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Phenotypic Detection and Drug Resistance Analysis of Extended-Spectrum β-Lactamases among *Escherichia coli* Isolates from Diseased Duck

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Abstract: Thirty two *Escherichia coli* isolates recovered from diseased ducks diagnosed with colibacillosis in China between 2010 and 2011 were characterized for detection of Extended-Spectrum β-Lactamases (ESBLs) by phenotypic screening and confirmatory test provided by the Clinical Laboratory Standards Institute and antimicrobial susceptibility profiles via agar disk diffusion and a broth doubling dilution method. Results showed 7 of the 32 *E. coli* isolates analyzed (21.87%) were found to be ESBL producers. The resistant rates of ESBL producers were >28.6% against third generation cephalosporins and semisynthetic penicillins and as high as 100% against amoxicillin, ampicillin and ceftiofur. All 32 isolates were sensitive to imipenem, meropenem and cefepime and the susceptible rates of all 32 isolates to β-lactam antibiotics in combination with enzyme inhibitors were higher than those of β-lactam antibiotics alone. Therefore, ESBL-producing isolates had multi-drug resistance and enzyme inhibitors can partially solve the drug resistance problem.

Key words: Duck, *Escherichia coli*, Extended-Spectrum Beta-Lactamases (ESBLs), phenotype, antibiotics susceptibility

INTRODUCTION

Escherichia coli is an important pathogen of animals and humans and it is also a common inhabitant of their intestinal tracts. Antimicrobials are valuable tools for the treatment of clinical disease and for the maintenance of healthy, productive animals. However, recent reports have discovered increased resistance to the antimicrobial agents commonly utilized for treatment (Altekruse *et al*., 2002; White *et al*., 2000; Yang *et al*., 2004). An increase in the rate of β-lactam resistance in *E. coli* isolates has been observed in the last few years, β-lactamase synthesis being the main mechanism of resistance to third generation cephalosporins such as ceftiofur among gram-negative bacteria. In recent years, there has been an increased incidence and prevalence of Extended-Spectrum β-Lactamases (ESBLs), enzymes that hydrolyze and cause resistance to oxyimino-cephalosporins and aztreonam. ESBLs which are predominantly derivatives of plasmid-mediated TEM or SHV β-lactamases, arise through mutations that introduce one or more amino acid substitutions that alter the configuration or binding properties of the active site, resulting in an expansion of the substrate range of the enzymes (Medeiros, 1997; Bradford, 2001). ESBL-Producing (ESBL-P) clinical isolates are frequently associated with nosocomial outbreaks (Gniadkowski *et al*., 1998; Pena *et al*., 1998) with production detected most commonly in *Klebsiella pneumoniae* (Coudron *et al*., 1997; Mabilat and Courvalin, 1990) in addition to other members of the Enterobacteriaceae family (Coudron *et al*., 1997) and *Pseudomonas aeruginosa* (Mugnier *et al*., 1996). ESBLs have been described for *E. coli* and this fact is a significant cause of concern (Bradford, 2001).

Several studies show the dissemination in China of ESBLs among human clinical isolates from hospitalized patients as well as from those in the community (Yu *et al*., 2007; Shen *et al*., 1999). Very few studies on the characterization of β-lactamases in *E. coli* isolates from sick animals (Brinas *et al*., 2005; Liu *et al*., 2007) exist to date. Thus, the aim of this study was to detect ESBLs among *E. coli* isolates from diseased ducks that were diagnosed with colibacillosis in China between 2008 and 2010 and determine the antimicrobial susceptibility profiles. The results presented herein may provide surveillance information for this specific region.

