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RESEARCH ARTICLE

Determination and molecular analysis of antibiotic resistance in Gram-negative enteric bacteria isolated from *Pelophylax* sp. in the Eastern Black Sea Region

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ABSTRACT

The aim of this study was to evaluate the prevalence and types of antimicrobial resistance among Gram-negative enteric bacteria isolated from *Pelophylax* sp. Fifty-four frogs were collected from six provinces in the Eastern Black Sea Region of Turkey. In the cloacal swab cultures, bacteria from 160 different colonies were identified by biochemical tests, automated systems, and matrix-assisted laser desorption ionisation-time of flight mass spectrometry. The antimicrobial susceptibility tests were performed by the disk diffusion method. The observed drug resistance rate was the highest to ampicillin and cefazolin, while the lowest against ciprofloxacin and tetracycline. In the molecular assays, bla_{TEM} (8 *Citrobacter* spp.), bla_{SHV} (2 *Escherichia coli*, 1 *Hafnia alvei*, and a *Serratia liquefaciens*), *tet*A genes (*E. coli* and *Klebsiella* spp.) and a class 1 integron without any gene cassette (*E. coli*) were detected. Among the strains, no plasmid-mediated quinolone resistance [*qnrA*, *qnrB*, *qnrS*, *qepA* and *aac (6 ′)-Ib-cr*] was found. However, two of three quinolone-resistant *Klebsiella* strains showed the novel amino acid substitution in the *gyr*A gene resulting in Ser83Asp and Asp87Glu.The clonality between *E. coli* isolates was also examined by pulsed-field gel electrophoresis. We consider that multidrug-resistant Gram-negative enteric bacteria in the intestinal microbiota of a cosmopolitan frog species might be a reservoir for antibiotic resistance genes.

KEYWORDS

Pelophylax, frog, enteric bacteria, drug resistance, integron

INTRODUCTION

The redundant use of antibiotics is a major health concern worldwide. Excessive use of antimicrobial agents in the past two decades is thought to have led to the rapid development of resistance. A multidisciplinary approach to clinical and preclinical evaluation and research is considered essential to control antibiotic resistance (Hosoglu et al., 2005). Antibiotic resistance is found not only in disease-causing bacteria but also in environmental microorganisms in terrestrial and aquatic environments in Turkey (Ozgumus et al., 2007, 2009). It is thought that commensal bacteria could serve as a reservoir of resistance genes that can be transferred to other commensal and pathogenic bacteria (Gonçalves et al., 2012).

The emergence and spread of antimicrobial-resistant bacteria in natural environments is a major concern for human and animal health. Bacteria with genes encoding resistance to these antimicrobials are found in numerous wildlife animals in different environments

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(Carroll et al., 2015). Amphibians are found everywhere in the world where the habitat is suitable (Wells, 2007). Thus, these species might be good indicators of biological variability and environmental changes (Sewell and Griffiths, 2009). *Pelophylax* spp., also known as water frogs, live both in terrestrial and aquatic habitats in Turkey, and are affected directly by habitat changes (Mahaney, 1994).

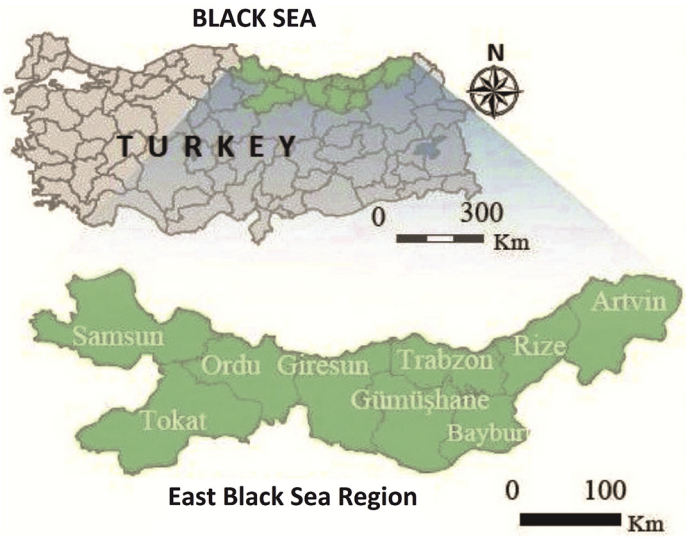
From Minnesota frogs and tadpoles (*Rana pipiens*), Hird et al. (1983) isolated 29 different *Enterobacteriaceae* species, mostly *Escherichia coli* and *Salmonella arizonae*. In addition, there were detected other Gram-negative bacteria such as *Aeromonas* spp., *Klebsiella* spp., *Morganella* spp., *Enterobacter* spp., *Serratia* spp., *Citrobacter* spp., *Yersinia* spp., *Providencia* spp., *Hafnia* spp. and *Proteus* spp. that could cause disease in humans. Acquisition of different resistance genes for enteric bacteria in amphibians is a known process from the aquatic environment (Minette, 1984). Bacteria with multidrug resistance in different environments are considered an important public health problem. These antimicrobial resistances are mostly accomplished by the transfer of drug resistance markers mediated by genetic elements such as integrons, transposons, and conjugative plasmids (Rowe-Magnus and Mazel, 2002). Integrons are genetic elements that allow gene cassettes to be inserted into the bacterial genome and expressed. Numerous studies have shown that enteric bacteria contribute to the spread of antibiotic resistance genes through class 1 and class 2 integrons. Class 1 and/or class 2 integrons have been reported in clinical isolates of the *Enterobacteriaceae* family and in bacteria from aquatic environments, as well (Fluit and Schmitz, 2004). However, integron studies on the bacteria isolated from the frogs, which represent carriers of drug resistance in both environments with their aquatic and terrestrial lives, are very limited. The integrons in different bacteria obtained from environmental samples might give us an idea about the use of antibiotics and the distribution of these antibiotics on earth.

This study is the first report to determine the drug resistance profile and prevalence of resistance genes in the Gram-negative bacteria in the enteric microbiota of a frog, *Pelophylax* sp. in the Eastern Black Sea Region of Turkey.

