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Distribution of Three OXA-Type β-Lactamase Encoded Genes in Avian Pathogenic *E. coli* Isolated in China

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Abstract: Two hundred and sixteen Avian Pathogenic *E. coli* (APEC) isolates were used for the determination of drug susceptibility to β-lactam and the detection of β-lactam drug resistance-related genes *OXA-1*, *OXA-5*, *OXA-31* using circular Disk Diffusion Method and PCR. Results showed that drug sensitivity rates among isolates were 190/216, 191/216, 162/216 to cefotaxime, ceftriaxone and ampicillin/sulbactam, respecively. The amplified gene fragments analysis of β-lactam drug resistance-related gene sequence of isolates showed 97%, homology with reference sequences from the GenBank. The OXA-1 and OXA-31 positive rates were only 7.4 and 10.6% but the OXA-5 positive rate was 30.6%. Seventy five strains carried only one kind of resistance genes, twelve strains carried two kinds of resistance genes and two strains carried all the three kinds of resistance genes. The integrated results of resistance gene detection and drug sensitivity test showed that the strains isolated in APEC on the rate of drug resistance testing and three resistance gene detection rate compared to the rate of aminoglycoside resistance and their drug resistance gene and the detection rate is not high but the growing development of bacterial resistance this is the alert source of bacteria in the prevention and treatment of poultry should pay close attention to rational drug use. In this study, the source for the understanding of avian bacteria β-lactam drug resistance mechanisms as well as generate a broader range of further drug resistance genes to provide a reference to epidemiological studies.

Key words: OXA-type β-lactamase gene, Avian Pathogenic *Escherichia coli* (APEC), distribution, strain, epidemiological studies

INTRODUCTION

Since, the discovery of penicillin in 1920's by Fleming but (Diggins, 1999) scientists have synthesized many new β-lactam antibiotics (Weaver *et al*., 1979; Neu, 1981; Snepar *et al*., 1981; Scribner *et al*., 1982; Imada *et al*., 1985; Benson and Nahata, 1988; Copper, 1992; Fineran *et al*., 2005; Horiuchi *et al*., 2006; Stein *et al*., 2009). Not long after however, there have been clinical relevant drug-resistant mainly Gram-negative bacteria but (Bondareva and Levitov, 1980; Guthikonda *et al*., 1987; Bedenic, 1999; Domenech-Sanchez *et al*., 1999; Munoz *et al*., 2003; Plhackova *et al*., 2003; Scaria *et al*., 2005; Drissi *et al*., 2008; Quiroga *et al*., 2009). Resistance mechanisms of Gram-negative bacteria resistance to β-lactam antibiotic were mainly due to the production of β-lactamases outer membrane protein changes and the active efflux mechanism. The production of β-lactamase is the most important reason of Gram-negative bacteria resistance to β-lactam antibiotics. OXA-type (benzoyl isoxazole penicillin hydrolase) enzymes are widespread and earlier reports showed that they mainly existed in Enterobacteriaceae and *Pseudomonas aeruginosa*. The main hydrolysis substrate of β-lactamases is oxacillin, so it was named OXA. In the late of 1980's, OXA was separated from serine-β-lactamase as an independent category. OXA-1 molecule is a monomer not a dimer is one of the sub-categories characterized as having an expanded OMEGA cyclopropyl near the β-lactam binding site. The OXA-31 and OXA-1 share >98% amino acid homology. The *OXA-31* gene is located in a non-bonding 300 kb plasmid cal type integron. Comparison tests carried between recombinant strains of OXA-1 and OXA-31 showed that the drug resistance characteristic was similar. The molecular weight of OXA-5 is about 27000 Da and it can hydrolyze oxacillin, methicillin and cloxacillin. When OXA-5 and extended-spectrum β-lactamases exist at the same time, the minimal inhibitory conce ntration of the experimental strains greatly increase. In order to explore, the cephalosporin drug resistance and its resistance

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Table 1: Drug sensibility test paper specifications and determinant criteria

