Original Article

Extended Spectrum Beta-lactamase Producing *Escherichia coli* Isolated from Infected Canines

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Abstract

Escherichia coli strains from dogs with different clinical signs were studied for their antimicrobial sensitivity profiles, extended spectrum beta-lactamase enzymes, and virulent genes. The 364 *E. coli* isolates originated from the skin (32.48%), urinary tract (31.32%), gastrointestinal tract (16.21%), reproductive system (7.69%), ear (6.05%), respiratory tract (4.94%), and other specimens (1.37%). Using the disk diffusion method, the *E. coli* were resistant to amoxicillin, amoxicillin-clavulanic acid, azithromycin, cephalexin, ceftriaxone, ciprofloxacin, doxycycline, enrofloxacin, norfloxacin, gentamicin, and sulfamethoxazole/trimethoprim at the rates of 84, 36, 41, 57, 38, 66, 73, 70, 64, 47, and 64%, respectively. Ninety-seven *E. coli* that were resistant to two or more beta-lactam drugs were chosen for investigation of their ESBL enzymes. Results showed that 36 isolates could produce ESBL enzymes by the combination disk test comprising one set of cefotaxime and cefotaxime-clavulanic acid and another set of ceftazidime and ceftazidime-clavulanic acid. Amplification of the ESBL-encoded genes of the 36 *E. coli* produced 35 isolates carrying *bla* CTX-M (543 bp) and 33 isolates harboring *bla* TEM (863 bp). The genes *bla*SHV and *bla*VEB were not found in the tested isolates by PCR. The *E. coli*-producing ESBL enzymes were further investigated for the virulent factors of Shiga-like toxin *E. coli* (*stx, eae,* and *hly*), Enteropathogenic *E. coli* (*bfp*) and Enterotoxigenic *E. coli* (LT, ST toxin). However, the isolates from the pets in this study did not present any of these virulent genes.

Keywords: antimicrobial resistance, dog, *E. coli*, ESBL, virulent factors

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Introduction

The genetics that encode antimicrobial resistance (AMR) and other virulent factors can be transmitted from one bacteria to another under selection pressure. Acquiring these genes enhances bacterial spread in the human and animal populations. Pets are one of the major sources for the transmission of pathogenic bacteria to humans. However, surveillance data of antimicrobial-resistant genes and emerging pathogenic strains in companion animal isolates are limited (Normand et al., 2000).

AMR related to the production of extended-spectrum beta lactamases (ESBL) is a particular problem in the handling of clinical infections. Beta-lactam antimicrobials are an important class of drugs used for the treatment of infection in both humans and companion animals. Resistance can arise by several mechanisms, including the acquisition of genes encoding beta-lactamases from other bacteria, alterations in the cell membrane permeability, and the over expression of endogenous beta-lactamases (Paterson and Bonomo, 2005).

E. coli is a good bacteria indicator for tracing resistance genes that could easily transfer in animals, humans and environment. The various *E. coli* pathogenic strains harbor a variety of virulent factors that cause different infections. The objective of the current study was to investigate the characteristics of antimicrobial profiles and virulent factors in suspected pathogenic *E. coli* isolated from dogs with different sites of infection.

Materials and Methods

Agar disk diffusion assay: A collection of *E. coli* isolated between 2010-2011, from sick dogs brought to the veterinary teaching hospital, was screened for antimicrobial sensitivity using the disk diffusion method following Performance Standard for Antimicrobial Susceptibility Testing M100-S19 (CLSI, 2009) and Performance Standard for Antimicrobial Disk and Dilution Susceptibility Test for Bacteria Isolated from Animals (M31-A3) (CLSI, 2008). Eleven antimicrobials were used and comprised amoxicillin (10 μg), amoxicillin/clavulanic acid (20/10 μg), azithromycin (15 μg), ceftriaxone (30 μg), cephalexin (30 μg), ciprofloxacin (5 μg), doxycycline (30 μg), enrofloxacin (5 μg), gentamicin (10 μg), norfloxacin (10 μg), and sulfamethoxazole/trimethoprim (23.75/1.25 μg). *E. coli* ATCC 25922 was used as the control strain. The NCSS (Number Cruncher Statistical System) program (Hintze, 2007) was used for data analysis of AMR and isolate origins.