MATERIALS AND METHODS

Collection of frogs and bacteria

Between May 2016 and July 2017, 54 juvenile, male and female *Pelophylax* sp. living in different ecological habitats (settlement area, industrial zone and ecological habitat) were collected from six cities (Samsun, Ordu, Giresun, Trabzon, Rize and Artvin) of the East Black Sea Coast of Turkey (Fig. 1). This study was conducted in accordance with the requirements of the Recep Tayyip Erdogan University Local Ethics Committee for Animal Experiments (Rize, Turkey) (approval number: 2020/41, 26 October 2020). The sex of each frog was determined according to the presence of an external vocal sac and a protruding pillow in the thumb. Those animals in which these secondary sexual characteristics were lacking or which were smaller than adults were considered juvenile (Erismis, 2011). After having rinsed the anal region with sterile distilled water, cloacal samples were taken from the frogs by the insertion of damped, sterile cotton swabs to 1–1.5 cm depth. Then the swabs were embedded in Luria-Bertani soft agar (1% tryptone, 0.5% yeast extract, 1% NaCl, 0.7% agar, pH 7.5) medium and transported to the lab on ice. Inoculation of the samples to Eosin Methylene Blue agar (Merck, Germany) plates selective for Gram-negative facultative anaerobe bacteria was carried out. Culture plates were incubated at 35 °C for 24 h. A total of 162 colonies showing different



SAMSUN
Yenikoseli village (settlement)
41° 13' 59.32" N, 36° 41' 31.35" E
Industry (industrial zone)
41° 14' 26.92" N, 36° 24' 43.36" E
Kizilirmak Delta Bird Sanctuary (ecological habitat)
41° 40' 12.67" N, 36° 02' 05.82" E

ORDU
Fatsa (settlement)
41° 02' 50.04" N, 37° 28' 38.03" E
Turnasuyu stream (ecological habitat)
40° 58' 30.58" N, 37° 59' 54.52" E
Solid waste plant (industrial zone)
40° 57' 28.90" N, 37° 56' 02.81" E

GIRESUN
Tirebolu shoreline (settlement)
41° 00' 16.52" N, 38° 50' 58.72" E
Espiye auto industry (industrial zone)
40° 56' 45.33" N, 38° 43' 15.38" E
Giresun Gobel plateau (ecological habitat)
40° 32' 38.68" N, 38° 27' 15.21" E

TRABZON
Besikduzu Kurbagalı stream (settlement)
41° 02' 55 23" N, 39° 14' 15.34" E
Yomra (industrial zone)
40° 57' 47.61" N, 39° 50' 30.88" E
Duzkoy way, Of (ecological habitat)
40° 52' 10.38" N, 40° 16' 41.39" E

RIZE
Yaylacilar village stream (settlement)
40° 58' 45.15" N, 40° 20' 32.60" E
Ikizdere way (ecological habitat)
40° 53' 30.27" N, 40° 26' 09.45" E
Engindere district (industrial zone)
41° 02' 35.10" N, 40° 34' 55.60" E

ARTVIN
Borcka Karagol Lake (ecological habitat)
42° 23' 07.90" N, 41° 54' 14.74" E
Hopa sundura district (settlement)
41° 22' 51 13" N, 41° 26' 56.62" E
Hopa nahseni district (industrial zone)
41° 23' 08.51" N, 42° 24' 58.22" E

Fig. 1. Map of the provinces where the frogs were collected and the geographic co-ordinates of the sampling points in the East Black Sea Region

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colour and morphology were randomly picked up from the cultures of the 54 cloacal swab samples. Identification at species level was performed by biochemical tests such as indole test, methyl-red and Voges-Proskauer test and citrate test, Vitek-2 (Biomérieux, France) and matrix-assisted laser desorption/ionisation (MALDI-TOF) mass spectrometer. Bacterial strains were preserved in Luria-Bertani broth liquid medium supplemented with 20% glycerol both at –20 and –80 °C.

For the differentiation of isolates belonging to the same bacterial species, an individual ID was formed. It consisted of the name of the isolation site (first letter), the type of the isolation site (first number: 1 – settlement, 2 – ecological habitat, 3 –industrial zone), the sex/age of the host (second letter: A – male, B – female, C – juvenile), and the serial number of the isolate at the same spot (second number).

Antimicrobial susceptibility testing

Antimicrobial susceptibilities of the bacterial strains were tested by the standard disk diffusion method according to the European Committee on Antimicrobial Susceptibility Testing guidelines (EUCAST, 2016). The strains were classified as susceptible, resistant or intermediate susceptible, with the last mentioned ones being considered susceptible. For the assays, commercial antibiotic discs (Oxoid, UK) were used on Mueller-Hinton Agar plates (Merck, Germany) and bacterial suspensions with the turbidity adjusted to a 0.5 McFarland standard. Plates were incubated at 35 °C for 24 h. The discs contained trimethoprim-sulphamethoxazole (1.25 µg/23 µg), streptomycin (300 µg), gentamicin (10 µg), ciprofloxacin (5 µg), sulbactam/ampicillin (10 µg/10 µg), imipenem (10 µg), ampicillin (10 µg), cefazolin (30 µg), cefuroxime (30 µg), ceftazidime (30 µg), tetracycline (10 µg), and chloramphenicol (30 µg). *E. coli* ATCC®25922™ was used as the quality control strain in susceptibility tests. We defined the multidrug-resistant strain as an isolate that was not susceptible to at least one agent in at least three antimicrobial classes (Magiorakos et al., 2012).

DNA isolation and PCRs for integrons and other resistance genes

Extraction of total DNAs from the bacterial strains was carried out by the boiling method (Ausubel et al., 1995). Briefly, 1.5-ml bacterial cultures were harvested by centrifugation (12,000 g for 5 min) at 4 °C, followed by rinsing twice with sterile distilled water. Then the suspension containing resuspended bacteria in 1 mL distilled water was boiled for 10 min, cooled on ice, and centrifuged as before. The supernatants were used as the DNA templates for the PCR reactions. All DNA samples were stored in aliquots at –20 and –80 °C for further experiments. Bacterial strains were screened for the presence of beta-lactamase genes (bla_{TEM} bla_{SHV} and bla_{OXA}), tetracycline-resistance genes (*tet*A to E), plasmid-mediated quinolone-resistance genes (*qnrA*, *qnrB*, *qnrS*, *qepA* and *aac (6′)-Ib-cr*) by specific PCRs. Class 1 and class 2 integron-specific reaction compositions and thermal cycling parameters were performed as previously described (Lévesque et al., 1995; White et al., 2001). Class 1 integron-containing *E. coli* KD39 and class 2 integron-containing *E. coli* KD36 (Ozgumus et al., 2009) strains were used for integron-specific PCRs as positive controls. To determine the putative chromosomal mutations in the quinolone-resistant strains, *gyr*A and *par*C genes were sequenced following the PCR amplifications. The primers, product sizes and annealing temperatures used for all PCRs are given in the represented references in Table 1. The PCR products were electrophoresed in 1–1.5% agarose gel containing 0.5 µg mL^{-1} ethidium bromide (Sigma, USA) and visualised with UV light.