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MATERIALS AND METHODS

Bacterial strains: The 32 *E. coli* isolates were recovered from the livers of diseased ducks raised on 32 different poultry farms in China, from February 2010 to January 2011. All *E. coli* organisms were isolated and purified on MacConkey agar and verified as *E. coli* using the Vitek system (Biology-mili. Co., France). The strains were maintained at -70°C until analysis. *E. coli* ATCC25922 was purchased from Beijing Ordinary Microbilogy Strain Store Center, Beijing, China. *Klebsiella pneumoniae* (ATCC700603) was purchased from Peking University Health Science Center.

Detection of ESBLs: The ESBL-P phenotypes of 32 isolates were determined by the standard disk diffusion test (CLSI, 2006). Organisms were thawed and subcultured twice on trypticase soy agar with 5% added sheep hemoglobin at 35°C. A suspension of the organism in 0.9% NaCl was prepared, adjusted to a 0.5 McFarland standard turbidity and spread onto Mueller-Hinton plates. For the screening procedure, the inhibition zone diameters around 30 μg aztreonam, 10 μg cefpodoxime, 30 μg ceftazidime, 30 μg cefotaxime and 30 μg ceftriaxone discs were measured. If the diameter of the inhibition zone was ≤27, ≤17, ≤22, ≤27 or ≤25 mm, respectively, the isolate was defined as a suspected ESBL producer and thus considered a candidate for confirmatory testing.

For the purposes of the study, confirmatory test was also performed as described by the Clinical Laboratory Standards Institute (CLSI, 2006) inhibition zones obtained by using disks that contained cefotaxime (30 μg) and ceftazidime (30 μg) were compared with those containing cefotaxime-clavulanic acid (30 and 10 μg) and ceftazidime-clavulanic acid (30 and 10 μg) (Oxoid, Madrid, Spain) on MH plates, respectively. *E. coli* ATCC 25922 and *K. pneumoniae* ATCC 700603 were used as negative and positive controls for ESBL production, respectively. Seeded plates were incubated at 35°C and read after 24 h. An organism was classified as having an ESBL-P phenotype if the zone of inhibition produced by at least one combination disk was ≥5 mm larger than that produced by the corresponding lone antibiotic-impregnated disk.

Antimicrobial susceptibility determination: Susceptibility testing to 28 antimicrobials (ceftriaxone, ceftiofur, cefotaxime, ceftazidime, cefepime, cefoperazone, cefoperazone/sulbactam, amoxicillin, amoxicillin/clavulanic acid, ampicillin, ampicillin/sulbactam, aztreonam, imipenem, meropenem, cefoxitin, amikacin, gentamicin, micronomicin, ciprofloxacin, enrofloxacin, gatifloxacin, sparfloxacin, levofloxacin, florfenicol, doxycycline, minocycline, fosfomycin and sulfamethoxazole) was determined in 32 *E. coli* isolates on Muller-Hinton agar by agar disk diffusion test that was performed according to the CLSI (2006). Minimum Inhibitory Concentrations (MICs) of ceftriaxone, amikacin, gatifloxacin and fosfomycin on 30 *E. coli* isolates were determined via the standard broth doubling dilution method on Muller-Hinton medium. Results were interpreted in accordance with CLSI (2006) standards. *E. coli* ATCC 25922 was used as quality controls in all of the MIC determinations.

RESULTS

Detection of ESBLs: Results for detection of ESBLs were shown in Table 1. Of 32 *E. coli* isolates, 7 isolates were found to be ESBL producers by an increase of 5 mm in the inhibition zone around the disc containing added clavulanic acid in confirmatory test.

