Isolation and Molecular Characterization of ESBL/pAmpC Producing *Escherichia coli* in Household Pigeons in Turkey

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ABSTRACT

In this study it was aimed to determine the prevalence of extended-spectrum β-lactamase (ESBL) and/or plasmidic AmpC β-lactamase (pAmpC) producing *Escherichia coli* among household pigeons in Hatay, Turkey. For this purpose, 150 cloacal swab samples were taken from apperently healthy household pigeons and the isolates were characterised by phylogenetic grouping, plasmid replicon typing, pulsed field gel electrophoresis (PFGE) and multilocus sequence typing (MLST) analysis. ESBL producing *E. coli* was detected in five (3.3%) of the cloacal swabs: all the strains carried the $bla_{\text{CTX-M-15}}$ gene together with $bla_{\text{TEM-1}}$ (four phylogenetic group B1 and ST446 and one phylogenetic group A and ST665). pAmpC producing *E. coli* was detected in one (0.7%) isolate and this isolate harbored the $bla_{\text{CMY-2}}$ (phylogroup B2 and ST4906). Three plasmid replicon types detected among the strains IncII/Iγ, IncFIB and IncFrepB were the most common. Three distinct PFGE pulsotypes were found among the isolates and multidrug resistance (MDR) phenotype was only observed in two isolates. The current study was, to the best knowledge of the authors, the first showing that household pigeons in Turkey are fecal carries of ESBL/pAmpC producing *E. coli* and a significant potential source of these bacteria for humans.

Key words: Pigeon; *E. coli*; ESBL; pAmpC; MLST; PFGE; Plasmid Replicon Typing.

INTRODUCTION

Frequent and misuse of antimicrobial agents for the treatment and prevention of bacterial infections have resulted in the emergence and spread of resistant bacteria (1). Emergence and increased frequency of extended spectrum β-lactamases (ESBL) and plasmidic AmpC β-lactamase (pAmpC) in the members of Enterobactericeae family has become worldwide problem affecting humans and animals (2). Recently, ESBL producing *Escherichia coli* has been reported at various prevalence rates in domestic and wild bird populations that is close proximity to human populations in urban areas (3-5). Previous studies have indicated that ESBL/pAmpC producing *E. coli* strains isolated from cattle (6), dogs (7), and chicken meat (8) in Turkey shared same enzyme types, detected commonly in human isolates (9). Human activities can influence occurrence of antimicrobial resistance in different habitats and animal species, such as environmental pollution with antimicrobials, usage of antimicrobials for treatment and prophylaxis (10).

Household pigeon breeding is common practice in rural and urban areas in Turkey. Information regarding carriage of antimicrobial resistance in pigeons is very limited in Turkey (11). Therefore, we aimed to determine the occurrence of ESBL/pAmpC producing *E. coli* in household pigeons and molecular characterization of the isolates.

MATERIALS AND METHODS

Ethical statement

The study was approved by the Animal Ethical Committee of Mustafa Kemal University (2013-7/7).