Pulsed-field gel electrophoresis

The clonality of the isolated 17 *E. coli* strains was investigated by pulsed-field gel electrophoresis (PFGE) using a CHEF-DR II (Bio-Rad, USA) (Durmaz et al., 2009). A pure culture was done on sheep blood agar. A loopful of bacteria was collected from the agar and suspended in 1 mL of cell suspension buffer (100 mM Tris-HCl, 10 mM EDTA). After adjusting turbidity of the cell suspension 0.7 to 1.0 absorbance at 590 nm by optical density measurement, the cells were embedded in low-melting agarose (Lonza, Switzerland). The bacterial cells in agarose plugs were lysed with cell lysis solution I (50 mM Tris-HCL, 50 mM EDTA) containing lysozyme (2.5 mg mL^{-1}) and proteinase K (1.5 mg mL^{-1}), and then with the cell lysis solution II (0.5 M EDTA, 0.1% sarcosyl with 400 µg mL^{-1} proteinase K), and finally the bacterial DNA was cut with XbaI restriction enzyme (Thermo Scientific, USA). Electrophoresis was performed in 1% agarose gel prepared in 0.5X TBE (Tris-borate-EDTA) buffer. The running temperature was 14 °C. Optimal run conditions for the separation of fragments were set as 20 h at 6 V cm^{-2} with an initial switch time of 5 s, a final switch time of 30 s and a 120° angle. The gel was stained with ethidium bromide (1 µg mL^{-1}) for 20 min, visualised under UV light using a transilluminator, and DNA band images were photographed using the gel imaging system (DNR MiniLumi, Israel). Cluster analysis of bacterial fingerprint profiles was done using Bionumerics (AppliedMaths, version 6.01). For determination of the molecular relationship among the isolated *E. coli* strains the optimised procedure of Tenover et al. (1995) was used.

Nucleic acid sequencing and bioinformatic analysis

DNA sequencing reactions of PCR products of *gyr*A, *par*C were analysed on an ABI 3130 automated capillary DNA sequence analyser (Applied Biosystems, USA) in the Karadeniz Technical University, Turkey. The sequence similarity searches were performed using the BLAST program (Altschul et al., 1990). For multiple sequence alignments (MSA) the program CLUSTAL W available on https://www.genome.jp/tools-bin/clustalw was used (Thompson et al., 1994). The nucleotide sequences determined in this study have been deposited in the GenBank.

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Table 1. Oligonucleotide primer sequences, amplicon sizes and annealing temperatures used in this study

Primer name	Primer sequence (5′ → 3′)	Gene	Product size (bp)	Annealing temperature (°C)	Reference
IntI1F	GGTCAAGGATCTGGATTTGG	*IntI1*integrase	500 bp	57	Machado et al. (2005)
IntI1R	ACATGCGTGTAAATCATCGTC				
IntI2F	CACGGATATGCGACAAAAAGGT	*IntI2* integrase	740 bp	57	Machado et al. (2005)
IntI2R	GTAGCAAACGAGTGACGAAATG				
5′-CS	GGCATCCAAGCAGCAAG	Variable region of class 1 integron	Variable	57	Lévesque et al. (1995)
3′-CS	AAGCAGACTTGACCTGA				
hep51	GATGCCATCGCAAGTACGAG	Variable region of class 2 integron	Variable	57	White et al. (2001)
hep74	CGGGATCCCGGACGGATGCACGATTTGTA				
TEM-F	ATGAGTATTCAACATTTCCG	bla_{TEM}	857 bp	55	Arlet et al. (1995)
TEM-R	CCAATGCTTAATCAGTGAGG				
SHV-F	TTATCTCCCTGTTAGCCACC	bla_{SHV}	796 bp	55	Arlet et al. (1997)
SHV-R	GATTTGCTGATTTCGCTCGG				
OXA-F	ACACAATACATATCAACTTCGC	bla_{OXA}	885 bp	60	Lim et al. (2009)
OXA-R	AGTGTGTTTAGAATGGTGATC				
tet(A)-1	GTAATTCTGAGCACTGTCGC	*tet*A	917 bp	58	Guardabassi et al. (2000)
tet(A)-2	CTGCCTGGACAACATTGCTT				
tet(B)-1	CTCAGTATTCCAAGCCTTTG	*tet*B	396 bp	55	
tet(B)-2	ACTCCCCTGAGCTTGAGGGG				
tet(C)-1	GGTTGAAGGCTCTCAAGGGC	*tet*C	589 bp	56	Schmidt et al. (2001)
tet(C)-2	CCTCTTGCGGGAATCGTCC				
tet(D)-1	AAACCATTACGGCATTCTGC	*tet*D	787 bp	56	Marshall et al. (1983)
tet(D)-2	GACCGGATACACCATCCATC				
tet(E)-1	AAACCACATCCTCCATACGC	*tet*E	278 bp	56	Marshall et al. (1983)
tet(E)-2	AATAGGCCACAACCGTCAG				
qnrA1	ATTTCTCACGCCAGGATTTG	*qnrA*	516 bp	54	Robicsek et al. (2006)
qnrA2	GATCGGCAAAGGTTAGGTCA				
qnrB1	GGMATHGAAATTCGCCACTG*	*qnrB*	264 bp	54	Cattoir et al. (2007)
qnrB2	TTTGCYGYYCGCCAGTCGA*				
qnrS1	GCAAGTTCATTGAACAGGGT	*qnrS*	428 bp	54	Cattoir et al. (2007)
qnrS2	TCTAAACCGTCGAGTTCGGCG				
gyrAWR	GCCATACCTACGGCGATACC	*gyrA*	344 bp	54	Kim et al. (2009)
gyrAWF	AAATCTGCCCGTGTCGTGGT				
parCWR	GCGAACGATTTCGGATCGTC	*parC*	168 bp	54	Kim et al. (2009)
parCWF	CTGAATGCCAGCGCCAAATT				
qepAF	AACTGCTTGAGCCCGTAGAT	*qepA*	596 bp	60	Kim et al. (2009)
qepAR	GTCTACGCCATGGACCTCAC				
accIbR	CTGGAATGCCTGGCGTGTTT	*acc(6′)-Ib-cr*	482 bp	54	Park et al. (2006)
accIbF	TTGCGATGCTCTATGAGTGGC				

* M = A or C; H = A or C or T; Y = C or T.