Antimicrobial susceptibility determination: Results of antimicrobial susceptibility determination were shown in Table 2 and 3. The ESBL-P *E. coli* isolates had lower rates of susceptible to third generation cephalosporins such as ceftiofur and ceftriaxone (≤42.9%) than those of ESBL-Nonproducing (ESBL-NP) *E. coli* isolates. 64% ESBL-NP *E. coli* isolates were susceptible to third generation cephalosporins except ceftiofur. The 96% ESBL-NP *E. coli* isolates were susceptible to cefoperazone in combination with sulbactam. However, 42.9% ESBL-P *E. coli* isolates were susceptible to ceftazidime, 28% ESBL-P *E. coli* isolates were susceptible to ceftriaxone. None of ESBL-P *E. coli* isolates was susceptible to both ceftiofur and cefotaxime. ESBL-P *E. coli* isolates had very significantly higher rates of resistant to semisynthetic penicillins than those of ESBL-NP *E. coli* isolates and were completely resistant (100%) to amoxicillin and ampicillin. ESBL-NP *E. coli* isolates had <65% resistant rate to amoxicillin and ampicillin. All isolates had significantly higher rates of susceptible to antimicrobial in combination with β-lactammase antibiotics inhibitors than alone. β-lactammase antibiotics inhibitors such as clavulanic acid and sulbactam protected the antibiotics from ESBLs hydrolysis and decreased the antibiotic resistance against bacteria. All isolates were susceptible to carbapenems (such as imipenem and meropenem) and monobactam cefoxitin. ESBL-P *E. coli* isolates were more resistant than ESBL-NP *E. coli* isolates to amikacin, gentamicin, micronomicin and gentamicin-micronomicin and their resistant rates ranged from 28.6-71.4%. All ESBL-P *E. coli* isolates and 64% ESBL-NP *E. coli* isolates were resistant

Table 1: Phenotypic confirmatory test of ESBLs among 32 *E. coli* isolates

No. of isolates	CTX[a] (CAZ)	CTX/CA (CAZ/CA)	ESBLs producer	Isolate No.	CTX (CAZ)	CTX/CA (CAZ/CA)	ESBLs producer
3	23 (25)	29 (30)	+[b](≥5)	19	20 (17)	25 (23)	+(≥5)
7	21 (15)	26 (22)	+(≥5)	23	22 (25)	23 (26)	-
9	21 (22)	27 (27)	+(≥5)	24	18 (20)	24 (25)	+(≥5)
12	22 (19)	25 (23)	-	28	21 (19)	26 (25)	+(≥5)
13	24 (22)	25 (22)	-	29	23 (21)	23 (24)	-
14	20 (18)	25 (24)	+(≥5)	30	22 (26)	23 (26)	-

[a]CTX: Cefotaxime; CAZ: Ceftazidime; CA: Clavulanic acid; [b]+: ESBLs-producer; −: ESBLs-nonproducer

Table 2: Antimicrobial susceptibility of 32 *E. coli* isolates by agar disk diffusion test