Antimicrobial agents	Code-named	Drug dosage of the paper contained (μg/film)	Inhibition zone diameter (R, M, S[1])
Ampicillin/sulbactam	AM/SU	10/10	≤Q11, 12-14, ≥R15
Cefotaxime	CTX	30	≤Q14, 15-22, ≥R23
Ceftriaxone	CRO	30	≤Q13, 14-20, ≥R21

[1]R, M, S, respectively, on behalf of drug-resistant, moderately sensitive and highly sensitive; Antibiotics are marked. NCCLS provided for the election will test the type of Enterobacter

Table 2: Primer sequences and PCR conditions of the test

Genes	Primer sequence (5-3)	Length (bp)	Name of strains	Serial No. of reference strain	PCR condition (°C/sec)		
					Denature	Anneal	Longation
OXA-1	FP:CC GGA TCC ATG AAA AAC ACA ATA CAT RP:GG GTC GAC TTA TAA ATT TAG TGT GTT	831	*E. coli.* GXQ	AF227505	94/60	52/60	72/60
OXA-5	FP:GC GGA TCC ATG AAA ACC ATA GCC GCA TAT RP:AT GTC GAC TTA GCC ACC AAT GAT GAT GCC	810	*E. coli.* QD6-1	AF347074	94/60	55/60	72/60
OXA-31	FP:CC GGA TCC ATG AAA AAC ACA ATA CAT ATC RP:CG GTC GAC TTA TAA ATT TAG TGT GTT TAG	830	*E. coli.* GXQ	AF294653	94/60	53/60	72/60

mechanism of APEC isolates, 216 APEC isolates were checked with cephalosporins drug susceptibility by drug sensitive slips method and three types of β-OXA lactamase encoding genes were detected by PCR.

MATERIALS AND METHODS

Bacterial isolates: The 216 APEC isolates were isolated on MaConkey plate from liver, lung, air sacs and yolk sacs of birds infected with colibacillosis collected from different poultry farms in China from 2003 to 2005. *E. coli* isolates were further identified by sugar fermentation, indole test and V-P test.

Drug sensibility and sensitivity tests: Three types of cephalosporin drug (Ampicillin/sulbactam, cefotaxime and Ceftriaxone) sensibility test papers were purchased from Hangzhou Tianhe Microorganism Reagent Co., Ltd. (Table 1). The 216 APEC isolates for drug susceptibility testing, references the use of a round disk diffusion test.

Reagents: MaConkey plates, LB agar plates and Luria broth were prepared according to the standard methods.

The PCR amplification of drug resistant genes: Bacterial DNA were prepared as earlierly described (Blanco *et al*., 2004; Jin *et al*., 2005; Sambrook *et al*., 1996). Briefly, after growing for overnight in LB at 37°C with shaking, the bacterial cultures were boiled for 20 min and the supernatants were used as DNA templates for PCR amplification after centrifugation for 10 min at 10000 g. The PCR amplification of different drug resistant genes was performed as previously described. PCR reactions were carried out in 50 μL final volume contained 50 ng of bacterial DNA, 0.5 μM of each primer (Table 2) 0.5 mM dNTPs, 2.5 mM Mg^{2+}, 5 μL 10 X buffer and 1 U of Taq DNA polymerase (Shanghai Sangon, China). The PCR products were analyzed on 1.0% agarose gel electrophoresis.

Purification and sequence analysis of PCR product: PCR products (OXA-1, OXA-5 and OXA-31) were purified with the Gel DNA Purification kit (Qiagen Co., Ltd.). DNA was directly sequenced using the Dideoxynucleotide Termination Cycle Method with an ABI3730XL sequencer using BigDye Terminator Version 3.1. Alignments of nucleotide sequences of isolates under study together with reference sequences from GenBank were performed with DNAStar Software.