RESULTS

A total of 162 bacterial strains were identified from the cloacal swab samples of *Pelophylax* frogs: 160 Gram-negative (68 *Citrobacter* spp., 25 *Enterobacter* spp., 17 *E. coli*, 14 *Raoultella* spp., 13 *Klebsiella* spp., 4 *Aeromonas* spp., 4 *Serratia* spp., 4 *Hafnia* spp., 3 *Kluyvera* spp., 2 *Pseudomonas* spp., 2 *Sphingomonas* spp., 1 *Morganella* spp., 1 *Stenotrophomonas* spp., 1 *Pantoea* spp. and 1 *Leclercia* spp.) (Table 2), and 2 Gram-positive bacteria. However, these latter were excluded from further examination.

The isolated Gram-negative bacteria presented drug resistance mainly to penicillins (ampicillin, sulbactam/ampicillin), to cephalosporins (cefazolin, cefuroxime, ceftazidime), and to carbapenems (imipenem), which are members of the beta-lactam class of antibiotics. The prevalence of resistance to ampicillin was 90%, and against the combination of sulbactam/ampicillin, it exceeded 50% among the studied strains. The resistance to cephalosporins was high but it was decreasing along with newer derivatives (first-, second- and third-generation) of drug molecules (58% to cefazolin, 34% to cefuroxime, and 25% to ceftazidime, respectively). In addition, moderate susceptibility to imipenem (31.5%) and streptomycin (18%) were present in the strains, while antimicrobial resistance of the strains to trimethoprim-sulphamethoxazole, gentamicin, tetracycline, ciprofloxacin, and chloramphenicol was low (Table 3). Among the 160 isolates, 9 (5.6%) showed resistance to none, 29 (18.1%) to one, 23 (14.3%) to two and 99 (61.8%) to three or more of the antimicrobial groups (Table 4).

Among the isolates that showed drug resistance, the presence of class 1 and/or class 2 integrons, of beta-

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Table 2. List of Gram-negative bacteria isolated from the frogs *Pelophylax* sp. living in different ecological habitats (settlement area, industrial zone and ecological habitat) of six cities (Samsun, Ordu, Giresun, Trabzon, Rize and Artvin) of the East Black Sea Coast of Turkey

Species	Number (%)
Citrobacter spp. (*C. freundii, C. braakii, C. amalonaticus*)	68 (42.5%)
Enterobacter spp. (*E. amnigenus, E. asburiae, E. cloacae complex*)	25 (15.6%)
Escherichia coli	17 (10.6%)
Raoultella spp. (*R. planticola, R. ornithnolytica*)	14 (8.6%)
Klebsiella spp. (*K. oxytoca, K. pneumoniae* ssp. *pneumoniae*)	13 (8.1%)
Aeromonas spp. (*A. sobria, A. hydrophila, A. veronii*)	4 (2.5%)
Serratia spp. (*S. fonticola, S. liquefaciens* group)	4 (2.5%)
Hafnia alvei	4 (2.5%)
Kluyvera cryocrescens	3 (1.9%)
Pseudomonas putida	2 (3.3%)
Sphingomonas paucimobilis	2 (3.3%)
Morganella morganii subsp. *sibonii*	1 (0.6%)
Stenotrophomonas maltophilia	1 (0.6%)
Pantoea spp.	1 (0.6%)
Leclercia adecarboxylata	1 (0.6%)
Total	160

lactamase genes (bla_{TEM}, bla_{SHV} and bla_{OXA}), of tetracycline-resistance genes (*tet*A to E), of plasmid-mediated quinolone-resistance genes (*qnrA*, *qnrB*, *qnrS*, *qepA* and *aac (6 ′)-Ib-cr*), and of putative mutations in *gyr*A and *par*C genes were screened by specific PCRs and sequencing.

The PCR for detection of integrase genes was positive only in *E. coli* strain S1C2 (from all 160) isolated from a juvenile frog collected in Yenikoseli village (settlement) close to Samsun city, adjacent to the Samsun Kizilirmak Delta Bird Sanctuary (Fig. 1). No amplification could be seen in the variable region of this class 1 integron, which suggested the lack of any cassette in it (Fig. 2). The integrase gene part of this integron was sequenced, and no mutation or gene polymorphism was detected (data not shown).

Beta-lactam resistant (resistance to ampicillin) strains were screened for the genes bla_{TEM}, bla_{SHV} and bla_{OXA} by PCR. Twelve isolates (8 *Citrobacter*, 2 *E. coli*, 1 *Hafnia alvei*, and 1 *Serratia liquefaciens*) possessed any of these drug resistance genes. No strain was found positive for the bla_{OXA} gene (Table 5).

Two (*E. coli* strain S1C2 and *Klebsiella* spp. O3C5) of four strains (*E. coli* strain S1C2, *Klebsiella* spp. O3C5, *Aeromonas sobria* T1A3 and *Morganella morganii* G3AR3) resistant to tetracycline were found to have a *tet*A gene (Table 5).

The *gyr*A and *par*C genes regions of three isolates (two *Klebsiella* spp. and a *Citrobacter koseri*) resistant to ciprofloxacin were sequenced for screening putative mutations responsible for quinolone resistance (Table 6).

Based on the deduced amino acid sequences, in the *gyr*A genes of *Klebsiella pneumoniae* A1B1 (from a female frog collected in Borcka Karagol Lake in the Artvin Province) and of *Klebsiella oxytoca* G1C3 (from a juvenile frog collected on the Tirebolu shoreline in Giresun city) serine amino acid in position 83 was replaced by aspartic acid (Ser83→Asp), and aspartic acid in position 87 was replaced by glutamic acid (Asp87→Glu); in addition, a Glu84→Val mutation was observed in the *par*C gene (Table 5). No novel mutations were observed in the *gyr*A and *par*C genes of the quinolone-resistant *C. koseri* strain A2B3 (from a female animal caught in Hopa Sundura district in Artvin Province) (Fig. 1). The nucleotide sequences determined in this study have been deposited in the GenBank and assigned the following accession numbers: MW119566 and MW119567.