	No. (%) of 25 ESBL-NP *E. coli* isolates[a]			No. (%) of 7 ESBL-P *E. coli* isolates		
Antibiotic	S	I	R	S	I	R
Ceftriaxone	17 (68)	5 (20)	3 (12)	2 (28.6)	1 (14.3)	4 (57.1)
Ceftiofur	0	2 (8)	23 (92)	0	0	7 (100)
Cefotaxime	18 (72)	7 (28)	0	0	2 (28.6)	5 (71.4)
Ceftazidime	21 (84)	2 (8)	2 (8)	3 (42.8)	2 (28.6)	2 (28.6)
Cefepime	24 (96)	1 (4)	0	7 (100)	0	0
Cefoperazone	16 (64)	5 (20)	4 (16)	1 (14.3)	1 (14.3)	5 (71.4)
Cefoperazone/Sulbactam	24 (96)	1 (4)	0	6 (85.7)	0	1 (14.3)
Amoxicillin	9 (36)	0	16 (64)	0	0	7 (100)
Amoxicillin/Clavulanic acid	10 (40)	8 (32)	7 (28)	1 (14.3)	2 (28.6)	4 (57.1)
Ampicillin	11 (44)	0	14 (56)	0	0	7 (100)
Ampicillin/Sulbactam	14 (56)	6 (24)	5 (20)	3 (42.85)	1 (14.3)	3 (42.85)
Aztreonam	24 (96)	0	1 (4)	3 (42.85)	1 (14.3)	3 (42.85)
Imipenem	25 (100)	0	0	7 (100)	0	0
Meropenem	25 (100)	0	0	7 (100)	0	0
Cefoxitin	25 (100)	0	0	7 (100)	0	0
Amikacin	19 (76)	3 (12)	3 (12)	3 (42.8)	2 (28.6)	2 (28.6)
Gentamicin	13 (52)	3 (12)	9 (36)	1 (14.3)	1 (14.3)	5 (71.4)
Micronomicin	12 (48)	4 (16)	9 (36)	3 (42.8)	2 (28.6)	2 (28.6)
Ciprofloxacin	10 (40)	4 (16)	11 (44)	0	1 (14.3)	6 (85.7)
Enrofloxacin	2 (8)	7 (28)	16 (64)	0	0	7 (100)
Gatifloxacin	10 (40)	4 (16)	11 (44)	1 (14.3)	1 (14.3)	5 (71.4)
Sparfloxacin	13 (52)	1 (4)	11 (44)	0	1 (14.3)	6 (85.7)
Levofloxacin	12 (48)	5 (20)	8 (32)	0	2 (28.6)	5 (71.4)
Florfenicol	9 (36)	4 (16)	12 (48)	1 (14.3)	0	6 (85.7)
Doxycycline	0	0	25 (100)	0	0	7 (100)
Minocycline	6 (24)	1(4)	18(72)	2(28.6)	0	5 (71.4)
Fosfomycin	20 (80)	1(4)	4(16)	4(57.1)	0	3 (42.9)
Sulfamethoxazole	0	1(4)	24(96)	0	0	7 (100)

[a]S: Susceptible; R: Resistant; I: Intermediate

Table 3: MIC distribution and susceptibility of 4 antibiotics on 30 *E. coli* isolates from diseased ducks

	Gatifloxacin (isolates)		Amikacin (isolates)		Ceftriaxone (isolates)		Fosfomycin (isolates)	
Antibiotic	No. of ESBL-NP	No. of ESBL-P	No. of ESBL-NP	No. of ESBL-P	No. of ESBL-NP	No. of ESBL-P	No. of ESBL-NP	No. of ESBL-P
MICs μg mL^{-1}								
>256	-	-	-	-	-	-	1 (R)	-
256	-	-	1 (R)	-	-	-	1 (R)	1 (R)
128	-	-	1 (R)	1 (R)	-	-	1 (I)	1 (I)
64	-	1 (R)	3 (R)	1 (R)	-	1 (R)	2 (S)	-
32	1 (R)	-	8 (I)	-	-	1 (I)	2 (S)	1 (S)
16	5 (R)	2 (R)	5 (S)	2 (S)	2 (I)	-	7 (S)	1 (S)
8	5 (R)	1 (R)	4 (S)	2 (S)	1 (S)	-	5	2
4	1 (I)	-	-	1 (S)	-	-	1 (S)	1 (S)
2	1 (S)	1 (S)	1 (S)	-	1 (S)	-	3 (S)	-
1	2 (S)	-	-	-	-	-	-	-
0.5	5 (S)	2 (S)	-	-	-	-	-	-
0.25	2 (S)	-	-	-	-	-	-	-
0.125	1 (S)	-	-	-	3 (S)	3 (S)	-	-
0.063	-	-	-	-	5 (S)	2 (S)	-	-
<0.063	-	-	-	-	11 (S)	-	-	-
Total	23	7	23	7	23	7	23	7
MIC_{50} (ug mL^{-1})	5.50	16.59	27.98	66.15	-	13.54	38.14	165.78
MIC_{90} (ug mL^{-1})								
Susceptible rate (%)	47.8% (11/23)	42.8 (3/7)	43.5% (10/23)	71.42 (5/7)	91.3% (21/23)	71.42 (5/7)	86.95% (20/23)	71.42 (5/7)
Resistant rate (%)	47.8% (11/23)	57.1 (4/7)	21.7% (5/23)	28.57 (2/7)	0% (0/23)	14.28 (1/7)	8.697% (2/23)	14.28 (1/7)