RESULTS AND DISCUSSION

Results of drug sensitivity tests showed that some strains were resistant against cephalosporin drugs: According to the results of drug sensitivity test, researchers can see (Table 3), the current main drug for prevention and treatment of the colibacillosis cefotaxime, ceftriaxone, ammonia benzyl-sulbactam also has a good antibacterial effect. But that can not be ignored is the drug-resistant strains have emerged and a considerable number of moderately susceptible strains is moving in the direction of the development of drug-resistant strains. Out of the 216 APEC strains under study, only five strains were found to be resistant to ampicillin/sulbactam, seven strains were resistant to cefotaxime and twenty fives strains were resistant to ceftriaxone. Moderate drugs susceptibilities to ampicillin/sulbactam, cefotaxime and ceftriaxone were observed in 21, 18 and 29 strains, respectively. On the other hand high sensitivity to cefotaxime (191) strains followed by ampicillin/sulbactam (190) strains and ceftriaxone (162) strains were observed (Table 3).

Table 3: Drug sensibility test paper specifications and determinant criteria

Antimicrobial agents	No. of high-sensitivity strain	No. of moderate-susceptivity strain	No. of drug-resistance strain
Ampicillin/Sulbactam (AM/SU)	190	21	5
Cefotaxime (CTX)	191	18	7
Ceftriaxone (CRO)	162	29	25

Table 4: Co-existence of β-lactam drug resistant genes

Combination of the resistant genes	Quantity	Percent
OXA-1	3	34.7
OXA-5	60	-
OXA-31	12	-
OXA-1+OXA-5	3	5.6
OXA-1+OXA-31	8	-
OXA-5+OXA-31	1	-
OXA-1+OXA-5+OXA-31	2	0.93
None of the three genes	89	58.77

β-lactam drug resistant genes: The three kinds of resistant genes were found with different rates among the APEC isolates. The detection of *OXA-1* and *OXA-31* gene-positive rates was relatively low 7.4 and 10.6%, respectively. However, the *OXA-5* gene detection rate was high reaching 30.6%.

Co-existence of β-lactam drug resistant genes: Results showed that 75 strains (34.7%) carried only one kind of the three resistance genes, 12 strains (5.6%) carry two kinds of resistance genes, only two strains carried all three kinds of resistance genes. The 127 strains (58.8%) were detected to have none of the three resistance genes (Table 4).

Sequence analysis: Alignments of nucleotide sequences (OXA-1, OXA-5 and OXA-31) of isolates under the study together with reference sequences from GenBank showed at least 97% homology. In clinical medicine because of its long-term use, cephalosporins drug resistance has shown to be a very serious problem but in the veterinary clinic, the use of cephalosporins is recently introduced.

This study showed that APEC isolates have been resistant to cefotaxime, ammonia benzyl-sulbactam, a small number of strains were resistant to two kinds of drugs, whereas *E. coli*. XZTSHD04072501 was found to have three kinds of drug tolerance. Due to the lack of an effective vaccine, *E. coli* treatment has been relying on drug control which makes bacterial drug-resistant to become a potential hazard. The spread of resistance genes to one drug or more and other factors in bacteria has resulted in a dramatic increase of drug-resistant strains is in the world.

Researchers are concerned about the source of the drug-resistant in isolates of bacteria and animal-derived drug-resistant strains. In this study, avian pathogenic *E. coli* isolates that carried the three kinds of anti-β-lactam drug resistance genes have been detected. The OXA-31 the highest (30.6%) detected gene. Such a high rate of resistance gene detection, explains that resistance of *E. coli* isolated from clinically infected animal to be more and more serious.

CONCLUSION

The OXA-1 is one of the main types of β-lactamases. Compared with clinical medicine in this study, the detection rate of gene *OXA-1* showed relatively low (7.4%) which may be related to long-term use of cephalosporins in clinical medicine but rarely in veterinary medicine during past few years. In fact in clinical medicine, the effect of a variety of cephalosporins drugs has been extremely limited but in veterinary clinic they still showing good effect. In this test, the result of drug sensitivity and gene detection rate maybe was good evidence.

As a preliminary study, only part of β-lactamase encoding genes epidemiology has been detected in APEC isolates in this study. In future, the more studies of drug resistance surveillance should be done to provide reference for the timely and accurate decision-making in veterinary medicine clinic.

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