Isolation and identification

A total of 150 cloacal swabs in Stuart Transport Medium (LP Italiana, 118898, Italy) from apparently healthy household pigeons were collected in three locations (Antakya, Kırıkhan and Reyhanlı) in Hatay, Turkey, between January 2014 and June 2014. Hatay is located in the Mediterranean basin in southern Turkey with an altitude of 85 m (36°12′N- 36°9′E). It is bordered by Syria in the east and south, and to the west is the Mediterranean sea. The Orontes river pass through the region. The region has a Mediterranean climate. Therefore, the summers are very hot, long and dry and the winters are cool and rainy. Annual precipitation is about 1092.2 mm and average temperature is 23.28 °C. The cloacal swabs were plated onto Eosin Methylen Blue (Merck, 101347, Germany) agar containing 2 µg/ml cefotaxime (Sigma-Aldrich, 22128, Germany) and incubated for 18 to 24 h at 35°C. When growth appeared on the plate, one typical colony was selected and passaged to Blood agar (Merck, 110886, Germany), to obtain pure culture. Identification of the isolates were done by biochemical tests and confirmed by polymerase chain reaction (PCR) using *E. coli* 16S rRNA spesific primers (E16S-F CCCCCTGGACGAAGACTGAC and E16S-R ACCGCTGGCAACAAAGGATA) (12). The PCR reaction was performed in 25 µl, containing 200 µM dNTPs, 2.5 µl 10× PCR Buffer [750 mM Tris-HCl (pH 8.8), 200 mM $(NH_4)_2SO_4$, 0.1% (v/v) Tween 20], 1.5 mM $MgCl_2$, 0.1 µM each primer and 1.25 U Taq polymerase (MBI Fermentas, Vilnius, Lithuania). The following PCR conditions were used: initial denaturation step at 95°C for 8 min, 30 cycles at 95°C for 30 s, annealing at 58°C for 30 s, and extension at 72°C for 30 s, ending with a final extension at 72°C for 7 min.

Antimicrobial susceptibility testing

Antimicrobial susceptibilities of ESBL/pAmpC producing *E. coli* isolates was determined by disk diffusion method in accordance with Clinical Laboratory Standards Institute (CLSI) criteria (13). The antimicrobials (Bioanalyse, Turkey) tested were: ampicillin (10 µg), amoxicillin-clavulanic acid (10/20 µg), nalidixic acid (30 µg), ciprofloxacin (5 µg), cefotaxime (10 µg), cefepime (30 µg), cefoxitin (30 µg), cephalothin (30 µg), aztreonam (30 µg), imipenem (10 µg), chloramphenicol (30 µg), gentamicin (10 µg), tobramycin (10 µg), amikacin (10 µg), kanamycin (30 µg), tetracycline (30 µg) and sulfametoxazole-trimethoprim (1.25/23.75 µg). Phenotypic confirmation of ESBL producers was done by double disk synergy (14) and disk combination method (13). Cefoxitin resistance was accepted as indication of pAmpC production (13). *E. coli* ATCC 25922 strain was used as quality control.

DNA isolation

DNA isolation was carried out using the boiling method as previously reported by Ahmed *et al*. (2007) (15). Briefly, an overnight bacterial culture (200 µl) was mixed with 800 µl of distilled water and heated at 100°C for 10 min at dry heating block (Benchmark). Then, it was centrifuged at 10 000 rpm for 10 min and the supernatant was transferred to new tubes and stored at -20°C for subsequent PCR analysis.

Determination of ESBL and pAmpC genes

Presence of ESBL genes (*bla*$_{TEM}$, *bla*$_{OXA}$, *bla*$_{SHV}$ and *bla*$_{CTX-M}$) were amplified by PCR as previously described (15). pAmpC genes were investigated using multiplex PCR as previously reported by Pérez-Pérez and Hanson (2002) (16) (Table 1). PCR reactions for the detection of ESBL genes were performed in a final volume of 50 µl containing of 5 µl PCR buffer, 0.5 µl each primer (100 pmol), 2 mM $MgCl_2$, 0.2 mM dNTP, 2 U Taq polymerase (Fermentas) and 10 µl of template DNA. PCR reactions consisted of initial denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 95°C for 45 s, annealing for 45 sn (56°C for ESBL genes, 64°C for pAmpC genes), extension at 72°C for 45 s. After the last cycle, a final extention at 72°C for 7 min was applied. PCR products were sequenced (Sanger sequencing) from both ends using sequencing primers for the determination of the exact subtypes of beta-lactamase genes (Medsantek, İstanbul, Turkey). Subsequently, the isolates found positive for CIT-like PCR amplicon were searched with *bla*$_{CMY-2}$ spesific primers (Zhao *et al*., 2001) (17).