[a]S: Susceptible; R: Resistant; I: Intermediate

to enrofloxacin and only 8% ESBL-NP *E. coli* isolates were susceptible to enrofloxacin, suggesting isolates had multi-drug resistance. About 32 isolates had 100% resistant rates to doxycycline and sulfamethoxazole. MIC_{max}, MIC_{50} and MIC_{90} of gatifloxacin for 30 *E. coli* isolates were 64, 5.50 and 16.59 μg mL^{-1}, respectively. Resistant rates of ESBL-P *E. coli* isolates and ESBL-NP *E. coli* isolates against gatifloxacin were 57.1 and 47.8%, respectively. MIC_{50} and MIC_{90} of amikacin for 30 *E. coli* isolates were 27.98 and 66.15 μg mL^{-1}, respectively. The 2 ESBL-P *E. coli* isolates and 1 ESBL-NP *E. coli* isolate were serious resistant to amikacin (MIC≥128, amikacin resistance breakpoints in *E.coli* might be defined by an MIC of 16 μg mL^{-1}). Susceptible rates of ESBL-P *E. coli* isolates and ESBL-NP *E. coli* isolate to ceftriaxone had 71.42 and 91.3%, respectively. MIC_{min} and MIC_{90} of ceftriaxone for 30 *E. coli* isolates were ≤0.063 and 13.54 μg mL^{-1}, respectively. However, 1 ESBL-P *E. coli* isolate showed serious resistant to ceftriaxone (MIC≥64, ceftriaxone resistance breakpoints in *E.coli* might be defined by an MIC of 8 μg m^{-1}). Resistant rate of ESBL-P *E. coli* isolates to ceftriaxone was 14.28%. Susceptible rates of ESBL-P *E. coli* isolates and ESBL-NP *E. coli* isolate to fosfomycin were 71.42 and 86.95%, respectively. Resistant rate of ESBL-P *E. coli* isolates against fosfomycin was 28.57%. MIC_{50} and MIC_{90} of fosfomycin for 30 *E. coli* isolates were 8.14 and 165.78 μg mL^{-1}, respectively.

DISCUSSION

Epidemic status of ESBLs-P *E. coli* isolates in clinical practice is very complex and different in different area and different period. Since, ESBLs was reported by Hu *et al.* (2005) in veterinary medicine in China (Hu *et al*., 2005), detection rate (between 7 and 26.7%) of ESBLs was less than that of ESBLs (between 33.5 and 46.2%) in human medicine. Detection rate (21.87%) of ESBL producing *E. coli* isolates in this study had an ascending tendency. It showed that the abuse of cephalosporins in Chinese veterinary practice may make speeds of *ESBLs* genes and incidence of ESBLs-producing bacteria increase resulting in improving detection rate (21.87%) of ESBL producing *E. coli* isolates.

The results in agar disk diffusion test showed ESBLs-NP *E. coli* isolates has high levels of susceptibility to third generation cephalosporins (except for ceftiofur), susceptible rate was 68 and the highest (96%) for cefoperazone. Susceptible rates of ESBLs-P *E. coli* isolates to third generation cephalosporins were lower (<42.9%). Resistant rates of ESBLs-P *E. coli* isolates and ESBLs-NP *E. coli* isolates against ceftiofur were 100 and 92%, respectively. Even if ESBLs-P isolates were *in vitro* susceptible to penicillins and cephalosporins, therapy on the clinical practice may be ineffectual. If an isolate is confirmed as an ESBL-producer by the CLSI-recommended phenotypic confirmatory test procedure, all penicillins, cephalosporins and aztreonam should be reported as resistant (CLSI, 2006). There are few reports in veterinary practice. Whether the third and four generation cephalosporins being susceptible *in vitro* in veterinary practice was used to therapy infection by ESBLs-P *E. coli* isolates are worth further studying.