Combination disk assay: Ninety-seven beta-lactam resistance *E. coli* were further tested for extended spectrum beta-lactamase enzymes using a combination disk assay comprising one set of cefotaxime (30 μg) and cefotaxime-clavulanic acid (30/10 μg), and another set of ceftazidime (30 μg) and ceftazidime-clavulanic acid (30/10 μg) following M100-S19 (CLSI, 2009). *K. pneumoniae* ATCC 700603 was used as the control strain. An increase in the diameter of 5 mm or more in each couple of antimicrobials was applied as the basis for evaluation of the ESBL-producing isolates. Three more antimicrobials consisting of cefotaxime (30 μg), ceftazidime (30 μg), and marbofloxacin (5 μg) were also disk-diffusion tested among these selected isolates.

Detection of ESBL genes: Isolates that produced ESBL enzymes were amplified for ESBL class A genes comprising *bla* CTX-M (5'-SCSATGTGCAGYACCAGTAA-3' and 5'-CCGCRATATGRTTGGTGGTG-3') (Gen Bank X92506), *bla* TEM (5'-ATGAGTATTCAACATTTCCG-3' and 5'-CTGACA GTTACCAATGCTTA-3') (U09188), *bla* SHV (5'-GGTTATGCGTTATATTCG CC -3' and 5' TTAGCGTTGCCAGTGCTC -3') (Gen Bank M59181) and *bla* VEB (5'-CGACTTCCATTTCCCGATGC-3' and 5'- CGACTTCCATTTCCCGATGC-3') (Gen Bank AF324833).

Screening virulent factors of STEC, ETEC, and EPEC: Pathogenic *E. coli* that produced ESBL enzymes were multiplex-PCR screened for shiga-like toxin *E. coli (*STEC) virulent factors including *stx*I *and stx*II genes, which encode shiga-like toxins I and II; *eae*, which encodes attachment protein; and hemolysin gene (*hly*) following Paton and Paton (1998). The ETEC virulent factors comprising heat labile enterotoxin (LT) and heat stable enterotoxin (ST) were amplified using multiplex PCR that had 3 sets of primers to detect the genes for LT, ST1a, and ST1b (Schultsz et al, 1994). Furthermore, *bfp* gene encoding bundle forming pili (the adherence factor of EPEC) were investigated (Gunzburg et al., 1995).

Results

Results of the agar disk diffusion method indicated that 364 *E. coli* isolates were resistant to amoxicillin (294, 84.24%,), amoxicillin/clavulanic acid (131, 36.49%), cephalexin (205, 57.26%), ceftriaxone (131, 37.64%), azithromycin (146, 40.78%), ciprofloxacin (234, 65.92%), enrofloxacin (251, 69.92%), norfloxacin (223, 63.90%), doxycycline (258, 72.68%,), gentamicin (153, 46.50%), and sulfamethoxazole/ trimethoprim (226, 64.02%) (Table 1).

Most of the *E. coli* isolates were derived from skin (118) and urinary tract (114) samples. The isolates from the skin presented the highest resistant rates against beta lactams while the *E. coli* from the urinary samples presented the highest resistant rates against the quinolone group. The isolates from the respiratory tract showed high resistance to both beta lactam and quinolone drugs; however, the number of respiratory samples was low (only 18).

Of the 36 isolates that produced ESBL enzymes, 19 were from the urinary tract samples (urine, urinary bladder flush or swab from dogs with cystitis), 13 isolates were derived from wounds, 2 isolates were from otitis samples, and the other two isolates were from nasal discharge and a fecal swab with enteritis.

The 36 isolates that yielded positive ESBL in two pairs of combination disks were amplified for detection of class A ESBL-resistant genes including the genes encoded with CTX-M, TEM, SHV, and VEB enzymes. Results showed that 35 *E. coli* encoded the

gene producing CTX-M and 33 isolates harbored the gene producing TEM. The genes producing either SHV or VEB were not found among the tested isolates.

All isolates producing ESBL in this study were resistant to cefotaxime and cephalexin, while 23 isolates (63.89%) were resistant to ceftazidime and 30 isolates (83.33%) resisted ceftriaxone (Table 2). Therefore, two groups of isolates that were ESBL phenotype positive (36) and ESBL phenotype negative (61) were χ^2 tested for comparison of their beta lactam antimicrobial resistance (AMX, AMC, CN, CTX, CAZ, CRO). Results showed that there was no significant difference between the two groups ($\chi^2 = 10.825$, df = 5, p value = 0.057).