Phylogenetic grouping

Phylogenetic grouping was determined by triplex PCR as described previously by Clermont *et al*. (2000) (18). The primers pairs used were: ChuA.1 (5′-GACGAACCAACGGTCAGGAT-3′) and ChuA.2 (5′-TGCCGCCAGTACCAAAGACA-3′), YjaA.1 (5′-TGAAGTGTCAGGAGACGCTG-3′) and YjaA.2 (5′-ATGGAGAATGCGTTCCTCAAC-3′), and TspE4.

Table 1: Primers used for PCR amplification of ESBL and pAmpC genes

Gene		Sequence (5′→ 3′)	Product size (bp)	Reference
****bla**TEM		ATAAAATTCTTGAAGACGAAA	1080	Ahmed *et al.* (2007)
		GACAGTTACCAATGCTTAATC		
***bla**SHV		TTATCTCCCTGTTAGCCACC	797	
		GATTTGCTGATTTCGCTCGG		
*****bla**SHV		CGG CCTTCACTCAAGGATGTA	927	
		GTGCTGCGGGCCGGATAAC		
****bla**OXA		TCAACTTTCAAGATCGCA	610	
		GTGTGTTTAGAATGGTGA		
***bla**CTX-M		CGCTTTGCGATGTGCAG	551	
		ACCGCGATATCGTTGGT		
*****bla** CTX-M		CCAGAATAAGGAATCCCATG	948	
		GCCGTCTAAGGCGATAAAC		
******bla**AmpC	MOXM	GCTGCTCAAGGAGCACAGGAT	520	Pérez-Pérez and Hanson (2002)
		CACATTGACATAGGTGTGGTGC		
	CITM	TGGCCAGAACTGACAGGCAAA	462	
		TTTCTCCTGAACGTGGCTGGC		
	DHAM	AACTTTCACAGGTGTGCTGGGT	405	
		CCGTACGCATACTGGCTTTGC		
	ACCM	AACAGCCTCAGCAGCCGGTTA	346	
		TTCGCCGCAATCATCCCT AGC		
	EBCM	TCGGTAAAGCCGATGTTGCGG	302	
		CTTCCACTGCGGCTGCCAGTT		
	FOXM	AACATGGGGTATCAGGGAGATG	190	
		CAAAGCGCGTAACCGGATTGG		

* Primers used for detection of ESBL genes
** Primers used for both detection and sequencing of ESBL genes
*** Primers used for sequencing
**** MOXM primer was targeted to MOX-1, MOX-2, CMY-1, CMY-8 - CMY-11; CITM primer to LAT-1 - LAT-4, CMY-2 - CMY-7 and BIL-1; DHAM primer DHA-1 and DHA-2; ACCM primer to ACC; EBCM primer to MIR-1T and ACT-1; and FOXM primer to FOX-1 - FOX-5b.

C2.1 (5′-GAGTAATGTCGGGGCATTCA-3′) and TspE4.C2.2 (5′-CGCGCCAACAAAGTATTACG-3′). PCR reaction was performed in a 25 µl of total volume containing 2.5 µl PCR buffer, 0.25 µl each primer (100 pmol), 2 mM $MgCl_2$, 0.2 mM dNTP, 1.25 U Taq polymerase (Fermentas) and 10 µl of template DNA. The PCR reaction was performed under the following conditions with slight modification: denaturation for 5 min at 94°C; 30 cycles of 30 s at 94°C, 30 s at 60°C, and 30 s at 72°C; and a final extension step of 7 min at 72°C. The isolates were assigned to phylogenetic groups according to presence or absence of the *chu*A (279 bp), *yja*A (211 bp) genes and DNA fragment TspE4.C2 (152 bp): *chu*A–, *yja*A–, TspE4.C2–, group A; *chu*A–, *yja*A–, TspE4.C2+, group B1; *chu*A+, *yja*A+, TspE4.C2+ group B2; *chu*A+, *yja*A–, group D.