Clonal relationship among the 17 *E. coli* strains as an indicator of faecal pollution were investigated by PFGE analysis (Fig. 3). A total of 17 strains were found to be 92% related according to the Tenover criteria (Tenover et al., 1995). These strains grouped into two clusters: one cluster (12 strains) contains four pulsotypes, while the other cluster (5 strains) is divided into two pulsotypes (Fig. 3).

DISCUSSION

Although antibiotic resistance is a major public health problem worldwide, this has been largely ignored in environmental studies. It is predicted that aquatic environments can be ideal places for acquiring and spreading antibiotic resistance via anthropogenic activities. It is now obvious that the presence of antibiotics acts as a stimulus for the development of antibiotic resistance, and is considered to contribute to the spreading of mobile genetic elements (Marti et al., 2014). Antibiotics and other pollutants, even just resistant bacteria and their metabolites reach aquatic environments through treated and untreated sewage waters, hospital waste, aquaculture waste, and agricultural discharges. Thus, water and its sediments play a leading role in the transfer, ecology, and evolution of antibiotic resistance genes (Taylor, 2011). The environment consists not only of water and soil but also of plants, animals, and microbes associated with the hosts' microbiota. Many studies have shown that antibiotic-resistant bacteria are isolated mainly from mammals, such as wild boar, rodents (Gilliver et al., 1999) and insects (Kadavy et al., 2000).

Frogs live in puddles and microbe-rich soil and mud. Hird et al. (1983) isolated *E. coli, Salmonella, Citrobacter, Klebsiella, Proteus, Aeromonas, Enterobacter, Serratia, Hafnia, Providencia, Morganella* and *Yersinia* species from frogs (*R. pipiens*) and tadpoles near Minnesota. In our study, members of 15 different species of Gram-negative bacteria were isolated, but the genera *Salmonella, Proteus, Providencia* and *Yersinia* were not found. Most of the isolated bacteria belonged to the *Citrobacter* genus that is well known as a human pathogen, as well (Ferranti et al., 2018). *Citrobacter* spp. are commonly found in the environment and

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Table 3. Incidence of the antimicrobial resistance of Gram-negative bacteria isolated from cloacal samples of the frogs *Pelophylax* sp. living in different ecological habitats of the East Black Sea Coast of Turkey

Species (n)*	Antibiotics**											
	SXT	S	GN	CIP	SAM	IMP	AMP	CZ	CXM	CAZ	TE	C
Citrobacter spp. (68)	1 (1.5%)	21 (30.9%)	3 (4.4%)	2 (2.9%)	42 (61.8%)	25 (36.8%)	63 (92.6%)	38 (55.9%)	25 (36.8%)	23 (33.8%)	0	0
Enterobacter spp. (25)	0	1 (4%)	0	0	17 (68%)	11 (44%)	22 (88%)	15 (60%)	11 (44%)	5 (20%)	0	0
Escherichia coli (17)	1 (5.9%)	2 (11.8%)	0	0	5 (29.4%)	1 (5.9%)	13 (76.4%)	9 (52.9%)	3 (17.6%)	2 (11.8%)	1 (5.9%)	0
Raoultella spp. (14)	1 (7.1%)	1 (7.1%)	0	0	5 (35.7%)	2 (14.3%)	14 (100%)	7 (50%)	2 (14.3%)	3 (21.4%)	0	0
Klebsiella spp. (13)	1 (7.7%)	2 (15.4%)	0	1 (7.7%)	4 (30.8%)	1 (7.7%)	12 (92.3%)	5 (38.4%)	1 (7.7%)	1 (7.7%)	1 (7.7%)	0
Aeromonas spp. (4)	0	0	0	0	4 (100%)	4 (100%)	4 (100%)	3 (75%)	1 (25%)	0	1 (25%)	0
Serratia spp. (4)	0	0	0	0	1 (25%)	0	3 (75%)	3 (75%)	1 (25%)	2 (50%)	0	0
Hafnia spp. (4)	0	0	0	0	4 (100%)	1 (25%)	4 (100%)	4 (100%)	3 (75%)	1 (25%)	0	0
Kluyvera spp. (3)	1 (33.3%)	0	0	0	0	1 (33.3%)	3 (100%)	3 (100%)	2 (66.6%)	0	0	0
Pseudomonas spp. (2)	2 (100%)	0	0	0	2 (100%)	0	2 (100%)	2 (100%)	2 (100%)	1 (50%)	0	2 (100%)
Sphingomonas spp. (2)	0	1 (50%)	1 (50%)	0	2 (100%)	2 (100%)	2 (100%)	2 (100%)	2 (100%)	1 (50%)	0	0
Morganella spp. (1)	0	0	0	0	1 (100%)	0	1 (100%)	1 (100%)	1 (100%)	0	1 (100%)	1 (100%)
Stenotrophomonas spp. (1)	0	1 (100%)	1 (100%)	0	1 (100%)	1 (100%)	1 (100%)	1 (100%)	1 (100%)	1 (100%)	0	0
Pantoea spp. (1)	0	0	0	0	0	1 (100%)	0	0	0	0	0	0
Leclercia spp. (1)	0	0	0	0	0	0	0	0	0	0	0	0
Total	7 (4%)	29 (18%)	5 (3%)	3 (2%)	88 (55%)	50 (31.5%)	144 (90%)	93 (58%)	55 (34%)	40 (25%)	4 (2.5%)	3 (2%)

*n, number of strains isolated. **SXT, trimethoprim/sulphamethoxazole; S, streptomycin; GN, gentamicin; CIP, ciprofloxacin; SAM, sulbactam/ampicillin; IMP, imipenem; AMP, ampicillin; CZ, cefazolin; CXM, cefuroxime; CAZ, ceftazidim; TE, tetracycline; C, chloramphenicol.