Table 1 Percentages of antimicrobial-resistant *E. coli* isolated from different samples of dogs using agar disk diffusion assay

Origin (N)	AMX	AMC	CN	CRO	AZM	CIP	ENR	NOR	DXC	GEN	STX
Skin (118)	91.3	38.05	65.81	46.09	37.61	63.25	66.67	61.54	76.27	53.7	66.67
Urinary (114)	86.11	38.05	57.14	35.78	47.79	73.45	76.11	71.03	75.68	45.45	66.97
Gastrointestinal (59)	73.68	32.76	48.28	27.59	39.66	63.79	65.52	60.34	70.69	42.11	58.62
Reproduction (28)	64.29	28.57	42.86	25.93	37.04	40.74	53.57	40.74	55.56	25.93	53.57
Ear (22)	81.82	31.82	54.55	40	27.27	54.55	68.18	54.55	57.14	40	61.9
Respiratory (18)	100	41.18	58.82	46.67	35.29	88.24	88.24	92.86	88.24	64.29	62.5
Other (5)	75	50	75	100	75	100	100	100	50	50	75
Total (364)	84.24 (349)	36.49 (359)	57.26 (358)	37.64 (348)	40.78 (358)	65.92 (355)	69.92 (359)	63.9 (349)	72.68 (355)	46.5 (329)	64.02 (353)

AMX, amoxicillin; AMC, amoxicillin/clavulanic acid; CN, cephalexin; CRO, ceftriaxone; AZM, azithromycin; CIP, ciprofloxacin; ENR, enrofloxacin; NOR, norfloxacin; DXC, doxycycline; GEN, gentamicin; and SXT, sulfamethoxazole/trimethroprim

Table 2 Comparison of antimicrobial-resistant percentages of positive and negative extended spectrum beta-lactamase-producing enzymes *E. coli*

E. coli (N)	AMX	AMC	CN	CTX	CAZ	CRO	AZM	CIP	ENR	MAR	DXC	GEN	STX
ESBL positive[a)] (36)	97.22 (35)	72.22 (26)	100 (36)	100 (36)	63.89 (23)	83.33 (30)	77.78 (28)	97.22 (35)	94.44 (34)	94.44 (34)	94.44 (34)	75 (27)	44.44 (16)
ESBL negative[a)] (61)	96.72 (59)	81.97 (50)	100 (61)	54.10 (33)	29.51 (18)	86.89 (53)	81.97 (50)	98.36 (60)	63.93 (39)	63.93 (39)	96.72 (59)	60.66 (37)	70.49 (43)
Total *E. coli* (97)	96.91 (94)	78.35 (76)	100 (97)	71.13 (69)	42.27 (41)	85.57 (83)	80.41 (78)	97.94 (95)	75.26 (73)	75.26 (73)	95.88 (93)	65.98 (64)	60.82 (59)

AMX, amoxicillin; AMC, amoxicillin/clavulanic acid; CN, cephalexin; CTX, cefotaxime; CAZ, ceftazidime; CRO, ceftriaxone; AZM, azithromycin; CIP, ciprofloxacin; ENR, enrofloxacin; NOR, norfloxacin; MAR, malbrofloxacin; DXC, doxycycline; GEN, gentamicin; and SXT, sulfamethoxazole/trimethroprim

[a)] ESBL positive/negative: extended spectrum beta-lactamase positive/negative in both combination disk tests using CTX and CTX-CLV, and CAZ and CAZ-CLV disks

Discussion

The variation of ESBL enzymes has led the researcher to identify the specific enzymes in each resistance outbreak. In particular, each enzyme has different mechanisms of action and is inhibited by different agents that impact on the selection of antimicrobial therapy. In this study, the characteristics of phenotypes and genotypes of the tested isolates correlated and clearly confirmed the report that CTX-M enzymes, in particular, caused resistance to cefotaxime and showed less efficiency against ceftazidime (Bradford, 2001). For a decade, CTX-M enzymes have been the dominant ESBLs in humans and it has been found that normally CTX-M encoding plasmids could carry *bla*TEM and other resistant genes including aminoglycosides, chloramphenicol, sulfonamides, trimethoprim, and tetracycline (Bonnet, 2004).

The consensus view regarding antimicrobial resistance showed that many severe clinical problems arose from the emergence of ESBL-producing Gram-negative isolates, for example the Shiga-like toxin *E. coli* (SHEC) serotype O104:H4 outbreak in Europe in 2010 (Frank et al., 2011). Therefore, the ESBL-producing *E. coli* were multiplex PCR tested for the detection of virulent genes of SHEC, ETEC, and EPEC. However, the virulent factors chosen in the current study were not found in all isolates. *E. coli* from pet dogs had higher resistant profiles but did not carry virulent factors that seriously harmed humans in contrast to the results for pig isolates (unpublished) which were also tested for these virulent factors.

