The Distribution of Cytotoxic Necrotizing Factors (CNF-1, CNF-2, CNF-3) and Cytolethal Distending Toxins (CDT-1, CDT-2, CDT-3, CDT-4) in Escherichia coli Isolates Isolated from Extraintestinal Infections and the Determination of their Phylogenetic Relationship by PFGE

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Backgrounds. Diagnostic markers of extraintestinal infection in Escherichia coli (E. coli) remain unclear in the literature. Extraintestinal pathogenic E. coli (ExPEC) is differentiated from other E. coli isolates in terms of virulence factors, such as host cell adhesion, invasion, cytotoxic necrotizing factor (CNF (cnf1-cnfl33)) and cytolethal distending toxin (CDT (cdtl-cdt4)) that are responsible for cell death. We aimed to investigate the frequency of CNF-CDT and the relationship between the clinical diagnosis and genotypes in E. coli isolates with different clinical origins. Methods. A total of 646 E. coli isolates (obtained from 645 patients) isolated from different infection sites other than the intestine were evaluated in aspects of the CNF, CDT virulence genes, phylogenetic grouping, and phylogenetic relationship by using PCR and PFGE. Results. At least one virulence gene was present in 156 (24%) of the 646 ExPEC isolates. We detected cnf1, cnf2, and cnf3, in 78, 12, and 20 ExPEC isolates, respectively. Also, cdtl, cdt2, cdt3, and cdt4 genes were present in 20, 4, 4, and 4 isolates, respectively. Some isolates harbored more than one gene, being cnf1-cnf3 (n = 6), cnf1-cdt1 (n = 4), and cdt1-cdt4 (n = 4). These 156 isolates were distributed into 106 large clusters by PFGE. Virulent ExPEC is primarily related to groups B2 (60%) and D (32%). Conclusion. To our knowledge, this study demonstrated the presence of cnf2, cnf3, cdt1, cdt2, cdt3, and cdt4 genes for the first time in the literature for Turkey. The widespread presence of the CNF gene in E. coli helps distinguish ExPEC from commensal isolates.

1. Introduction

Escherichia coli (E. coli) is one of the important members of the human colon microflora. E. coli can easily transfer virulence and resistance genes from other bacteria in the intestinal flora. Some genotypes and serotypes of E. coli may cause pathologies in the intestinal tract, ranging from mild secretory diarrhoea to inflammatory mortal dysenteric diarrhoea. However, their colonization in sterile areas of the body, outside of the intestinal system, is always considered to be the cause of clinical pathology. Extraintestinal pathogenic E. coli (ExPEC) causes urinary tract infections, newborn meningitis, sepsis, osteomyelitis, pneumonia, surgical site infections, and infections in other extra-intestinal areas. Although infections caused by ExPEC have low morbidity, it is becoming an increasingly important endemic problem due to its fatal course and prolonged hospital stay [1, 2].
Important virulence mechanisms that distinguish ExPEC from other *E. coli* isolates include the ability to adhere, invasion into host cells/tissues, defects in host defense, and secreted effectors that affect host cellular functions directly. These effectors are composed of a functional toxin and damage the cell cycle, which is the main process of the host cell. Two important toxins/factors have been defined in *E. coli* species: the first one is cytoxic necrotizing factor (CNF), consisting of *cnf1-cnf3*, which is a Rho-GTPase-targeting toxin, and the second one is cytolethal distending toxin (CDT), consisting of *cdt1-cdt4*, which is a genotoxin that may cause DNA damage in target cells. While CNFs promote DNA replication without cytokinesis, CDTs block mitosis [3, 4]. These toxins-factors are encoded by mobile elements (e.g., the island of pathogenicity, plasmids) [5].

ExPEC-related infections, which have a wide range of disease spectrum from urinary tract infections to fatal bacteraemia, have increased from 17.8% to 65.3% in Turkey in the last 15 years [6]. This increase may be due to ExPEC isolates, which have undergone the pressure of irrational antibiotic use and gained new virulent characteristics with variable genetic elements from different bacteria to survive. There are a limited number of clinical-epidemiological studies showing that ExPEC isolates have different colonisation and virulence factors that affect the prognosis of the diseases compared with intestinal *E. coli*. These virulence factors are encoded by gene polymorphism.