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Table 4. The number/proportion of the bacterial isolates showing resistance to none, one, two, three or more groups of antimicrobial agents

Bacterial species	Resistance to antimicrobials/n*			
	None	One	Two	Three or more
Citrobacter spp.	4/68 (5.8%)	9/68 (13.2%)	6/68 (8.8%)	49/68 (72%)
Enterobacter spp.	1/25 (4%)	6/25 (24%)	3/25 (12%)	15/25 (60%)
Escherichia coli	2/17 (11.7%)	6/17 (35.2%)	3/17 (17.6%)	6/17 (35.2%)
Raoultella spp.	0/14	5/14 (35.7%)	4/14 (23.5%)	5/14 (35.7%)
Klebsiella spp.	0/13	2/13 (15.3%)	6/13 (46.1%)	5/13 (38.4%)
Aeromonas spp.	0/4	0/4	0/4	4/4 (100%)
Serratia spp.	1/4 (25%)	0/4	1/4 (25%)	2/4 (50%)
Hafnia spp.	0/4	0/4	0/4	4/4 (100%)
Kluyvera spp.	0/3	0/3	0/3	3/3 (100%)
Pseudomonas spp.	0/2	0/2	0/2	2/2 (100%)
Sphingomonas spp.	0/2	0/2	0/2	2/2 (100%)
Morganella spp.	0/1	0/1	0/1	1/1 (100%)
Stenotrophomonas spp.	0/1	0/1	0/1	1/1 (100%)
Pantoea spp.	0/1	1/1 (100%)	0/1	0/1
Leclercia spp.	1/1 (100%)	0/1	0/1	0/1
Total	9/160 (5.6%)	29/160 (18.1%)	23/160 (14.3%)	99/160 (61.8%)

* Total number of the isolates.

are considered an indicator of water contamination. They are also found in different organs of diseased animals, including mammals, birds, reptiles, and amphibians (Wang et al., 2000).

Hacioglu and Tosunoglu (2014) isolated bacteria belonging to the genera *Aeromonas, Plesiomonas, Vibrio, Citrobacter, Enterobacter, E. coli, Klebsiella, Edwardsiella, Hafnia, Proteus, Providencia* and *Pseudomonas* from the salamanders, frogs and reptiles collected in Kavak delta (Canakkale) in Turkey. Seven to 46% of the isolated bacteria were resistant to all antibiotics tested (tobramycin, kanamycin, amoxicillin, oxytetracycline, cefmetazole, gentamicin, furazolidone, erythromycin, cefoxitin, ampicillin, cefotaxime, and chloramphenicol). Boman (2000) also isolated coliform bacteria resistant to nalidixic acid, rifampicin and streptomycin from frogs. The incidence of multidrug resistance in the bacteria we isolated is indicative of anthropogenic pollution in the area where the frogs live.

We found that the Gram-negative bacteria (fermentative and/or non-fermentative) isolated from the *Pelophylax* sp., known as a cosmopolitan species, were highly resistant to antibiotics.

We detected three bla_{TEM}-containing strains (*Citrobacter freundii* T2C2, *Citrobacter braakii* A1A1 and *H. alvei* A1C1) resistant to ampicillin/sulbactam among the strains isolated from the frogs in Trabzon and Artvin counties. We suppose that the strains might have an inhibitor-resistant TEM gene (bla_{IRT}), because bla_{IRT} coding TEM-type enzymes has been reported to be related to β-lactamase inhibitors such as clavulanic acid, sulbactam and tazobactam (Chaibi et al., 1999).

Although no statistical analysis of the prevalence of drug resistance in the studied bacteria was performed, it has been reported that the intestinal flora of frogs and tadpoles could gain antimicrobial resistance genes from aquatic environments (Minette, 1984). The basis of this hypothesis is that frogs and tadpoles live in nature as coprophages, and the food as stool remains in the intestinal tract for a longer time, allowing microbial digestion to occur.

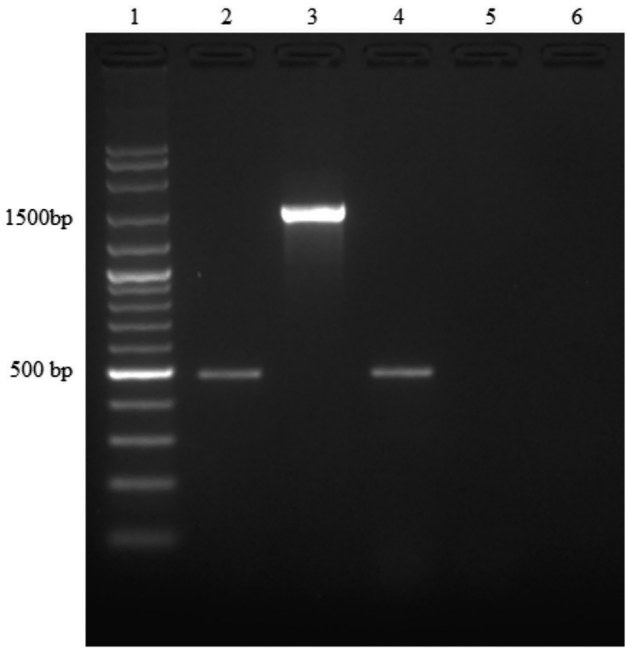


Fig. 2. Agarose gel electrophoresis analysis of the integrons. 1. VC 100 bp Plus DNA ladder (Vivantis, Malaysia); 2. PCR amplicon of the integrase gene (*IntI1*) in *Escherichia coli* KD39 (Ozgumus et al., 2009); 3. PCR amplicon of the variable region of the class 1 integron in *Escherichia coli* KD39 (Ozgumus et al., 2009); 4. PCR amplicon of the integrase gene (*IntI1*) in *Escherichia coli* S1C2; 5. PCR analysis of the *IntI1* gene of the variable region of the class 1 integron in *Escherichia coli* S1C2 (no amplification); 6. PCR analysis of the integrase gene (*IntI1*) in an integron-negative control strain (*Escherichia coli* ATCC25922) (no amplification)

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Table 5. The properties of the β-lactam-resistant strains containing resistance genes detected by PCR from the frogs collected in different ecological habitats (settlement area, industrial zone and ecological habitat) of six cities (Samsun, Ordu, Giresun, Trabzon, Rize and Artvin) of the East Black Sea Coast of Turkey