ESBLs-P *E. coli* isolates were completely resistant to amoxicillin and ampicilin, ESBLs-NP *E. coli* isolates had also high levels of resistance. This was because amoxicillin and ampicilin were universally used in veterinary practice, resulting in bacteria having resistance. Table 2 showed ESBLs-P *E. coli* isolates had higher susceptibility to compound preparations of amoxicillin/clavulanic acid, ampicillin/sulbactam than amoxicillin and ampicillin alone. It showed that enzyme inhibitor may protect β-lactam antibiotics from ESBLs hydrolysis. So, infection by ESBLs-P *E. coli* isolates is treated, compound preparations of β-lactam antibiotics and enzyme inhibitor are used according to results of antimicrobial susceptibility. ESBLs-P *E. coli* isolates and ESBLs-NP *E. coli* isolates were all higher susceptible to imipenem, meropenem, cefoxitin and cefepime, etc.

Multi-resistant bacterium is caused by plasmid-mediated ESBLs. In the study, ESBLs-P *E. coli* isolates had higher resistant rates (28.6~100%) to aminoglycosides, quinolones and sulfonamides and were higher than these of ESBLs-NP *E. coli* isolates and multi-drug resistance. This was in accord with that reported in human clinical medicine. Plasmid carrying ESBLs resistant gene harbors resistant genes of other β-lactam antibiotics such as quinolones and sulfonamides. Treatment frequency of *E. coli* from duck used by aminoglycosides, quinolones and sulfonamides was too high, resulting in elevation of itself drug-resistance. Susceptible rates of ESBLs-P *E. coli* isolates and ESBLs-P *E. coli* isolates against florfenicol (14.3, 36%) and doxycycline (0, 0%) were lower while their resistant rates were very high. This may be induced by that florfenicol and doxycycline are used to treat infection of *E. coli* from diseased ducks to produce a large number of drug-resistance bacteria.

The results of gatifloxacin, amikacin and fosfomycin by a broth doubling dilution method were the same as those by agar disk diffusion test. Susceptible rates and resistant rates of ESBLs-P *E. coli* isolates and ESBLs-NP

E. coli isolates almost were the same. Compared with that reported previously (Decousser *et al*., 2002), MIC_{50} and MIC_{90} of gatifloxacin for 30 tested isolates showed decreased susceptibility. Decreased susceptibility to gatifloxacin may be caused by multi-drug resistance and selective pressure produced by extensive use of gatifloxacin. MIC_{max} of amikacin was 256 μg mL^{-1}, 1 of 3 drug resistance isolates was an ESBLs-P *E. coli* strain. It showed also the ESBLs-P *E. coli* isolate had multi-drug resistance to aminoglycosides. Susceptible rates (71.42, 86.95%) of ESBLs-P *E. coli* isolates and ESBLs-NP *E. coli* isolates against fosfomycin were higher and resistant rates were lower. MIC_{50} and MIC_{90} of fosfomycin for 30 tested isolates were 38.14 and 165.78 μg mL^{-1}, respectively.

There was no cross resistance or low levels of cross resistance between fosfomycin and other drugs. It showed fosfomycin had strong activity of antibacterial in accord with those by agar disk diffusion test. The results of ceftriaxone by a broth doubling dilution method were not the same as those by agar disk diffusion test. Susceptible rates (71.42, 91.3%) of ESBLs-P *E. coli* isolates and ESBLs-NP *E. coli* isolates against ceftriaxone were higher than those by agar disk diffusion test (28.6, 68%). Experimental condition was one of main factors that affect susceptible rate, other factors are worth further studying.

CONCLUSION

ESBL-producing isolates had multi-drug resistance and enzyme inhibitors can partially solve the drug resistance problem.

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