We designed this study because of the rapid increase in ExPEC-related infections in recent years, the irrational use of antibiotics, and the limited number of studies on this topic in Turkey. We aimed to investigate the frequency of CNF-CDT and the relationship between the clinical picture and genotypes in *E. coli* isolates with different clinical origins. Thus, it was to detect a specific marker for diagnosing noncommensal *E. coli*.

### 2. Materials and Methods

#### 2.1. Collection, Isolation, and Identification of the Specimens.

A total of 646 clinical samples obtained from 645 patients were sent from different departments to the Microbiology Laboratories of Çukurova University and Adana Regional Hospitals, Turkey, between September 2014 and April 2016. These 646 ExPEC isolates were isolated from 465 outpatients and 180 inpatients. Also, 80% of all specimens were from the urinary tract, and most of 645 of them were from the urology and paediatric departments. The remaining specimens were collected from other medical departments and belonged to wounds, blood, sputum, tracheal aspirate, sterile body fluid specimens (cerebral spinal fluid and bone marrow), and vaginal swabs. All isolates of *E. coli* isolated from different infection sites other than the intestine during the study period were included consecutively in the present study. In addition, from one patient with the diagnosis of urosepsis, two specimens were obtained; one specimen belonged to blood, and the other one belonged to urine. The median age of patients was 45 years (a range between newborn to 82 years). Urine samples belonged to 80 males and 442 females; other specimens, such as wounds and blood, belonged to 107 males and 16 females. The samples were cultured on blood agar and Endo agar plates (Merck, Germany) at 37°C for 24 hours. Isolates were first evaluated by Gram staining to examine the morphology of colonies and biochemical test characteristics. Isolates were confirmed phenotypically by the IMVIC test.

#### 2.2. Storage and General Processes.

*E. coli* isolates were kept at −70°C in broth containing 20% glycerol. These isolates were also genotypically confirmed by PCR in the gene region (*uidA*) encoding the Beta-D-glucuronidase enzyme found structurally in *E. coli* [7]. All isolates of *E. coli* were evaluated in terms of the CNF and CDT virulence genes, phylogenetic grouping, and phylogenetic relationship. The *uidA* gene and *cdt1*, *cdt2*, *cdt3*, and *cdt4* genes were amplified in simplex PCRs. However, a multiplex PCR protocol was used to investigate the *cnf1*, *cnf2*, and *cnf3* genes [8, 9]. In addition, the Triplex PCR was used for the phylogenetic grouping of ExPEC isolates [10]. After that, the phylogenetic relationship of ExPEC isolates was investigated by the XbaI-PFGE [11].

#### 2.3. PCR Processing.

DNA extraction of all isolates was performed mechanically with a Mickle cell disruptor (The Mickle Lab. Engineering Co. Ltd., Gomshall, Surrey, UK). The amplicons were obtained by PCR using previously published primers (Table 1), *uidA*, *cnf1*, *cnf2*, *cnf3*, *cdt1*, *cdt2*, *cdt3*, *cdt4*, and extracted DNA samples.

The PCR amplification was carried out in a total volume of 25 μl. The PCR mixture was constructed as follows: 12.5 μl PCR master mix (1x without MgCl2, 4 μl MgCl2 (25 mM), 1 μl dNTP (200 μM each nucleotide), 1 μl each primer, Taq DNA polymerase 0.2 μl U (5 U/μl), template DNA 5 μl (approximately 50 ng), and distilled water by completing to 25 microliters.

The *uidA* gene and *cdt1*, *cdt2*, *cdt3*, and *cdt4* genes were amplified in a single PCR reaction in one tube. An in-house PCR method is used for the amplification of *CDT* gene regions.

A multiplex PCR protocol was used to determine the *cnf1*, *cnf2*, and *cnf3* genes. The PCR processes were applied for the amplification of toxin genes (Table 2).

The PCR products were separated on a 2% agarose gel for *cdt* and *uidA* and a 1.8% agarose gel for CNFs and monitored using a Kodak UV transilluminator (Kodak, New York, USA).

Since this was one of the rare studies in Turkey, a positive control isolate for CNF-CDT genes could not be obtained. After reviewing the literature, positive isolates for amplification were obtained by trying different PCR mixtures and PCR cycles. Then, we used our own isolates as positive controls for the following PCR tests.