Strain*	Antibiotic resistance phenotype**	Resistance gene
Citrobacter spp. T1B4	AMP	bla_{TEM}
Citrobacter spp. T1B5	AMP	bla_{TEM}
Citrobacter freundii T2C2	S, SAM, IMP, AMP, CXM	bla_{TEM}
Escherichia coli T3C2	AMP	bla_{TEM}
Citrobacter braakii A1A1	SAM, AMP, CZ, CAZ	bla_{TEM}
Serratia liquefaciens group A1B3	AMP, CZ	bla_{TEM}
Hafnia alvei A1C1	SAM, AMP, CZ, CXM	bla_{TEM}
Escherichia coli S1C1	AMP	bla_{TEM}
Citrobacter freundii R1B1	AMP, CZ, CXM, CAZ	bla_{SHV}
Citrobacter freundii R1B4	SAM, AMP, CXM, CAZ,	bla_{SHV}
Citrobacter freundii T1A1	SAM, IMP, AMP	bla_{SHV}
Citrobacter spp. O3C3	SAM, IMP, AMP	bla_{SHV}
Klebsiella spp. O3C5	AMP, TE	*tet*A
Escherichia coli S1C2	SXT, S, AMP, CZ, CAZ, TE	*tet*A

* It consists of the name of the isolation site (first letter), the type of isolation site (first number: 1 – settlement, 2 – ecological habitat, 3 –industrial), the sex/age of the host (second letter – A-male, B – female, C – juvenile), and the serial number of the isolate at the same spot (second number). **SXT, trimethoprim/sulphamethoxazole; S, streptomycin; GN, gentamicin; CIP, ciprofloxacin; SAM, sulbactam/ampicillin; IMP, imipenem; AMP, ampicillin; CZ, cefazolin; CXM, cefuroxime; CAZ, ceftazidim; TE, tetracycline; C, chloramphenicol.

Table 6. Amino acid substitutions in *gyr*A and *par*C genes in ciprofloxacin-resistant strains

Strain*	Antibiotic resistance phenotype**	Amino acid substitutions	
		*gyr*A	*par*C
Klebsiella pneumoniae A1B1	GN, CIP, IMP, AMP, CZ, CXM, CAZ	Ser83→Asp and Asp87→Glu	Glu84→Val
Klebsiella oxytoca G1C3	CIP, AMP, CZ	Ser83→Asp and Asp87→Glu	Glu84→Val

*It consists of the name of the isolation site (first letter), the type of isolation site (first number: 1 – settlement, 2 – ecological habitat, 3 –industrial), the sex/age of the host (second letter – A-male, B – female, C – juvenile), and the serial number of the isolate at the same spot (second number). **SXT, trimethoprim/sulphamethoxazole; S, streptomycin; GN, gentamicin; CIP, ciprofloxacin; SAM, sulbactam/ampicillin; IMP, imipenem; AMP, ampicillin; CZ, cefazolin; CXM, cefuroxime; CAZ, ceftazidim; TE, tetracycline; C, chloramphenicol.

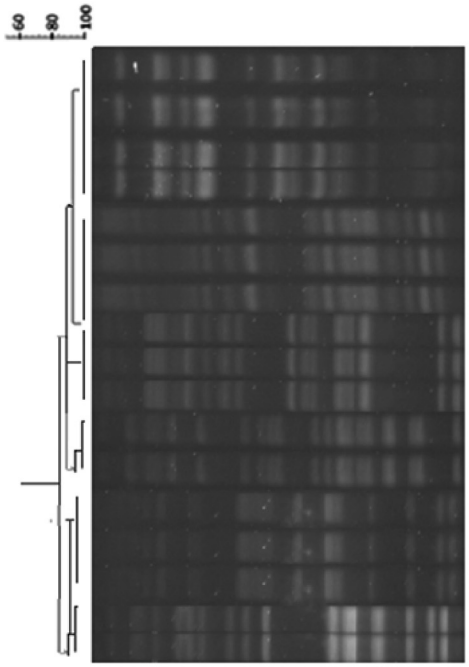
Bacteria from the rectal swabs of female, male and juvenile individuals from three different locations were taken for this study to achieve 'homogeneous' sampling. After performing antimicrobial susceptibility tests, we determined that 80.9% of the strains from males, 70.6% of the strains from females and 78.3% of the strains from juveniles were multidrug resistant.

Class 1 integrons were originally detected on transposons such as *Tn*21 (Grinsted et al., 1990). Class 2 integrons have been found in *Tn*7 and contained dihydrofolate reductase (*dhfr*) gene cassettes and carried three classical gene cassettes, such as *dfrA1*, *sat2* and *aadA1*, which provide resistance to trimethoprim, streptomycin and streptomycin/spectinomycin, respectively (Bryne-Bailey et al., 2011). Cissell (2006) recorded a class 1 integron with low prevalence in *E. coli* obtained from ponds accessible to cattle. At the same time, *E. coli* strains without integron could be isolated from amphibians collected in the same pools. Although antibiotic resistance in *E. coli* was common in and adjacent to areas with cattle farms, this resistance was not correlated with the presence of class 1 integrons. In our study, the finding of an integrase gene-carrying *E. coli* strain S1C2 with an empty integron implies that these bacterial strains have the potential to become multidrug resistant by capturing antibiotic resistance genes from other bacteria in the environment, even passing them between terrestrial and aquatic ecosystems.

The tetracycline-resistance genes *tet*A and/or *tet*B in different species of Enterobacteriales of aquatic origin have been shown to be well expressed and resulted in high-level resistance (Ozgumus et al., 2009). In the present study, two strains (*Klebsiella* spp. O3C5 and *E. coli* S1C2) were found to have *tet*A gene (Table 5). Our previous study showed that *tet*A-mediated tetracycline resistance is widespread in tetracycline-resistant *E. coli* strains isolated from the drinking water (Ozgumus et al., 2007). In another study conducted with bacteria isolated from fish farms, *tet*A and *tet*B genes were detected in oxytetracycline-resistant *Yersinia ruckeri* strains (Balta et al., 2010). The coliform pathogens could obtain resistance genes to deactivate various antibiotics through horizontal gene transfer of the resistance plasmids from bacterial populations in aquatic environments (Sandalli et al., 2010). In this way, microorganisms of aquatic origin can become pathogenic for humans, which can interfere with the success of antimicrobial treatment.