Plasmid incompatibility (Inc) group detection

Plasmid incompatibility (Inc) group detection was performed following the method described by Carattoli *et al.* (2005) (19). To detect the incompatibility group, PCR amplification was done with the primers specially designed corresponding to 18 different replication origins of each incompatibility group as described in the method. For this purpose, 5 multiplex and 3 simplex PCR analyses were carried out. The PCR reaction mixture was as follows: 2,5 µl 10× PCR buffer, 2 mM $MgCl_2$, 0.25 µl each primer (100 ρmol), 200 µM dNTP, 2 U Taq polymerase (Fermentas) and 10 µl template DNA in 25 µl of total volume. The same PCR conditions were applied for both multiplex- and simplex PCR analyses, except FrepB incompatibility group. PCR amplification conditions were: initial denaturation for 5 min at 94°C; 30 cycles of denaturation at 94°C for 1, annealing at 60°C for 30 s, extention at 72°C for 1 min; and a final stage of extention at 72°C for 5 min. The FrepB-simplex-PCR was performed with an annealing temperature of 52°C. When positive result was obtained from the mPCR, simplex-PCR was performed with the corresponding primers to confirm the PCR product.

Pulsed field gel electrophoresis (PFGE) analysis

Clonality of the isolates was determined using pulsed field gel electrophoresis (PFGE) following the method suggested by Durmaz *et al.* (2009) (20). PFGE analysis was performed using the restriction enzyme *Xbal* in the Molecular Microbiology Research and Application Laboratory, Ministry

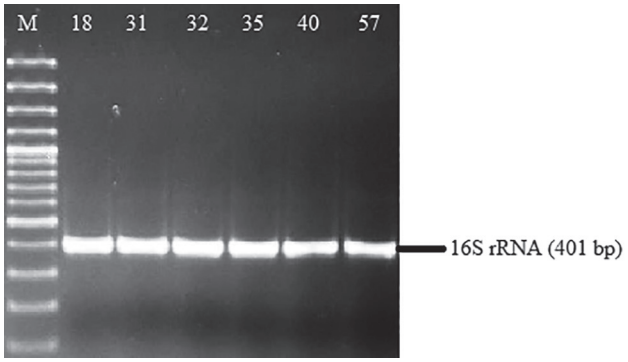

Figure 1: PCR amplification of 16S rRNA gene (401 bp)

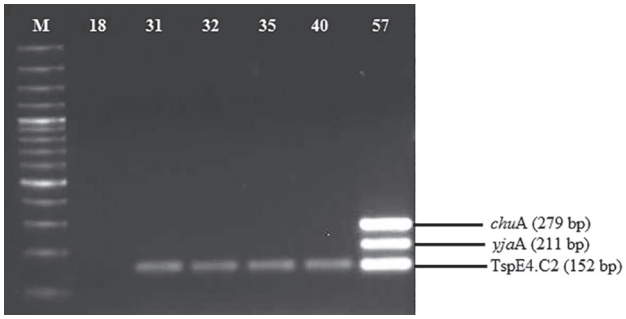

Figure 2: Triplex PCR profiles of the isolates. Lane M: 100 bp plus molecular marker (Fermentas), Lane 18: group A, Lane 31, 32, 35, 40: group B1, and Lane 57: group B2.

of Health, Public Health Agency of Turkey (Ankara, Turkey). The relatedness between the isolates was evaluated according to the criteria previously described by Tenover *et al*. (1995) (21). Isolates exhibiting identical PFGE patterns were considered to be genetically indistinguishable, isolates with a difference of 1 to 3 bands as closely related, isolates with a difference of 4 to 6 bands as possibly related. Multilocus sequence typing of the isolates were performed following the scheme previously described by Wirth *et al*. (2006) (22) and seven housekeeping gene (*adk*, *fum*C, *gyr*B, *icd*, *mdh*, *pur*A and *rec*A) were amplified and sequenced for each isolate. The allele of each gene were determined based on *E. coli* MLST database (http://mlst.warwick.ac.uk/mlst/dbs/Ecoli) and sequence types (ST) were obtained by entering the designated allele numbers via the same database.