#### 2.4. Phylogenetic Classification.

We investigated the phylogenetic analyses of 156 ExPEC isolates by the Triplex PCR method, which was defined by Clermont previously [10].
Table 1: Oligonucleotide primers are used in this study.

| Primer | Oligonucleotide sequence (5′–3′) | Specificity | PCR product size (bp) | References |
|--------|---------------------------------|-------------|-----------------------|------------|
| uidA F | ATGCCAGTCCAGGGTTTTTGC           | uidA        | 102                   | Abid and Al-Zuwainy [7] |
| uidA R | AAATGTGGGGTCTAATTAGGAAAGTG      |             |                       |            |
| cnf1 F | GGGGAAAGTACAGAAGAATTAA          | cnf1        | 1111                  |            |
| cnf1 R | TTGGCGTCACCTCACCAGT             |             |                       |            |
| cnf2 F | TATCATACGGGAGGAAGGAGCACC        | cnf2        | 1240                  | Dubois et al., [8] |
| cnf2 R | GTACCAATAAGACAATAATTTCCG         |             |                       |            |
| cnf3 F | TAACGTAAATTAGCAAGAAGA           | cnf3        | 757                   |            |
| cnf3 R | GTCTTCATTACTTCTACGT             |             |                       |            |
| cdt1 F | CAATAGTCGCCACAGGAGA             | cdt1        | 411                   |            |
| cdt1 R | ATATAACAGAAACACCCA             |             |                       |            |
| cdt2 F | GAAAGTAAATGGAATATAAATGTCCG      | cdt2        | 556                   | Toth et al., [9] |
| cdt2 R | TTTGTGTGCCGGCGCTGTTGAA          |             |                       |            |
| cdt3 F | GAAAGTAAATGGAATATAAATGTCCG      | cdt3        | 555                   |            |
| cdt3 R | TTTGTGTGCCGGCGCTGTTGAA          |             |                       |            |
| cdt4 F | CCTGATGGTTCAGGAGGTGGTTC         | cdt4        | 350                   |            |
| cdt4 R | TTTGTCAGAATCTATACCT             |             |                       |            |
| chuA F | GACGAACCAACGGTCAGGAT            | chuA        | 279                   | Clermont et al., [10] |
| chuA R | TGCGGCACGTACAAAGAC          |             |                       |            |
| yjaA F | TGAATGTCAGGAGACGCTG             | yjaA        | 211                   |            |
| yjaA R | ATGAGAATGCGGTCCTCCTACAC        |             |                       |            |
| TspE4C2 F | GAGTAATGTCGGGGCATTCA       | TspE4C2     | 152                   |            |
| TspE4C2 R | CGCGGCAACAAAGATATTACAG     |             |                       |            |

Table 2: Thermal cycles for eleven primers are used in this study.

| No | Step                  | uidA       | CNFs      | CDTs      | chuA, yjaA, tspe4.c2 |
|----|-----------------------|------------|-----------|-----------|----------------------|
| 1  | Initial denaturation  | 94°C/5 min | 94°C/3 min| 94°C/5 min| 94°C/30 min          |
|    | Denaturation           | 94°C/30 sec| 94°C/1 min| 94°C/1 min| 94°C/30 sec          |
| 2  | Annealing             | 63°C/30 sec| 60°C/1 min| 60°C/1 min| 60°C/30 sec          |
|    | Extension             | 72°C/1.5 min| 72°C/1 min| 72°C/1 min| 72°C/1.5 min         |
| 3  | Final extension       | 72°C/5 min | 72°C/7 min| 72°C/10 min| 72°C/5 min          |

E. coli isolates belonged to four groups (A, B1, B2, and D) based on the presence of the chuA and yjaA genes and the DNA fragment (TspE4.C2) (Table 1) by the Triplex PCR (Table 2).

2.5. Xbal Pulsed-Field Gel Electrophoresis. PFGE was performed to determine DNA profiles of CNFs-CDTs producing isolates using the Xbal enzyme as reported previously [11]. This method has been used for Klebsiella, E. coli, P. aeruginosa, and Acinetobacter (KEPA). Epidemiological relationships between isolates were assessed by studying the PFGE patterns of genomic DNA after restriction by Xbal. Xbal-PFGE is the gold standard for determining the clonal distribution of ExPEC isolates.