Quinolone resistance occurs in three ways: (1) mutation in DNA replication target enzyme genes (DNA gyrase, topoisomerase IV), (2) plasmid-mediated quinolone resistance (PMQR) (*qnr* gene family), and (3) loss of membrane-bound porin or ejection of the drug by the efflux pump (enzymatic modification gene *acc-(6′)-Ib-cr* and efflux pump gene *qepA*) (Jacoby, 2005). Fluoroquinolone resistance is commonly associated with DNA gyrase and topoisomerase IV gene mutations. Structural changes caused by mutations in the quinolone resistance-determining regions (QRDR) of the *gyr*A and *par*C genes reduce the affinity of fluoroquinolones to these enzymes (Moon et al., 2010). Previous studies demonstrated that the mutations in the QRDR of *gyr*A and *par*C genes, at nucleotide positions 248 and 259/260 of *gyr*A resulting in Ser83 and Asp87 substitution and mutations at 238/239 and 250/251 nucleotide sites of *par*C resulting in Ser80 or Glu84 changes, are mainly responsible for

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Strains	Antibiotic resistance phenotype	Sampling Location
T3C1	AMP	Trabzon Duzkoy way, Of (ecological habitat)
T3C2	AMP	Trabzon Duzkoy way, Of (ecological habitat)
T3C3	AMP	Trabzon Duzkoy way, Of (ecological habitat)
R3B12	-	Rize Engindere district (industrial zone)
G3C1	SAM, AMP, CAZ, CZ, CXM	Giresun Gobel plateau (ecological habitat)
G3C2	SAM, AMP, CZ	Giresun Gobel plateau (ecological habitat)
S2A2	IMP, CZ	Samsun industry (industrial zone)
R2C3	SAM, AMP, CZ, CXM	Rize Ikizdere way (ecological habitat)
O1B2	AMP	Ordu Fatsa (settlement)
O1B3	SAM, AMP, CZ	Ordu Fatsa (settlement)
S2A1	S, CZ	Samsun industry (industrial zone)
S1C1	AMP	Samsun Yenikoseli village (settlement)
S1C2	SXT, S, AMP, CAZ, CZ, TE	Samsun Yenikoseli village (settlement)
S3B1	-	Samsun Kizilirmak Delta Bird Sanctuary (ecological habitat)
G3C3	SAM, AMP, CZ	Giresun Gobel plateau (ecological habitat)
S1C3	AMP	Samsun Yenikoseli village (settlement)
S3B3	SAM, AMP, CZ, CXM	Samsun Kizilirmak Delta Bird Sanctuary (ecological habitat)

Fig. 3. Pulsed-field gel electrophoresis profiles of the 17 *Escherichia coli* isolates. 1st cluster (from T3C1 to S1C1); Pulsotype 1 (four strains), Pulsotype 2 (three strains), Pulsotype 3 (three strains), Pulsotype 4 (two strains). 2nd cluster (from S1C2 to S3B3); Pulsotype 1 (three strains), Pulsotype 2 (two strains)

fluoroquinolone resistance (Cullen et al., 1989; Villa et al., 1996). In this study, three strains (Table 5) were found to be resistant to a fluoroquinolone, ciprofloxacin. In water samples taken from the northern rivers of Turkey, we earlier reported the isolation of a *K. pneumoniae* strain (KD100) carrying the *qnrS* gene, which was the first report of plasmid-mediated quinolone resistance of environmental origin in Turkey (Ozgumus et al., 2009). However, we found no *qnr* genes in this study. Three quinolone-resistant strains were detected among our isolates. Two *Klebsiella* spp. showed the same novel amino acid changes both in *gyr*A and *par*C genes (Table 5). However, no mutations of these genes were found in the quinolone-resistant *C. koseri* A2B3 strains. This latter resistance may be the result of the mutation in the *gyr*B, which is seen less frequently. These data demonstrate a strong correlation between the accumulation of mutations in *gyr*A and *par*C and increases in resistance to fluoroquinolones reported previously (Webber and Piddock, 2001).

Faecal pollution of water resources is an important public health problem as it causes people to be exposed to pathogens. Therefore, these waters should be constantly monitored and appropriate protection measures should be taken. Faecal indicator bacteria are used to monitor pollution in environmental waters and identify public health risks. *E. coli* has been used as an indicator of faecal pollution for a long time (Napier et al., 2017). According to the PFGE analysis, the similarity between the *E. coli* strains isolated from samples collected from different areas with various grades of anthropogenic contamination was quite high.

Although the bacterial strains from the frogs were highly resistant to the tested antibiotic groups, transmissible genetic elements such as conjugative plasmids (e.g. PMQR) and/or integron gene cassettes (e.g. cassette arrays) coding for the resistance to antimicrobials could not be detected, except for an *E. coli* strain with empty class 1 integron.

This finding draws attention to the need for long-term monitoring of intestinal bacteria carrying mobile genetic elements in cosmopolitan frog species. Thus, we can follow changes in microbial populations in the animal microflora, and their interaction with the human microflora in terms of genetic elements coding for resistance to clinically important antimicrobials.

Resistant bacteria are present in the male, female, and juvenile individuals living in all studied environments, which is considered an indicator of human-induced pollution. The pathogenicity and phylogenetic classification of faecal coliforms, especially those of human and animal origin, such as *E. coli*, should be elucidated by further studies. For this, the underlying genetic mechanisms of drug resistance determined by mobile genetic elements or different virulence genes, and the molecular epidemiology of the strains carrying these genetic elements should be investigated and analysed. These exploratory studies should be expanded across the country and even the world as a global surveillance. As all the isolated bacterial species, not just *E. coli* might be potential human pathogens, further studies are needed to identify their potential virulence factors and genotypic relationships.

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identification of several strains at species level by the use of MALDI-TOF mass spectrometry. For sharing his knowledge and technical opportunities with us in our DNA sequence analysis assays, we would like to thank Prof. Ersan Kalay and Research Assistant Idris Er (Medical Biology Department of Karadeniz Technical University). We thank Associate Professor Alper Karagoz (Department of Molecular Biology and Genetics in Usak University, Usak, Turkey) for assistance with the dendrogram analysis of pulsed-field gel electrophoresis results. We would also like to thank Zihni Acar Yazici for his critical reading and editing of the manuscript. This study was supported by the Recep Tayyip Erdogan University Scientific Research Projects Co-ordination Unit (Project Number TSA-2016-526).

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