RESULTS

Following inoculation on Eosin Methylen Blue agar with cefotaxime (2 µg/ml), *E. coli* colonies were detected in 6 (4%) of 150 cloacal swab samples. Biochemical tests and PCR amplification of the 16S rRNA gene confirmed all isolates as *E. coli* (Figure 1). Out of 6 cefotaxime resistant *E. coli* isolates, five (83.3%) isolates showed ESBL phenotype, while remaining one isolate (16.7%) exhibited a pAmpC phenotype.

The results of antimicrobial susceptibility of ESBL/pAmpC producing *E. coli* isolates are given in Table 2. All isolates were resistant to ampicillin, amoxicillin-clavulanic acid, cefotaxime and ceftazidime, but susceptible to imipenem. Resistance rates for aztreonam and cefoxitin were 83.3% and 16.7%, respectively. Resistance to non-β lactam antimicrobials was observed only in two (33.3%) isolates.

Following PCR amplification and sequencing of ESBL/AmpC producing *E. coli* strains, five (3.3%) carried $bla_{\text{CTX-M-15}}$ and one $bla_{\text{CMY-2}}$. In addition to ESBL/AmpC genes, all isolates harbored $bla_{\text{TEM-1}}$.

The majority of ESBL/pAmpC producing *E. coli* isolates were assigned to phylogenetic group B1. The remaining two isolates belonged to phylogenetic groups A and B2 (Figure 2).

IncII/Iγ, IncFIB, IncFrepB was the most common

Table 2: Characteristics of the ESBL/pAmpC producing *E. coli* isolates

Isolate	Resistance Phenotype[a]	Phylogroup	Beta-lactamase type(s)	Plasmid replicon type	Pulsotype	MLST[b]
18	AM, AMC, CTX, CAZ, C, S, NA, CIP, TE, STX	A	CTX-M-15, TEM-1	II/Iγ, FIB, FIC, A/C, FrepB	I	ST665
31	AM, AMC, CTX, CAZ, ATM	B1	CTX-M-15, TEM-1	II/Iγ, FIB, FrepB	I	ST446
32	AM, AMC, CTX, CAZ, ATM	B1	CTX-M-15, TEM-1	II/Iγ, FIB, FrepB	I	ST446
35	AM, AMC, CTX, CAZ, ATM	B1	CTX-M-15, TEM-1	II/Iγ, FIB, FrepB	I	ST446
40	AM, AMC, CTX, CAZ, ATM	B1	CTX-M-15, TEM-1	II/Iγ, FIB, FrepB	II	ST446
57	AM, AMC, CTX, CAZ, FOX, ATM, NA, TE	B2	CMY-2, TEM-1	FIB, FrepB	III	ST4906

[a] AM: ampicillin, AMC: amoxicillin-clavulanic acid, CTX: cefotaxime, CAZ: ceftazidime, ATM: aztreonam, C: chloramphenicol, S: streptomycin, NA: nalidixic acid, CIP: ciprofloxacin, TE: tetracycline, STX: trimethoprim-sulfamethoxazole

[b] MLST: multi locus sequence typing

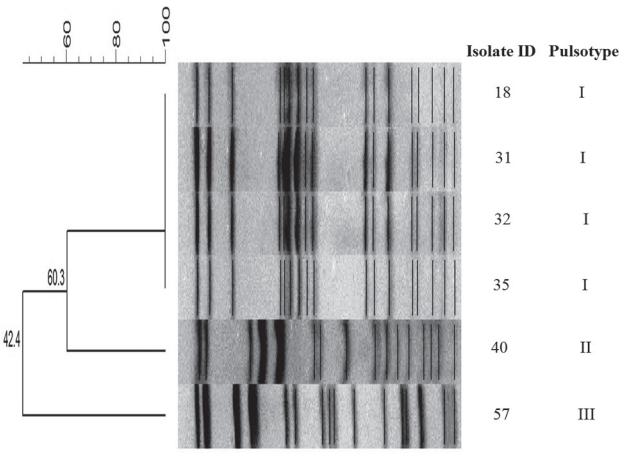

Figure 3: Dendogram showing PFGE analysis of ESBL/pAmpC producing isolates

plasmid replicon profile occuring in four isolates. IncII/Iγ, IncFIB, IncFIC, IncA/C, IncFrepB and IncFIB, IncFrepB profiles were found in one isolate for each (Table 2).

