.4 (10/41)
Cefoxitin	9.8 (4/41)	17.1 (7/41)	73.2 (30/41)
Cefaclor	48.8 (20/41)	12.2 (5/41)	39.0 (16/41)
Ceftriaxone	43.9 (18/41)	19.5 (8/41)	36.6 (15/41)
Cefotaxime	34.2(14/41)	26.8 (11/41)	39.0 (16/41)
Ceftazidime	34.2 (14/41)	24.4 (10/41)	41.5 (17/41)
Cefepime	7.3 (3/41)	4.9 (2/41)	87.8 (36/41)
Imipenem	0 (0/41)	0 (0/41)	100 (41/41)
Aztreonam	41.5 (17/41)	22.0(9/41)	36.6(15/41)

[a]The disc diffusion standard for drug resistance against antibiotics recommended by the CLSI was used to evaluate drug resistance of the isolates based on size of inhibition zone

corresponding sequences available in the GenBank database [ID: AY956315 (TEM gene), AY293071 (CTX-M gene) and EF650037 (SHV gene)].

Distribution of Multiple β-lactam Antibiotic Resistance Genes in Avian *E. coli* Isolates

Of 41 *E. coli* isolates, 48.8% (20/41) carried a single resistance gene; 22.0% (9/41) had two resistance genes and 2.4% (1/41) had three resistance genes. In addition, a total of 11 isolates accounting for 26.8% (11/41) had no target gene (Table 3).

Discussion

This study isolated 41 avian *E. coli* strains and detected their resistance to β-lactam antibiotics. The results showed that the resistance to penicillins and first generation cephalosporins was serious. The amount of resistance strains to 2nd and 3rd generation cephalosporins and monocyclic lactams was close to that of sensitive strains. The most of isolated strains were extremely sensitive to 4th generation cephalosporins and carbapenems. These results indicate that the avian *E. coli* had different drug resistance to different types of β-lactam antibiotics. Therefore, this drug-resistance regularity is directly related to the clinical use time and range of β-lactam antibiotics. The longer use time, the larger use range, and the more serious resistance.

Table 3: Detection of β-lactam resistance genes

Number	Commensal gene	Positive rate (%)
0	–[a]	26.8(11/41)
1	TEM	39.0(16/41)
	CTX-M	7.3(3/41)
	SHV	2.4(1/41)
2	TEM + CTX-M	22.0(9/41)
	TEM + SHV	0(0/41)
	CTX-M + SHV	0(0/41)
3	TEM+CTX-M+SHV	2.4(1/41)

[a]negative result

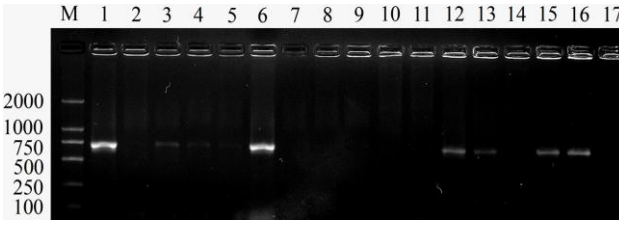

Fig. 1: PCR products of TEM gene
M: DNA Marke;Lanes 1-17:1-17 strains;Lanes 1,3-6,12,13,15,16 have the specific bands (719bp)

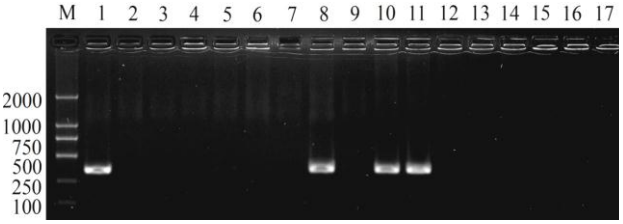

Fig. 2: PCR products of CTX-M gene
M: DNA Marke;Lanes 1-17:1-17 strains;Lanes 1,8,10,11 have the specific bands (365bp)

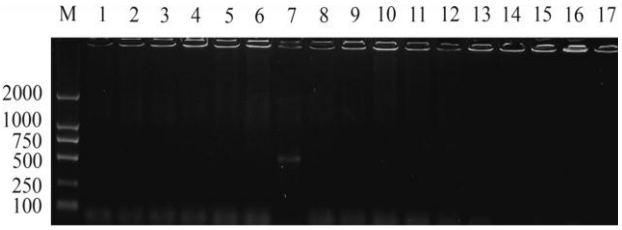

Fig. 3: PCR products of SHV gene
M: DNA Marke;Lanes 1-17:1-17 strains;Lanes 7 have the specific bands (502bp)

CLSI has reminded that the *E. coli* may be transformed from sensitive strain into resistance strain with the long time use of 3rd generation cephalosporins, and this transformation can be produced within 3–4 d after treatment (CLSI, 2011). It was found that the ESBLs producing strains are not only resistant to 2nd and 3rd generation cephalosporins generally but also to fluoroquinolones, tetracyclines and aminoglycoside (Tian *et al.*, 2011). Fu *et al.* (2007) have reported that ESBLs producing strains and *Klebsiella pneumoniae* are only sensitive to carbapenems including imipenem and meropenem. Moreover, the β-lactamase

inhibitors such as clavulanic acid or tazobactam can reduce the minimal inhibitory concentration (MIC) of drugs.

The resistance genes were detected by PCR, and 73.2% (30/41) of *E. coli* isolates carried ESBLs gene and they were ESBLs producing strains. The TEM gene had the highest positive rate, up to 63.4% (26/41). The positive rate of CTX-M and SHV were 31.7% (13/41) and 4.9% (2/41), respectively. There were 10 strains carrying more than two kinds of resistance genes. It was verified that the β-lactam antibiotics resistance of avian *E. coli* in North China is mainly mediated by TEM and CTX-M genes. The previous study has reported that the TEM gene accounts for 53% in *E. coli* (Xiao *et al.*, 2005). The TEM gene was amplified in ESBLs producing strains, but no SHV gene was found (Zhang *et al.*, 2009). SHV gene is mainly carried by *K. pneumoniae*. However, it was amplified in avian *E. coli*, indicating that the resistance genes can transfer and exchange between different species of bacteria.

Conclusion

Studies on the drug-resistance and resistance gene of avian *E. coli* can guide clinical medication in North China and provide reference for research of β-lactam antibiotics resistance regularity of *E. coli*.

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