3. Results

A total of 646 specimens isolated from the extraintestinal region of the patients within 18 months were included in this study. Of the 646 samples, 522 were urine; of the remaining were 54 wounds, 28 blood, 21 sputum, 6 tracheal aspirates, 12 sterile body fluids, and 3 vaginal swabs (Figures 1 and 2).
Clinic and Polyclinics: Numbers

- Cardiovascular surgery service: 6
- General surgery outpatient clinic: 5
- Endocrinology service: 5
- Orthopedic service: 5
- Gastroenterology service: 5
- Orthopedic service: 5
- Pediatric outpatient clinic: 212
- Urology outpatient clinic: 216
- Surgical intensive care: 60
- Obstetrics and gynecology: 34
- Infection diseases: 32
- Internal medicine service: 25
- Nephrology outpatient clinic: 32
- Infectious diseases: 32
- Surgical intensive care: 60
- Newborn unit: 8
- Internal medicine service: 25

Table 1: Distribution of 645 patients by clinics and polyclinics.

At least one virulence gene was detected in 156 (24%) of the 646 ExPEC isolates. We detected cnf1, cnf2, and cnf3, in 78, 12, and 20 ExPEC isolates, respectively (Figures 3(a)–3(e)). cdt1, cdt2, cdt3, and cdt4 genes were present in 20, 4, 4, and 4 of the isolates (Figures 4(a)–4(d)), and at least two factors cnf1-cnf3, cnf1-cdt1, and cdt1-cdt4 existed in 6, 4, and 4 of the isolates, respectively (Table 3). These 156 isolates were distributed in 106 large clusters by PFGE (Figures 5 and 6). Virulent ExPEC is primarily related to groups B2 (60%) and D (32%).

One hundred sixteen (18%) of these isolates harbored one of the genes encoding CNF: cnf1 (n = 78), cnf2 (n = 12), cnf3 (n = 20), and cnf1 + cnf3 (n = 6) (Figures 3(c) and 3(e)). However, 36 (5.4%) isolates had CDT only: cdt1 (n = 20), cdt2 (n = 4), cdt3 (n = 4), cdt4 (n = 4), and cdt1 + cdt4 (n = 4) (Figures 4(a)–4(d)). Interestingly, four (0.6%) had both toxin factors.

According to the clinical origins of 646 ExPEC isolates, the CDT gene family was found to be 3.6% in the urinary tract samples and 13.7% in the nonurinary isolates. The CNF gene family was at an 18% rate in the urinary tract samples (Table 3).

In 156 ExPEC isolates in which at least one virulence factor had been detected, the most frequently isolated gene was cnf1, with a rate of 50% + 6.3% in binary combinations, followed by cdt1, with a rate of 12.8% + 5% in binary combinations (Table 4).

The distribution of CND and CDT genes of 156 ExPEC isolates according to the origin of the isolate as urinary and nonurinary samples is presented in Table 5. Of the 156 ExPEC isolates, 121 (78%) were of urinary tract origin, while 35 (22%) were extraurinary. The most common virulence genotypes in 121 urinary tract specimens were those of the CNF gene family, with a rate of 84.3%. In the urinary samples, the range of the CDT gene family was 18.3%. The positivity rate of both CNF and CDT genes at the same time in urine samples was only 2.4% (Table 5). While 68 (87.2%) of 78 cnf1-positive isolates originated from the urinary tract, 10 (12.8%) of them were isolated from other extraintestinal sites. On the other hand, among the CDT genes (cdt1, cdt2, cdt3, and cdt4), the most common isolate in urine samples was cdt1, with a rate of 10% (12/121) (Table 5).

The phylogenetic analyses of 156 ExPEC isolates revealed that these isolates consisted of four main phylogenetic groups, including A, B1, B2, and D. In 156 ExPEC isolates, the most frequently analysed phylogenetic group was B2 (60.8%), followed by D (30.7%), A (5.12%), and B1 (3.2%) (Table 6).

Of 68 cnf1 isolates originating from the urinary system, while 38 were in the B2 and 30 were in the D phylogenetic group. No cnf1 isolate was found in the A phylogenetic group. Only three isolates of cnf1 originating from the nonurinary sites were in the B1 phylogenetic group (Table 6).