The multidrug-resistant *E. coli* in this study were resistant to common drugs used in humans and animals including beta lactam and quinolone groups. The results indicated that a high proportion of the bacteria isolated from pet dogs could produce ESBL enzymes, although only the 1st and 2nd cephalosporin groups had been used for treatment in dogs at the veterinary teaching hospital at that time. These might result from the transfer of mobile genetic elements that carried cassettes of various resistant genes. Under selection pressure, multi-resistant bacterial species survived in the environments. Fortunately amoxicillin/clavulanic acid, ceftriaxone (the third

generation cephalosporin) and azithromycin (semi-synthetic macrolide) presented higher sensitive rates for these bacteria. To control the dissemination of resistant strains, prudent use of clinically important antimicrobials is necessary. To assist effective surveillance of AMR in public health, indicator bacteria isolates from animals and humans in different countries should be tested routinely for antimicrobial sensitivity. To combat bacterial septicemia, it is essential to identify the virulent factors of septic strains.

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บทคัดย่อ

เชื้ออีโคไลที่สร้างเอนไซม์เบตาแลคแตมแบบขยายแยกจากสุนัขป่วย

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การศึกษารูปแบบการดื้อยา เอนไซม์เบตาแลคแตมแบบขยาย และยีนก่อโรคของเชื้อ *Escherichia coli* ที่แยกได้จากตัวอย่างสุนัขที่มีอาการทางคลินิกแตกต่างกัน ในจำนวน *E. coli* รวม 364 เชื้อ ร้อยละ 32.48 แยกได้จากตัวอย่างผิวหนัง ร้อยละ 31.32 จากทางเดินปัสสาวะ ร้อยละ 16.21 จากทางเดินอาหาร ร้อยละ 7.69 จากระบบสืบพันธุ์ ร้อยละ 6.05 จากหู ร้อยละ 4.94 จากทางเดินหายใจ และร้อยละ 1.37 จากตัวอย่างอื่นๆ โดยอัตราการดื้อยา amoxicillin ยา amoxicillin-clavulanic acid ยา azithromycin ยา cephalexin ยา ceftriaxone ยา ciprofloxacin ยา doxycycline ยา enrofloxacin ยา norfloxacin ยา gentamicin และยา sulfamethoxazole/trimethoprim เท่ากับร้อยละ 84 36 41 57 38 66 73 70 64 47 และ 64 ตามลำดับ จากนั้นคัดเลือกเชื้อ *E. coli* ที่ดื้อต่อยาเบตาแลคแตมมากกว่าหรือเท่ากับ 2 ชนิด จำนวน 97 เชื้อ มาศึกษาการสร้างเอนไซม์ ESBL ด้วยวิธีทดสอบ combination disks ที่มีแผ่นยา 2 คู่ คู่แรก คือ cefotaxime และ cefotaxime-clavulanic acid และคู่ที่สอง คือ ceftazidime และ ceftazidime-clavulanic acid จากการทดลองพบว่า *E. coli* 36 เชื้อสามารถสร้างเอนไซม์ดังกล่าวได้ จึงนำมาตรวจหาชนิดของยีนที่สร้าง ESBL ด้วยวิธี PCR พบว่ามี 35 เชื้อที่มียีน *bla* CTX-M (543 bp) และ 33 เชื้อที่มี *bla*TEM (863 bp) แต่ไม่พบยีน *bla*SHV และ *bla*VEB ในเชื้อกลุ่มนี้ นอกจากนี้ยังได้นำเชื้อที่สามารถสร้างเอนไซม์ ESBL มาตรวจหายีนก่อโรคของเชื้อ Shiga-like toxin *E. coli* (*stx, eae,* และ *hly*) Enteropathogenic *E. coli* (*bfp*) และ Enterotoxigenic *E. coli* (สารพิษ LT และ ST) แต่เชื้อ *E. coli* ซึ่งแยกจากตัวอย่างสุนัขในการศึกษานี้ไม่พบยีนก่อความรุนแรงเหล่านี้

คำสำคัญ: การดื้อยา สุนัข อีโคไล เบตาแลคแตมแบบขยาย ปัจจัยก่อความรุนแรง

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