PFGE and MLST analysis revealed three pulsotypes (Figure 3) and three STs (ST665, ST446, ST4906) among ESBL/pAmpC producing *E. coli* isolates (Table 2).

DISCUSSION

Misuse and overuse of antimicrobials for different purposes such as treatment, prophylaxis and feed additives in animals have resulted in the emergence and spread of antimicrobial resistant bacteria, and has become a major concern in Turkey and in the world (23, 24). Many studies have been performed to investigate antimicrobial resistance of *E. coli* isolates from different animal species in Turkey such as cattle (6), dogs (7) and broilers (25), however, up until now, there has been only one study to determine antimicrobial resistance in pigeons in Turkey (11). Having pigeons is a popular hobby in rural and urban areas in Turkey. Therefore, determining the current status of antimicrobial resistance is of importance.

In this study, the prevalence of ESBL/AmpC producing *E. coli* were found to be 4% (6/150). In addition, 5 isolates had *bla*$_{\text{CTX-M-15}}$ genotype and one isolate *bla*$_{\text{CMY-2}}$. This genotypes were also reported by previous studies in cattle (6), dogs (7), broilers (24), chicken meat (8) and humans (9) in Turkey, indicating widespread distribution of these genotypes. Similar observation was reported by Hasan *et al.* who found prevalence of ESBL producing *E. coli* isolates as 5% (7/150) in Bengladesh, and all isolates had *bla*$_{\text{CTX-M-15}}$ (5). To the best of knowledge of the authors, the current study is first reporting existence of CTX-M-15 and CMY-2 from *E. coli* in household pigeons in Turkey.

ESBL/AmpC producing isolates have frequently been reported to be resistant to other classes of antimicrobials (26, 27). Because the genes encoding the ESBL/AmpC usually located on large plasmids, and these plasmids also carry genes conferring resistance to agents such as aminoglycosides, trimethoprim, sulphonamides, tetracyclines and chloramphenicol (28). In this study, only two isolates showed resistance to non-beta lactam antimicrobials such as chloramphenicol, streptomycin, tetracycline, nalidixic acid, ciprofloxacin and trimethoprim-sulfamethoxasole. Another study carried out by Aşkar *et al.* in Kırıkkale, reported higher resistance rates to ampicillin/sulbactam (70%), oxytetracycline (64%) and nalidixic acid (49%) among *E. coli* isolates (11). The occurrence of resistant bacteria in pigeons can be possibly explained in two different ways: (i) ingestion of resistant bacteria from their surrounding environment via contaminated feeds, and (ii) development of resistance to antimicrobials in pigeons due to antibiotic usage for different purposes such as prevention and treatment by humans.

PFGE and MLST revealed presence of three clonal lineage where each clone shared different a phylogenetic group, plasmid replicon type and antimicrobial resistance patterns (Table 2). ST446 found in pigeon was reported among foodborne shiga toxin-producing *E. coli* in Germany (29). Recently, Day *et al.* (30) reported ST665 with IncII/Iγ and IncA/C carrying plasmids among ESBL producing *E. coli* isolates from poultry in Europe. In this study, the isolate belonging to ST665 also carried similar plasmid replicon types. ST4906 was previously reported as a human pathogen in France (http://mlst.warwick.ac.uk/mlst / dbs/E.coli).

In this study, for the first time, it has been shown that household pigeons are reservoir of ESBL/pAmpC producing *E. coli* strains in Turkey. This indicates the possibility of the transmission of this bacteria from pigeon to human or vice versa due to their close proximity. Based on these results, there appears to be a need for further studies to determine distribution and prevalence of resistant bacteria in different animal species and environments.

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