To determine the phylogenetic relationships of 156 ExPEC isolates, whole-genome DNA fragment polymorphism analysis was performed with XbaI-PFGE (Figure 5). According to this method, based on 80% similarity, 156 ExPEC isolates were collected in 106 clusters. Four of the test isolates (Z23, Z25, Z36, and Z38) had five members; eight of them (A, E, G, Z60, Z64, Z80, Z83, and Z88) had three members; and 15 of them had two members (C, F, I, M, O, R, Z2, Z3, Z5, Z6, Z24, Z56, Z58, Z73, and Z76) (Figure 6). In addition, 82 isolates were unrelated, and each of them was present as a "singleton" (Figure 6). The isolates accumulated in the single-member cluster and the same subcluster had different virulence factors. Two specimens were obtained from one patient with a diagnosis of urosepsis; one was blood, and the other was urine. These two specimens were distributed in different clusters and had different virulence factors. While the sample sourced from blood was positive in terms of cdt1 virulence factor and showed Z polymorphism, the other sourced from urine was negative in terms of any virulence factor and showed Z10 polymorphism, using the PFGE method. The clustering of the virulence genotype is shown in "Figure 7" as a simple representative dendrogram.

The data that support the findings of this study are available on request from the corresponding author (Cansu Onlen Guneri). The data are not publicly available due to participant privacy.
4. Discussion

To our knowledge, this is the first study that showed cnf2, cnf3, cdt1, cdt2, cdt3, and cdt4 genes in Turkey. We used CNF- and CDT-specific primers to determine the prevalence of CNF-CDTs, which were proven to increase the colonization of ExPEC isolates outside the intestinal tract. There are a limited number of clinical-epidemiological studies in the literature that suggest CNF-CDT causes changes in host cell functions, damage to epithelial cells, and potent virulence factors that may lead to tissue pathologies, such as stimulating the inflammatory response [9].

While the rate of CDT found in this study was consistent with some previously reported studies [8, 9], there were also
studies reporting low rates, such as 0.9–2.5% [12, 13]. The different CDT gene ratios between our study and others may be explained by the difference in the origins of the study materials (Table 3). In this study, the CDT genes were higher in nonurinary tract specimens compared with urinary tract samples. Also, approximately 80% of the samples included in our study were urine. It could be explained by the high prevalence of E. coli isolates in urinary tract infections by the easy colonization of the intestinal isolates by invasion into the urinary system through the neighbourhood relationship. It could also be explained by the fact that they can cause disease in the host under different stress factors because they can easily colonize without the need for an extra virulence factor.

Besides the CDT gene, we also investigated all CDT alleles (cdt1, cdt2, cdt3, and cdt4). We found that the frequency of cdt1 and cdt4 was higher than that of cdt2 and cdt3 in all ExPEC isolates (Table 4). Previously published studies also reported that the cdt1 and cdt4 alleles were positive, while the cdt2 and cdt3 alleles were negative [8, 12–15]. These results suggest that the cdt1 and cdt4 alleles of the CDT genes show very common and close homology and may have been derived from a common ancestor by phage transduction.

On the other hand, the rates of CNF genes and alleles that we detected in the present study could be similar to some studies; their rates were between 5–34% [16, 17], and such a
One of the most important results of this study was the high levels of CNF genes, especially in urine samples. The results of the present study were higher than in most other studies. In other studies, these rates have changed between 16.5% and 61.9% [9, 12, 13, 18, 19]. In a study conducted in Turkey by Bozcal et al., the cnf1 ratio was 12% in ExPEC isolates obtained from blood samples [20]. When only considering blood samples in our study, this rate was 11% (3/28). We can say that our results were consistent with Bozcal's study. These results suggest that cnf1 can be a specific marker for virulent isolates, especially for the urinary tract and sepsis-related ExPEC.

In the present study, cnf1 was the most common virulence factor compared to cnf2 and cnf3. We can explain this by the fact that cnf2 and cnf3 are of animal origin and rare in humans. However, in our study, the rates of cnf2 and cnf3 were higher than in previous studies [9, 16, 17, 19, 21].

The phylogenetic distributions of ExPEC isolates in our study were similar to previous studies. According to these results, we can say that ExPEC originated predominantly from phylogroup B2 and, to a lesser extent from, group D [9, 10, 12, 16, 17, 22]. However, in the study conducted by Bozcal et al., in ExPEC isolates obtained from blood samples, these rates were D (38.14%), A (29.89%), B2 (20.61%), and B1 (11.34%) [6]. The results of our work are inconsistent with the Bozcal study. It may depend on the fact that the samples in our study were from different sites and the high number of specimens studied.

Finally, to understand the CNF-CDT positive E. coli isolates that evolved from deletion, recombination mutations, or evolved from an ancestor strain and acquired the wide range of outcomes may be attributed to unreported patient characteristics.

Table 4: The distribution of toxin genes in CDT- and CNF-positive 156 ExPEC isolates.

| Virulence factor | cnf1 | cnf2 | cnf3 | cnf1-cnf3 | cdt1 | cdt2 | cdt3 | cdt4 | cdt1-cdt4 | cnf1-cdt1 |
|------------------|------|------|------|-----------|------|------|------|------|-----------|-----------|
| Total (n = 156)  | 78   | 12   | 20   | 6         | 20   | 4    | 4    | 4    | 4         | 4         |
| n/total          | 78/156 | 12/156 | 20/156 | 6/156     | 20/156 | 4/156 | 4/156 | 4/156 | 4/156     | 4/156     |
| %                | 50   | 7.6  | 12.8 | 3.8       | 12.8 | 2.5  | 2.5  | 2.5  | 2.5       | 2.5       |

Table 5: The distribution of factor-toxin genes of 156 ExPEC isolates by clinical origin/the virulence factor distributions in the urinary and nonurinary tract foci in CDT- and CNF-positive ExPEC isolates.

Table 6: The distribution of 156 ExPEC isolates virulence genes by phylogenetic group and clinical origin.
ExPEC feature, we used PFGE in these ExPEC isolates. The results of PFGE in the present study regarding similarity rates and clusters have shown differences from studies conducted by Oloomi and Bouzari [23] and Mora et al. [24]. In our study, in the aspect of the XbaI-PFGE results, the phylogenetic relationships of ExPEC isolates were found to be very weak. ExPEC isolates with the same phylogenetic properties may have different virulence characteristics (Figure 7). As shown in the results section of our study, two ExPEC isolates obtained from one patient from different clinical samples at the same time may have originated from different ancestors evolutionarily. We could explain this situation by saying that most extraintestinal *E. coli* infections have been acquired from the community. The characteristics and spread of virulent ExPEC isolates should be monitored by molecular surveillance and limited by vaccine studies.

5. Limitations

There were some limitations to the present study. The first limitation was the lack of detailed data on clinical features, although the diagnosis of the patients was presented. Thus, we could not compare the clinical course and antibiotic susceptibility of the patients with positive virulence genes to negative ones. The second limitation was that the virulence genes we detected in extraintestinal *E. coli* isolates could also be investigated in intestinal isolates. The third limitation was that we could not show antitoxin in susceptible cell types like Vero by cell culture studies. Finally, standard controls could not be obtained due to the limited number of similar studies in our country. Despite efforts for international cooperation, we could not have the standard isolates.

6. Conclusion

To our knowledge, we showed *cnf*2, *cnf*3, *cdt*1, *cdt*2, *cdt*3, and *cdt*4 genes for the first time in Turkey. The widespread presence of the CNF gene in *E. coli* can help distinguish ExPEC from commensal isolates. These genes may also be a specific marker for noncommensal *E. coli*. In particular, the presence of *cnf*1 increases the *E. coli* colonization in the urinary system and could be a characteristic virulence factor for the uropathogenic *E. coli* isolates. Virulence factors can be distributed depending on phylogenetic groups (*A*, *B1*, *B2*, and *D*), and these phylogroups may be used to distinguish between pathogen and nonpathogen isolates.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon reasonable request.

Ethical Approval

This study was conducted with the approval of the Çukurova University Faculty of Medicine Non-interventional Clinical Research Ethics Committee (Date: 10.10.2014 and Decision No: 35-2).

Disclosure

The funding body had no contribution to the study design, data analysis, interpretation, and in writing of the manuscript. A preprint has previously been published [25]

Conflicts of Interest

The authors declare that there are no conflicts of interest.
Authors’ Contributions

Cansu Onlen Guneri collected samples, cultured the isolates, and performed DNA extraction, PCR, and PFGE. Fatih Koksal and Cansu Onlen Guneri designed this study. Fatih Koksal, Cansu Onlen Guneri, Suna Kızılyıldırım, and Togrul Nagiyev supervised the practical work and data management. Cansu Onlen Guneri and Fatih Koksal wrote the manuscript. All the authors approved the final version of this manuscript.

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